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Sourcebook in Forensic Serology, Immunology, and Biochemistry

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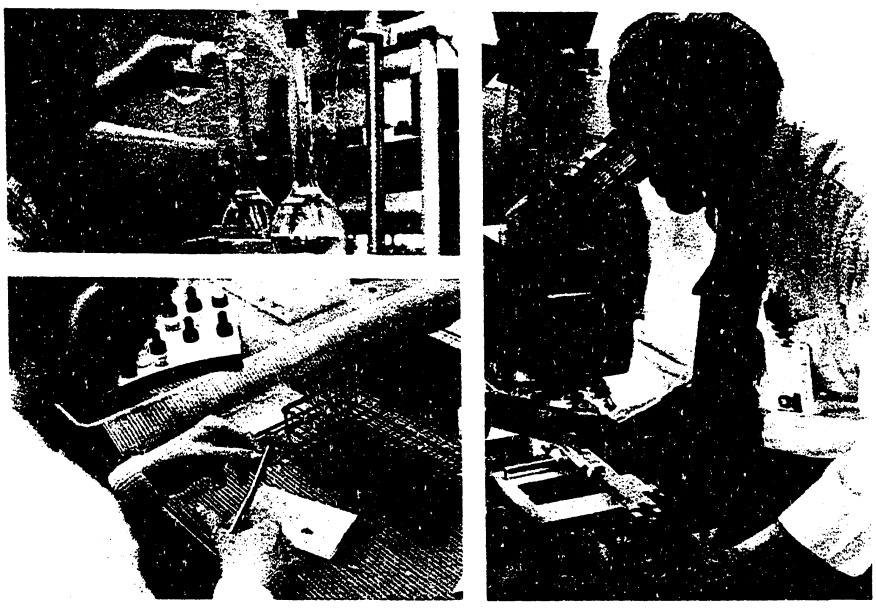

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U.S. Department of Justice
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Sourcebook in Forensic Serology, Immunology, and Biochemistry

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with 1989 Update

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Sourcebook Errata

On page 17, the equation following line 11, after the words "The above equation may be rewritten:" should read as follows:

$$[ES] = \frac{k_1}{k_2 + k_3}\,[E][S]$$

The equation following lines 16 and 17, after the words "Solving for ES and substituting in the previous equation yields" should read as follows:

$$\frac{v_o}{V_{max}} = \frac{k_1}{k_2 + k_3}\,\frac{[E]\,[S]}{[E_t]}$$

On page 426, lines 29 and 30 giving the correspondences of different PGM subtype nomenclatures should read as follows:

1- = a3 = 1F; 1+ = a1 = 1S; 2- = a4 = 2F; and 2+ = a2 = 2S.

PREFACE TO THE REPRINT EDITION

In the six years or so since this *Sourcebook* appeared, the stock of copies was exhausted. There were indications from a number of sources, however, that there was still a demand for copies and that the book still has a valuable place as a reference work in forensic biology. Accordingly, the National Institute of Justice is to be commended for its decision to reprint the book on a demand basis through the National Criminal Justice Reference Service. In this respect, I am grateful to Mr. James K. Stewart, Director of the Institute, and to Dr. Richard M. Rau, manager of the Forensic Science and Criminal Justice Technology Program. As a result of their continued support, this book will continue to be available to those interested in it.

It was not realistic to consider undertaking a complete and systematic update and review of all the literature that has appeared since the book's publication. I was asked, however, to provide a brief summary of some of the more recent information in the field in this new Preface, and that is its major purpose. Because the literature grows with such rapidity, a number of more recent reviews as well as some specific papers are cited here and an effort has been made to related these to specific subject areas covered in the book. In addition, an introduction to the rapidly developing field of molecular biology and DNA analysis that has recently become a part of forensic biology is given, and some references provided. DNA analysis has become a part of forensic serology in the relatively few years since the book was written.

Selected Recent Material on Sourcebook Subjects

Several more recent reviews cover blood and body fluid identification in stains, species determination and blood and body fluid stain grouping [1-3], and the application of genetic markers including HLA to parentage testing has also been reviewed [4-6]. Volume 1 of *Advances in Forensic Science* [7] contains a number of review chapters by noted authorities on various subjects: Divall, on menstrual blood identification (cf. §8.1); Katsumata and Oya, on feta and neonatal blood identification (cf. §§8.2 and 8.3); Suzuki and Oya on semen identification in stains (cf. §10); Fiori, on body fluid grouping (cf. §19.10.5); Benciolini and Cortivo, on ABO grouping of human hair (cf. §19.10.7.1); Carracedo, as well as Pascali, on isoelectric focusing and its applications in serum group protein typing (cf. §§ 40-43 and 45); Tumosa, on the occurrence of ABH antigens in infrahuman species; Newall, on typing HLA antigens in bloodstains (cf. §46.7.2.); and Smith, on detecting drugs in bloodstains (cf. §50.2.2).

In the area of semen identification, there have been a number of newer developments. The original description of and earlier papers on γ-seminoprotein (γ-Sm) are discussed in §10.10. Similarly, the original work on seminal protein p30 may be found in §10.14. It is highly likely (though perhaps not proven) that γ-Sm and p30 are identical to one another, as well as to "human prostate antigen" (sometimes abbreviated PA) [8]. The γ-Sm protein has been further characterized biochemically and its amino acid sequence determined [9-11]. An ELISA assay using anti-γ-Sm has been developed for use wi seminal stains [12]. Similarly, an ELISA has been developed for p30 and shown to be applicable to the investigation of seminal stains an vaginal swabs in sexual assault cases [13].

Another smaller seminal protein of prostatic origin, called β-microseminoprotein (β-MSP) has been isolated and extensively characterized by Hara and his collaborators in Japan [14-17]. I am indebted to Prof. Dr. Mitsuwo Hara at the Kurume University School of Medicine for making copies of his more recent work available. Another human seminal protein of seminal vesicle origin, known as MHS-5, has been purified and a monoclonal antibody prepared against it [18]. The monoclonal anti-MHS-5 has been used as the basis for an ELISA test for human semen identification.

The theory underlying absorption-inhibition testing as well as a novel two-dimensional A-I method are discussed in a paper by Lee and collaborators [19]. More recent material on the biochemical genetics of and relationships between ABO, Lewis, Secretor and related antigens (cf. § 19.9) may be found in reviews by Watkins and by Oriol and coworkers [20-22]. Extensive reviews of the application of the polymorphic isoenzyme (and other) systems in forensic serology (cf. Unit VI) have been published by Sensabaugh [23-26].

The U.S. population data for various genetic marker systems that are included in the book have been updated and analyzed in a series of three papers [27-29]. In addition, several papers have discussed the application of population genetic marker data to stain typing information as might be obtained in particular case situations [24, 30-32].

The forthcoming third volume of *Advances in Forensic Science* [33] offers reviews of several important subjects, in addition to its extensive coverage of DNA typing (about which more below). Schanfield extensively reviews immunoglobulin allotyping (cf. §44), Bütler reviews and updates the Ag system (cf. §45.1.2), and Mayr reviews the application of HLA typing in disputed parentage cases (cf. §46.7.1).

In addition, Fletcher reviews enzyme-linked immunsorbent assay (ELISA as applied to forensic blood and body fluid identification and grouping problems. ELISA applications have come along sufficiently recently that there is nothing about them in the book.

Molecular Biology and DNA Typing

Evidence that DNA is in fact the genetic material, the structure of the nucleic acids, and the manner in which DNA controls protein synthesis are briefly reviewed in §1.2.2 of this book. In the past decade or so, extraordinary advances have been made in the field of molecular biology. These advances have enabled the development of wha is often called genetic engineering. Perhaps the most significant advances in molecular biology from the point of view of forensic biology have been: (1) the discovery and characteri- ion of a large variety of restriction endonucleases and their widespread availability; (2) the discovery and refinement of techniques for cloning manageable-sized fragments of DNA into vectors; (3) the discovery of restriction fragment length polymorphisms and the availability of human DNA probes for their detection; and (4) the description and refinement of polymerase chain reaction techniques, and their use in connection with allele specific oligonucleotide probes.

The large array of restriction endonucleases (restriction enzymes; RE) allows very large DNA to be cleaved into smaller, manageable fragments for subsequent characterization and/or manipulation. Knowledge of the RE cleavage recognition sequences in DNA has meant that sequence information is available about the ends c the fragments produced.

Some of the REs produce blunt ends, but many others produce "jagged" cuts in double stranded DNA producing fragments in which a few bases from one strand protrude as a single strand beyond the terminus of the other strand. These few base single stranded ends are sometimes called "sticky," because if another piece of double stranded DNA with a complementary single stranded sticky end is produced, the two fragments of DNA can be recombined into a single doble stranded molecule using appropriate ligases. Variations of this procedure form the basis of genetic engineering. Using these techniques, fragments of human DNA can be introduced into vectors (usually plasmids or cosmids). Then, by subsequent cloning, these human DNA fragments can be reproduced in any desirable quantity in perpetuity, and in addition they can be isolated and recovered from the vectors.

In recent years it has been recognized that the human genome contains substantial segments of repetitive sequence DNA [34,35]. Some repetitive DNA occurs in the form of relatively short, highly repeated sequences that have been called 'minisatellites.' Certain minisatellite loci have been found in the human genome at which there is substantial variation between individuals in the number of times the core sequence is repeated. If a RE is used to cleave DNA outside the repeat region, fragments of differing size are produced according to the number of repeats occurring in the region. Human DNA loci of this kind are termed "variable number of tandem repeat" or "VNTR" loci. Separation of the RE-digested VNTR fragments according to size by electrophoresis, transfer of the fragments to a nitrocellulose or nylon membrane, and hybridization with a labeled human DNA probe that recognizes the core sequence produces banding patterns that are characteristic of the individual from whom the DNA came.

This phenomenon is called "restriction fragment length polymorphism" or "RFLP" and is the basis of most current "DNA typing" as it is applied in forensic serology.

In 1980, Botstein, White, Skolnik and Davis [36] recognized that RFLP could be used as a basis for genetic mapping, and this approach has indeed yielded considerable information [37]. Wyman and White [38] soon described a highly polymorphic VNTR locus, and a large number of other such loci are now known. Not much notice of these developments was taken by the forensic science community until Jeffreys described several multilocus probes [39] that could be employed to produce what were described as DNA 'fingerprints' [40]. The importance of these findings in terms of their applications both to disputed parentage problems and to individual identification problems was quickly recognized [41,42].

In just the past few years, Jeffreys has further characterized the multilocus probes [43] and cloned a series of single locus probes recognizing several of the loci detected by the original probes [44-46]. These probes are used exclusively by Cellmark Diagnostics in their DNA typing work in the U.S. Other DNA probes are used by the Lifecodes Corp. in their DNA typing work, and some information about their probes and procedures has been published [47-49]. A number of DNA probes from GenMark are available through Promega, and still other probes are available from Collaborative Research, Inc. Recently, the FBI Laboratory initiated DNA typing in casework after a lengthy research and development effort aimed at selecting a typing system, appropriate probes, and validating the procedures that are to be used [50-51].

Another DNA analysis procedure that has already found limited application in forensic serology, and is certain to be significant in the future, is the polymerase chain reaction (PCR) technique. PCR was developed by Erlich and collaborators at the Cetus Corp. in California [52-53]. With PCR, specific sequences of DNA can be replicated to produce hundreds of thousands to millions of copies provided specific primers are available. The primers are constructed from knowledge of the sequences flanking the region of interest. PCR has been applied to the diagnosis of genetic disorders [54] and to the analysis of polymorphism at specific subregions of the *HLA* locus [55]. DNA analysis of the HLA-DQα polymorphism has been described in single human hair roots [56]. Many samples of forensic interest are limited in quantity and may also have been subjected to environmental conditions that degrade the DNA. As a result, RFLP analysis may not be possible. PCR techniques are attractive for forensic analysis because so little DNA is required for analysis, and experience has shown that some samples which were unsuitable for RFLP analysis could be analyzed using PCR procedures. At the present time, PCR procedures are used in conjunction with allele-specific oligonucleotide (ASO) probes. The loci currently detected do not show the degree of polymorphism exhibited by RFLP loci. The information obtained at present from PCR analysis is thus very valuable as an exclusionary tool, but less valuable in inclusionary cases. Efforts are underway in many laboratories, however, to develop primers that will enable PCR amplification of VNTR loci [57]. Further research and development will be necessary to evaluate the forensic applications of PCR techniques, as very few forensic laboratories have had much experience with PCR at the present time.

Further information about DNA typing and its forensic applications may be obtained from a number of currently available sources. Volume 3 of *Advances in Forensic Science* [33] will have ~~eight~~ seven chapters on DNA polymorphisms and their forensic applications. The Banbury Conference on forensic applications of DNA has papers by a number of authorities in the field [58]. The FBI Forensic Science Research and Training Center at Quantico has sponsored two major symposia on the forensic applications of DNA [59,60]. The proceedings of the first of these symposia are available on videotape, and the proceedings of the second will be published. An extensive report on forensic applications of DNA typing is currently in preparation by the Office of Technology Assessment of the U.S. Congress, and should be delivered sometime in 1989.

DNA typing is undoubtedly the most exciting development in forensic serology in many years, and arguably the most exciting development ever. It will take some time for techniques and procedures to be worked out and tested on a relatively wide scale. The need for standardization of methodology and for some general agreement on procedures for the interpretation of RFLP typing results has recently been discussed [58,61]. In the next few years, molecular and forensic biologists working together will undoubtedly establish guidelines and standards for reliable and reproducible DNA typing procedures that can be widely employed in the analysis of both disputed parentage and identification cases.

West Haven, CT
July, 1989

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FOREWORD

The National Institute of Justice is pleased to publish this important reference work for forensic serologists. The late John O. Sullivan, manager of the Institute's forensic science program from 1975 to 1981, played a key role in encouraging and supporting development of this publication. It is a particularly fitting legacy of Mr. Sullivan's contributions to advancing the state of the art in the forensic sciences.

James K. Stewart
Director

PREFACE

For a number of years, I have thought it would be desirable to have available a comprehensive review of the literature of the many subjects that now comprise forensic serology, immunology and biochemistry. My appointment as a Visiting Fellow in the National Institute of Law Enforcement and Criminal Justice (now the National Institute of Justice) in 1976 afforded me the opportunity to prepare this review. I trust that the product may be a useful reference work for forensic serologists working in various laboratories, particularly in this country.

I have taken a more or less historical approach to each of the major subjects, in part because I thought it would provide continuity, and in part because I thought it would be more interesting. Accordingly, the different subject areas are discussed from the time of their origins in the published literature up to the present time. Much of the material is now of purely historical interest, and does not represent the current understanding of the subjects. I hope that the distinctions between older notions of purely historical interest, and current ones, have been clearly made.

There are many excellent reviews of the subjects covered here by specialists in those fields. They treat the various topics more comprehensively and better than I have been able to do, and I have cited them in the reference lists. In this work, I have attempted to treat all the subjects of interest in present-day forensic serology, and to combine the historical developments, the essential background information, and the forensic applications under the same cover.

This work has been entitled a "sourcebook", because it is quite simply a narrative review of the scientific literature. Because I regard this book primarily as a guide to the published literature, careful attention has been paid to the accuracy of the reference lists which appear at the end of each unit.

The book is divided into a total of nine units. The first unit consists of background material in serology, immunology, biochemistry, genetics and methods that are employed in the field. I was persuaded that this material should be included, and that it might serve a useful purpose. Units II and III have to do with the identification of blood and body fluids, respectively, and Unit IV has to do with species determination. These make up most of the identification sections. Units V, VI and VII have to do with the different classes of genetic markers in blood and body fluids, and make up most of the individualization sections. Unit VIII is concerned with the sexing of bloodstains, and with efforts to individualize blood using non-genetic markers. Unit IX consists of a set of translations of original papers of historical interest in the field. The rationale for the translations set is discussed in the Preface to that unit, which is self-contained. The eight units of the sourcebook are further divided into sections and subsections.

References are compiled at the end of each unit. Because of the large number of references, some consistent bibliographic style had to be selected, and in arriving at these conventions, I have made an effort to provide as much information as possible for readers who wish to find particular references. An effort was made to consult every reference which is cited here. References which could not be examined have a notation of the source that was used. These are indicated as "cited by" or "through". If another reference contains similar information to the one cited, or an abstract of it, I have indicated this fact with the words "and see".

The A.I.B.S. convention has been followed in citing all the references [Council of Biological Editors, Committee on Form and Style: *CBE Style Manual,* 3rd ed., American Institute of Biological Sciences, Washington, D.C., 1972]. References are cited in the text by the name(s) of the author(s) and the year the paper was published. The use of the name(s) and year as part of a sentence constitutes a citation. Papers written by more than two authors are cited in the text by the last name of the first author, and "*et al.*", followed by the year. In cases where the same author(s) wrote

several papers in the same year, they are distinguished in the text and in the reference lists by lower case arabic letters, e.g. 1971a, 1971b, etc. In some cases, a senior author with two or more coauthors, not always the same people, wrote more than one paper in a given year. The year and lower case letter convention is used to distinguish these, even though the full list of names on the papers is not the same. Thus, for example, if A. Smith, B. Jones and C. Williams wrote a paper in 1960, and A. Smith, B. Jones, C. Johnson and D. Williams had another paper in the same year, the former would be cited in the text as "Smith *et al.,* 1960a", the latter as "Smith *et al.,* 1960b". The arabic letters are used in the reference list as well as in the text in these cases. The reference lists are in strict alphabetical order by first letter of last name of first author, including institutional authors. Editorials are cited as "Editorial", unless they were signed, and it was clear who wrote them. In the older literature, first name(s) or initial(s) of authors were not always given. There was a tendency to use titles. Authors' initials which are given in parentheses in the reference lists were supplied, and did not appear in the original article. Titles of articles are given in full in the original language, except in cases where the original language does not use the Latinic alphabet. I have tried to retain accent and diacritical marks in citing authors' names and article titles. Russian and Japanese journals generally provide an English translation of the names of authors and the title of the article. I have usually given these in English. Transliteration of author names and article titles from sources in languages using Cyrillic alphabets follow the *U.S. Government Printing Office Style Manual* (1973). Abbreviations of journal titles have been taken from *Bibliographic Guide for Editors and Authors,* American Chemical Society, Washington, D.C., 1974, or from *BIOSIS List of Serials,* BioSciences Information Service of Biological Abstracts, Philadelphia, PA, 1976. In cases where these sources did not provide a standard abbreviation, I have followed the guidelines given in ANSI Standard Z39.5-1969 (R1974) of the American National Standards Institute in arriving at the usage which appears.

In some libraries, foreign journals are catalogued according to their foreign titles. Where I encountered this practice, footnotes were added to the reference lists giving the appropriate information. Similarly, many journals have undergone title changes over the years, many have been superceded by other journals, and some have been divided up into a number of separate parts, and so forth. In cases where I thought these changes might cause difficulty in locating an article in a library, I have added explanatory bibliographic footnotes. The principal Russian medicolegal journal Судебно-медицинская Експертиэа is uniformly cited in the reference lists as "Sud. Med. Ekspert.". Journal title abbreviations are set in italic type, and volume numbers are in boldface type. In many cases, journals have been issued in several series over the years. Sometimes, the original volume numbering was dropped when a new series was issued, but in other cases it was retained. The series in which the cited volume number appeared is given in parentheses following the volume number. "N.S." means "new series" and this series is always the second one. In German language journals, the word "Folge" indicates a series; thus, "N.F." means "neue Folge", "3F" means third "Folge", and so on. If the original volume number was retained in the journal, even though a subsequent series designation was being used, both designations are given. For example, "**21** (2 ser. **6**)" means that the piece is the 21st volume of the journal, and is also the 6th volume of the second series. An arabic numeral in parentheses following the volume number is the *number* of the journal within the particular volume (or over-all). Thus, "**14**(12)" indicates volume 14, number 12. I included this in some cases because it was common in the older literature to cite references by number only, rather than by volume and page number. Thus an author might cite "*Berl. Klin. Wochenschr.,* **1906**, No. 6". I would cite this reference as "*Berl. Klin. Wochenschr.* **43**(6): pages". In this way, a reader could verify that the two papers were the same, though cited differently. Full pagination for each article has been given as called for by the A.I.B.S. convention. A single page number indicates that the reference occupies only one page. Deviations from these conventions are in the direction of giving more information about the reference. I hope that the use of well defined conventions, and explanatory footnotes where they seem to be necessary, will help readers to find references in which they are interested more easily than I was able to do in many cases.

Papers in the reference lists marked with the symbol ¶ have been translated into English as part of the translations set, which appears as Unit IX.

The term "substrate" is sometimes used in forensic serology to mean the object or material upon which a stain was deposited. The term also has the technical biochemical meaning of the reactant(s) in enzyme-catalyzed reactions. I have restricted the use of "substrate" to the biochemical meaning. Objects or materials upon which stains have been deposited are "substrata" (singular: "substratum").

In many of the respective sections dealing with genetic marker systems, I have compiled as much U.S. population data as I could find with a reasonable amount of effort. Some criteria had to be used in selecting and presenting this data. Since this book was prepared with forensic serologists in this country in mind, I have included only U.S. population data. I also decided, arbitrarily, not to include any data published before 1950. The data are presented in tables in essentially the same form as given by the original author(s). The only additions I have made are percentages of individuals representing various phenotypes, in cases where the author(s) gave only numbers. I have not tried to calculate numbers if the author(s) presented percentages. The population sampled is described in the terms used by the original author(s). At the present time, the single, most comprehensive reference work on population data ever compiled is the 1976 edition of *The Distribution of the Human Blood Groups and Other Polymorphisms,* by Mourant, Kopeć and Domaniewska-Sobczak (cited in the text as Mourant *et al.,* 1976). No one seriously interested in human blood group population data can do without this reference. In the older literature, a comprehensive tabulation was prepared by W.C. Boyd in 1939. ABO and MN frequencies for many of the world's populations which had been studied were given.

Because this book took considerably longer to complete than was originally anticipated, some more recent references may be cited in later units, and not in earlier ones, even though they contain information on the subjects covered in both places. I have made some effort to remedy this problem in revision, but may not have succeeded entirely.

A large number of people have been helpful to me in many different ways in the course of this project. I take pleasure in acknowledging their help and assistance in the remainder of the preface. I am grateful to the following for granting their kind permission to use material from figures and tables in published sources: American Association for the Advancement of Science [publishers of *Science*]; Dr. V. A. McKusick; W. B. Saunders & Co.; Elsevier Sequoia, S.A.; Prof. Dr. Hiroshi Hirose in Japan; Interscience Publishers, Division of John Wiley & Sons; and Rutgers University Press.

The following were very helpful in locating, reproducing and/or obtaining permissions to reprint the photographs of well-known investigators: A special word of thanks to Lucinda Keister, Curator of the Photographic Collection, National Library of Medicine. She was extremely helpful and cooperative throughout the entire project. Col. Frank R. Camp, Jr. provided me with the photographs of L. Hirszfeld, F. Schiff, R. R. Race and Ruth Sanger, and I am very grateful to Dr. R. R. Race who took time to grant me permission to publish the last-mentioned of these pictures. Vergesellschaft Otto Spatz, München, kindly granted permission to reprint the photograph of Prof. Dr. O. Prokop, which originally appeared in *Muenchener Medizinische Wochenschrift.* Edizioni Minerva Medica, Torino, kindly granted permission for reprinting the photograph of Prof. L. Lattes which originally appeared in *Minerva Medicolegale.*

As the project was largely bibliographic, I could not have managed it without the assistance of many people associated with various libraries I used. Morton Goren and Lavonne Wienke of the LEAA Library were very helpful in obtaining interlibrary loan materials. I owe a special debt of thanks to Mr. Albert Berkowitz and his staff at the National Library of Medicine in Bethesda. They provided me with space to work, and a most congenial environment in which to do so, for more than two years time. The NLM staff treated me as a colleague during may stay in the library. I would particularly thank Doralee Agayoff, Jeanne Crosier, Edith Blair, Paula Strain, Maxine Henke, Peggy Beavers, Richard Mumford, John Broadwyn, Dr. Stephen Kim, Charlotte Kenton, and Dorothy Hanks. All of them went out of their way to assist me in

various ways. Mr. Stanley Lewicki and Mr. Alexander Draznowski helped me in the translations of languages of which I have no knowledge. Frank Liebersky was helpful in translating foreign languages, and in locating documents in the collection as well.

I want to thank the Chief of the Stack and Reader Division, U.S. Library of Congress, for providing me access to the collection.

A number of people provided me with references and translations to which I would otherwise not have had access. Col. Kiel in Texas sent me translations of several articles from Chinese. Mr. Walter Hoetzer of the International Association for Identification provided me with several articles from *Identification News*. Mr. Richard Tanton of West Palm Beach, FL, provided me with a translation of an article from Czechoslovakian. The libraries of Toulouse University in France, and of the Julius-Maximilian University in Germany, sent me copies of theses. Dr. Patricia Tippett kindly allowed me to consult her doctoral thesis when I was in London, and spent some time talking with me as well. Many colleagues and friends made me aware of references as the project progressed, and I want to thank all of them collectively.

My fellow visiting fellows at the National Institute of Justice were wonderful colleagues for two years, and they have since become good friends. It was indeed a pleasure to have been associated with them: Abraham Miller, Paul Wice, Joan Jacoby and Kent Miller.

I want to thank Gary Siglar and W. C. Stuver for supplying me with population data for U.S. cities prior to its publication elsewhere, and for granting me their permission to use the data in the tables of this book.

I am very grateful to Dr. Peter Shenkin of the Department of Mathematics, John Jay College of Criminal Justice of the City University of New York, for working with me as a co-author for section 1.2.8.

A number of people agreed to read various sections of the manuscript during its preparation, and they gave me many useful and constructive suggestions for improvements and modifications. In this respect, I want to thank Joanne Slaunwhite, Frances Gdowski, W. C. Stuver, Dr. John Thornton, Dr. Henry Lee, Dr. Charles Tumosa, Dr. Robert Shaler, Dr. Cornelius McWright, Dr. George Sensabaugh and Dr. Patrick Lincoln. Dr. Lincoln was exceptionally helpful with his comments on a number of major sections, and both he and Dr. Barbara Dodd have been extremely kind and helpful to me over the years in many ways. In expressing my thanks to these expert readers, I hasten to point out that the responsibility for any remaining deficiencies and shortcomings is mine alone.

I am very grateful to the National Institute of Justice for the support provided to me for this project. Without it, the project would certainly never have been undertaken. I am grateful to my Government project monitors, Winifred L. Reed and the late John O. Sullivan, for their patience and support over the several years. They not only tolerated my presence for two years, but have always been supportive and understanding about the many delays the project has experienced.

I reserve very special praise and gratitude for Maureen Swift and for Danice Gomien. Ms. Swift typed the entire manuscript, in some cases more than once. Ms. Gomien prepared all the figures and tables. Both of them navigated hundreds of pages of difficult material skillfully and well, somehow managing to make sense out of the many curious symbols and usages that are found in this field. There is no doubt that the work could not have been completed but for their continuing cooperation and perseverance.

West Haven, Connecticut
December, 1980

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SECTION 10. IDENTIFICATION OF SEMEN

SECTION 11. IDENTIFICATION OF SALIVA

SECTION 12. IDENTIFICATION OF URINE

SECTION 19. THE ABO AND SECRETOR SYSTEMS

FIGURES

TABLES

UNIT I
BACKGROUND MATERIAL

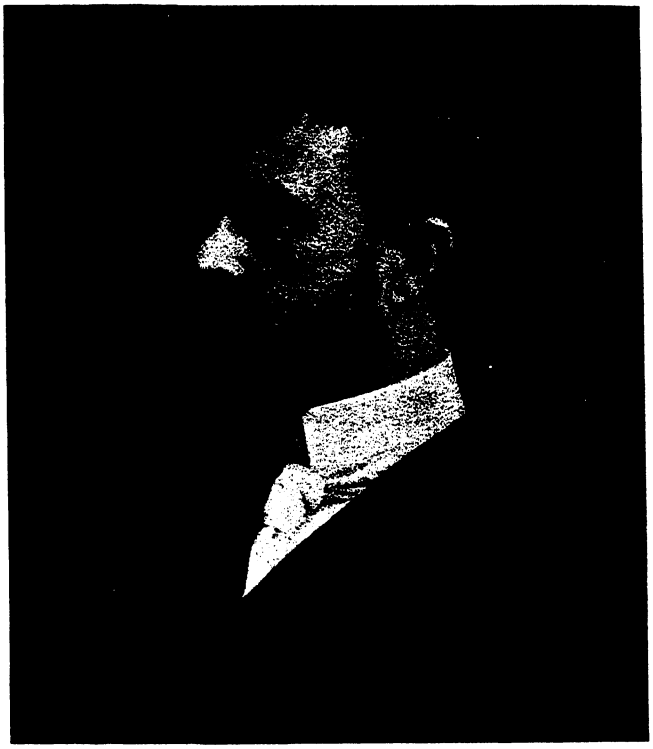
Forensic serology, if taken in its broadest sense to include all aspects of the medico-legal examination of blood and body fluids, draws its methods and the basis of its activities from a number of traditional intellectual disciplines. Foremost among these are biochemistry, immunology and genetics.

This being a sourcebook, an effort has been made to provide background on the various methods and concepts discussed as they come up. As a result, many aspects of biochemistry, genetics and immunology are discussed in connection with the various procedures and tests which are used in forensic practice. Such an approach necessarily presents much of the material of a particular unified body of knowledge in a rather disjoint manner. Unit I has been included in order to provide some background material in the unified disciplines in a more organized fashion. This treatment is brief and topical. No effort has been made to provide detailed documentation of the background material, for that approach would be the equivalent of preparing a sourcebook in one of these related fields. References are given, many of them to standard texts, review articles, and so forth. Just as detailed documentation was not in order for this unit, neither was complete treatment of the various disciplines. Topics for inclusion were selected on the basis of their relevance to forensic serological tests or methods. A few topics which are not particularly relevant to medico-legal practice, have been briefly discussed for the sake of completeness.

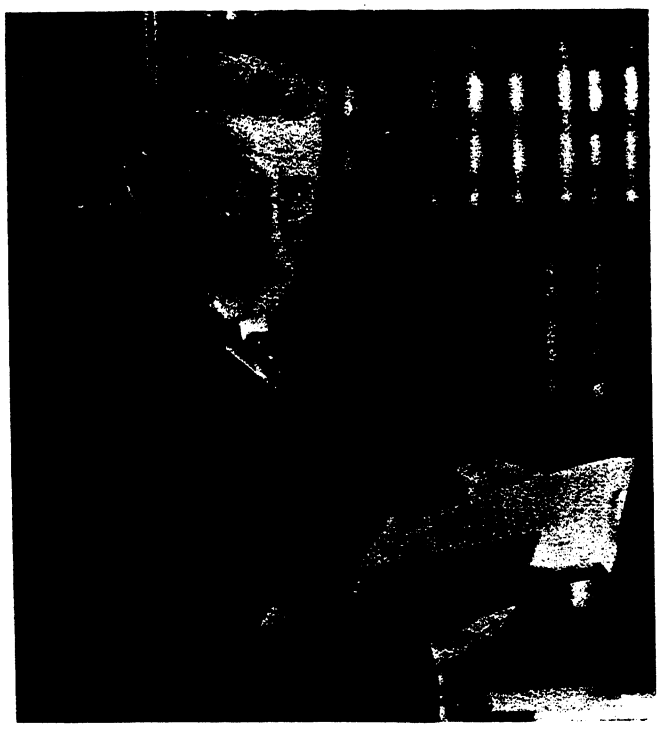
The material included in the background chapters is not regarded as being essential to the use of the succeeding units of the Sourcebook. Cross references have been included, where relevant, however, so that readers who may wish to consult the background material on a particular subject in some context will be able to locate it without undue difficulty.

Unit I consists of two sections, the first consisting of background material in biochemistry, immunology and genetics and the second consisting of background material of various selected methods which are widely used in forensic serology, immunology and biochemistry.

Gregor Mendel 1822–1884
Courtesy National Library of Medicine



Thomas Hunt Morgan 1866–1945
Courtesy National Library of Medicine



Paul Ehrlich 1854–1915
Courtesy National Library of Medicine

Jules Bordet 1870–1961
Courtesy National Library of Medicine

SECTION 1. BACKGROUND MATERIAL IN BIOCHEMISTRY, GENETICS AND IMMUNOLOGY

1.1 Biochemistry

1.1.1 Classes of biologically important organic compounds

In this section, the structures of the major classes of compounds of interest in biochemistry will be described. These are carbohydrates, lipids, amino acids and proteins and nucleotides and nucleic acids. Vitamins and coenzymes could comprise another category, but will not be discussed in great detail here. Some specialized types of molecules are discussed in other units, where germane. Porphyrins and hematin compounds, for instance, are discussed in section 4.1

1.1.1.1. Carbohydrates. The carbohydrates are polyhydroxy aldehydes and ketones. The aldehydes are called *aldoses,* and the simplest of these is glyceraldehyde, a three-carbon compound. There are four-, five-, six- and seven-carbon compounds as well. Dihydroxyacetone is the simplest of the ketones, called *ketoses,* and there are likewise important four-, five- and six-carbon compounds in the group. These simplest carbohydrates, called *monosaccharides,* often have one or more asymmetric carbon atoms, giving rise to 2^n stereoisomers, where n is the number of the asymmetric carbon atoms in the molecule. With aldohexoses, for example, where n = 4, there are 16 possible stereoisomers, 8 of which are mirror images of 8 others. The pairs of compounds which are mirror images are designated D- and L-, and the D-sugars may be thought of as being derived from D-glyceraldehyde in terms of absolute configuration. Similarly, the L-sugars may be thought of as being derived from L-glyceraldehyde. The D-series of three-, four-, five- and six-carbon sugars is shown in Fig. 1.1. Formulas used in this figure are called Fisher projection formulas, and can adequately represent the D- and L-forms of the compounds. In solution, however, D-glucose or any other sugar, may show different behavior with respect to the rotation of plane polarized light. This phenomenon comes about because the molecule exists in solution as a cyclic hemiacetal, for which two structures are possible. These are usually represented by so-called Haworth formulas, as shown for glucose in Fig. 1.2. Aldohexoses normally form six membered rings through a C_1-C_5 interaction, while ketoses, such as fructose, form five membered rings through a C_2-C_5 interaction. The C_1 which can have its hydroxyl group oriented above or below the plane of the ring is called the anomeric carbon. The two optically distinct forms, α- and β-, are called anomers. The six-membered rings are termed *pyranoses* in systematic nomenclature, while the five-membered rings are called *furanoses,* by analogy to pyran and furan.

More complicated carbohydrates are, for the most part, polymers of monosaccharides. They may be homopolymers, and can vary in size from disaccharides (two monomeric units) to very large molecules (polysaccharides) like starch or glycogen. Different bonding arrangements between monomeric units are possible. Bonds in which the C_1 of one unit is attached to the C_4 of the next are relatively common. These are called 1→4 linkages. If the C_1 (anomeric carbon) is in the α-configuration, the bond is called α1→4, while if it is in the β-configuration, the bond is β1→configuration, the bond is β1→4. Figures 1.3 and 1.4 show maltose, which has an α1→4 bond, and cellobiose, which has a β1→4. Both consist of glucose units. Long chains may be formed too, such as that of amylose (Fig. 11.1). Branched polymers such as amylopectin (Fig. 11.2) are possible, and are characterized by α1→6 bonds at the branch points.

1.1.1.2 Lipids. The lipids are perhaps the most structurally diverse class of compounds. They are considered together primarily on the basis of their solubility in organic solvents. As a class, lipids are nonpolar, hydrophobic materials. The simple lipids are neutral triglycerides and waxes. Triglycerides consist of glycerol, with three moles of esterified fatty acid. The fatty acids are simple aliphatic organic acids, derived from alkanes or alkenes. They may be saturated (having no double bonds in the chain) or unsaturated (one or more double bonds in the hydrocarbon chain). The most important fatty acids are the C_{12} through C_{20} saturated compounds, the C_{16} acid with a 9,10-double bond and the C_{18} acids with either one, two or three double bonds. The structure of a triglyceride is shown in Fig. 1.5. Waxes are esters in which both the acid and the alcohol moieties have long hydrocarbon chains.

The more complex lipids may be phosphoglycerides, sphingolipids, glycolipids, steroids or carotenoids. Phospholipids are derivatives of phosphatidic acid (Fig. 1.6). The phosphoric acid residue may be esterified again to various compounds, as indicated in Fig. 1.7. The most common compounds which are found as the "X" in Fig. 1.7 are glycerol, inositol, ethanolamine, choline and serine. The compounds thus formed are then called phosphatidyl glycerol, phosphatidyl inositol, and so forth. These are actually classes of compounds, since the fatty acid residues can vary.

Sphingolipids are those derived from sphingosine (Fig. 1.8). An example is sphingomyelin (Fig. 1.9) which occurs in nervous tissue. Glycolipids are compounds consisting of carbohydrate and lipid moieties in covalent linkage. They are very important in some animal cell membrane structures. Major classes of glycolipids are the cerebrosides and the gangliosides. Complete hydrolysis of cerebrosides yields sphingosine, one or two moles of fatty acid and a simple sugar, usually glucose or galactose. Gangliosides are

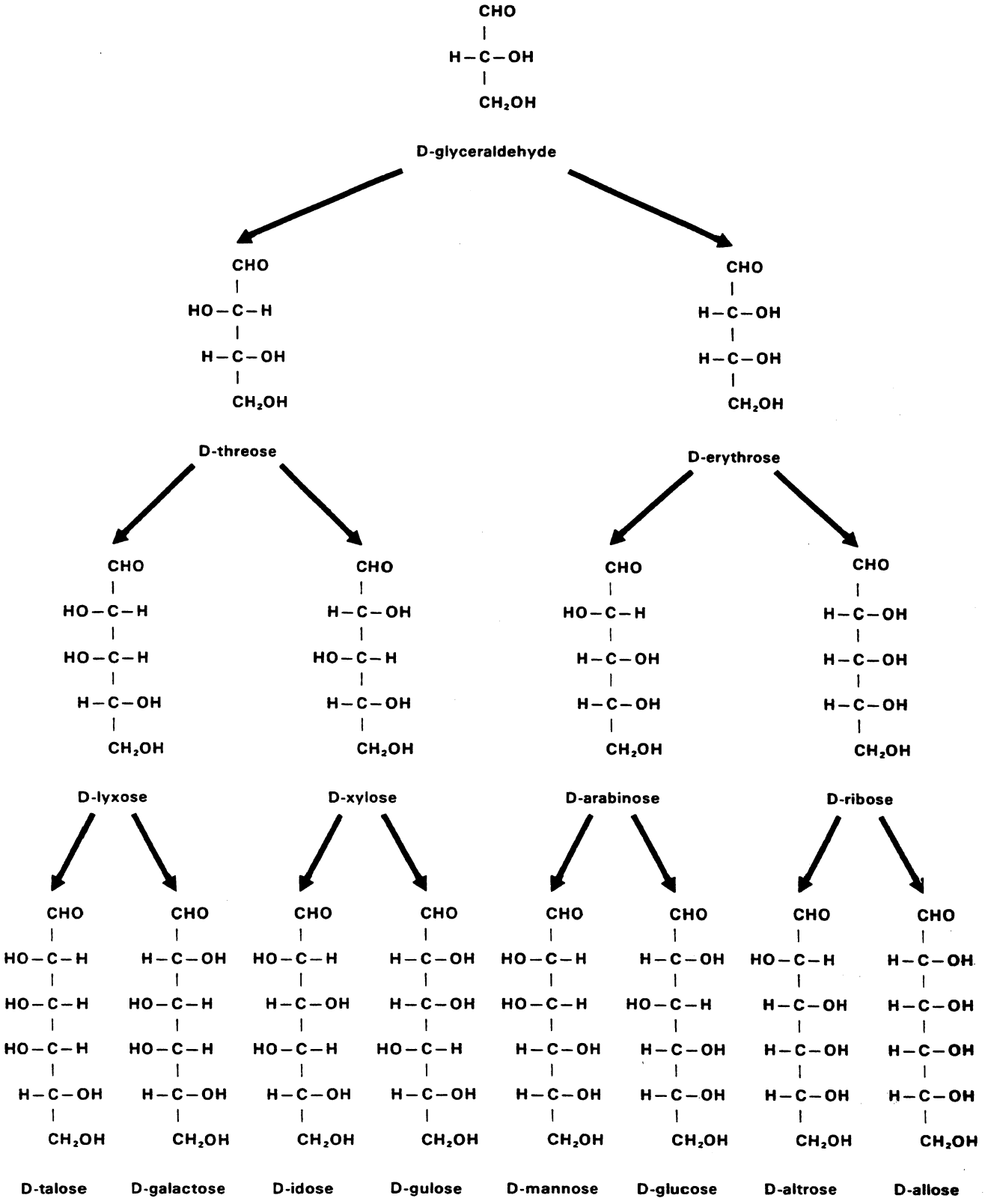
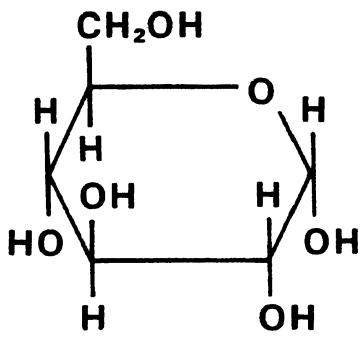
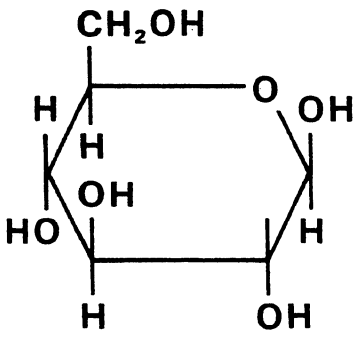


Figure 1.1 D - Series of aldoses



α-D-glucopyranose



β-D-glucopyranose

Figure 1.2 Haworth formulas for the anomers of glucose

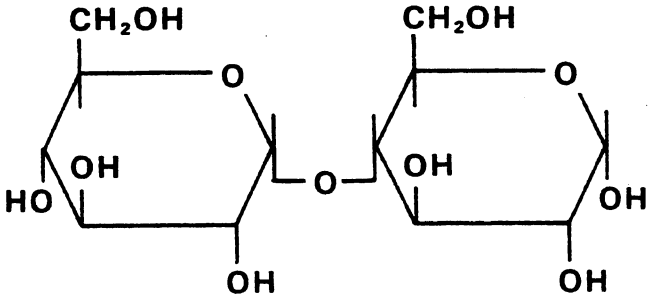


Figure 1.3 Maltose

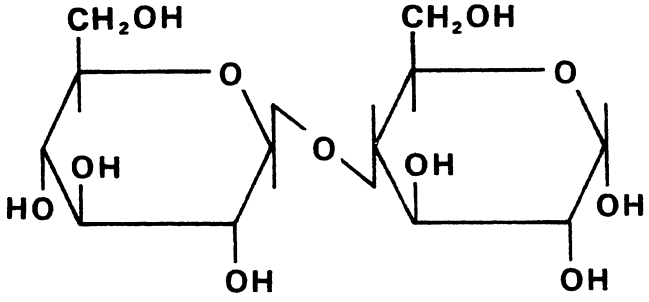


Figure 1.4 Cellobiose

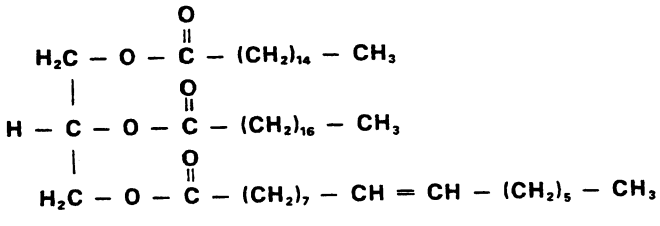


Figure 1.5 A Triglyceride

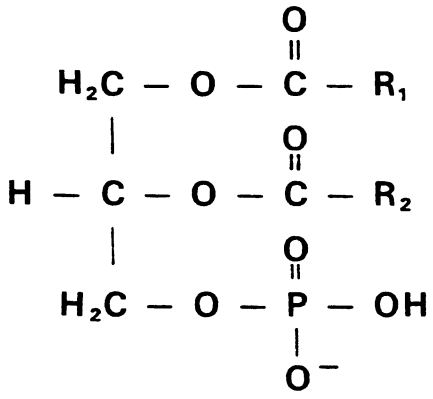


Figure 1.6 A phosphatic acid

derivatives of cerebrosides and contain a more complex carbohydrate moiety, such as N-acetylated amino hexoses or neuraminic acid. The latter is a modified C_9 hexose (Fig. 1.10 and is also called sialic acid.

Steroids and sterols comprise another class of lipids. They are characterized structurally by a fused hydrocarbon ring system known as perhydrocyclopentanophenanthrene (Fig. 1.11). The conventional ring numbering system is also given in the figure. The most abundant sterol in animals is cholesterol (Fig. 1.12). The major male and female sex hormones, testosterone (Fig. 1.13) and estradiol (Fig. 1.14) belong to this class of compounds as well.

Carotenoids may be of two kinds, carotenes and xanthophylls, the latter being most abundant in plants. Carotenes are members of a group of compounds called isoprenoids, because they are derived from isoprene

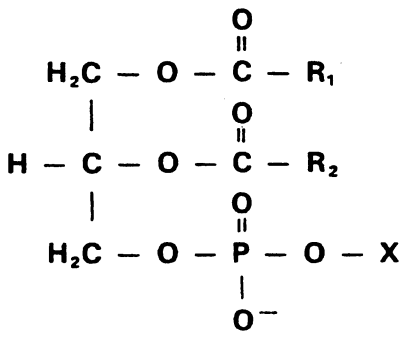


Figure 1.7 A phosphatidyl derivative

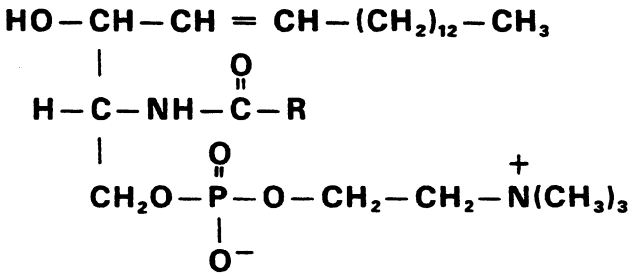


Figure 1.9 A sphingomyelin

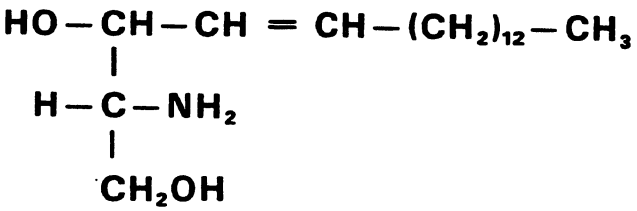


Figure 1.8 Sphingosine

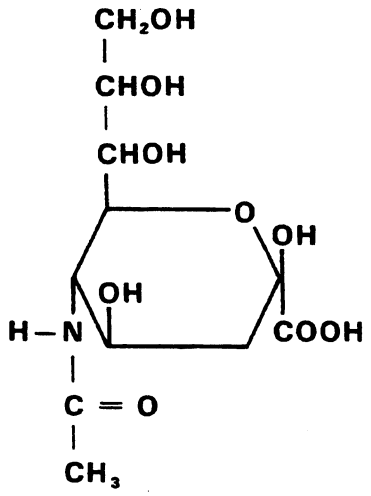


Figure 1.10 N-acetyl neuraminic acid

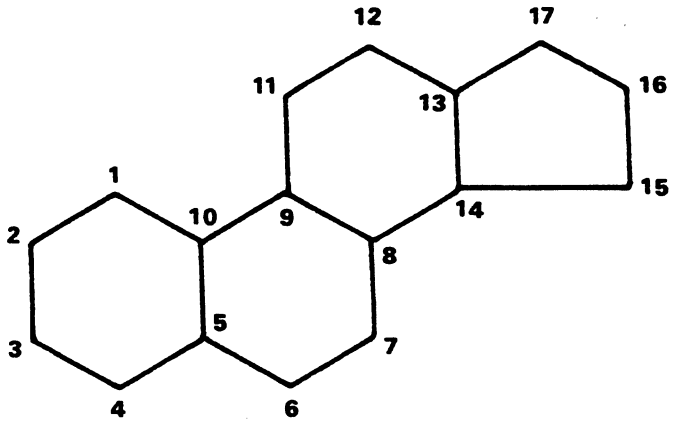


Figure 1.11 Perhydrocyclopentanophenanthrene

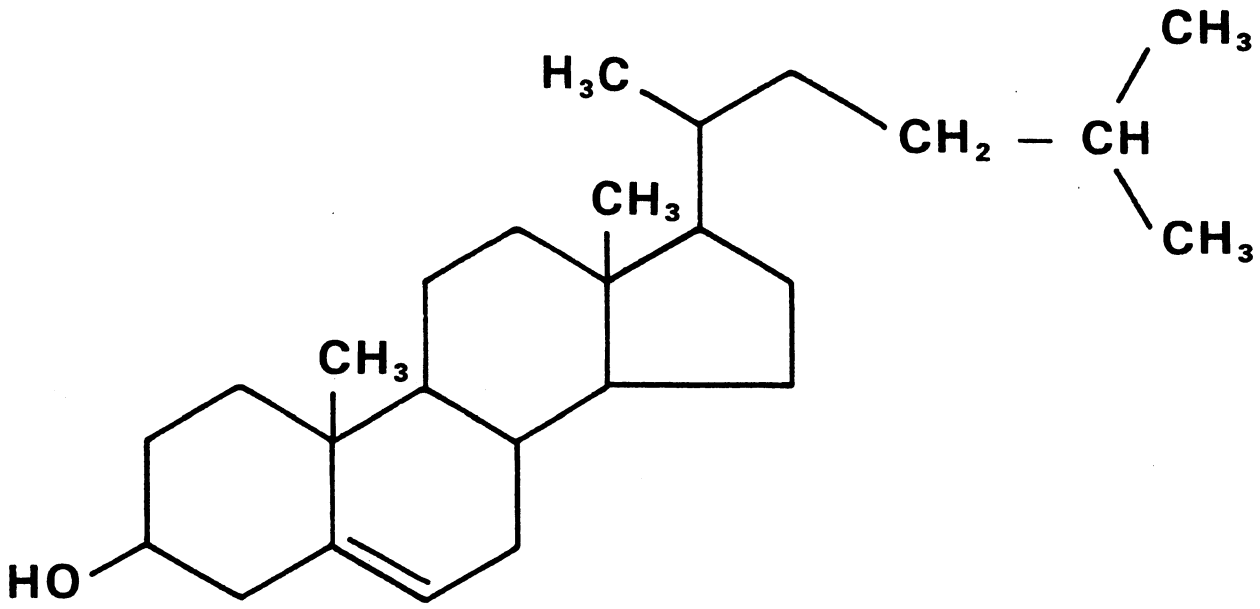
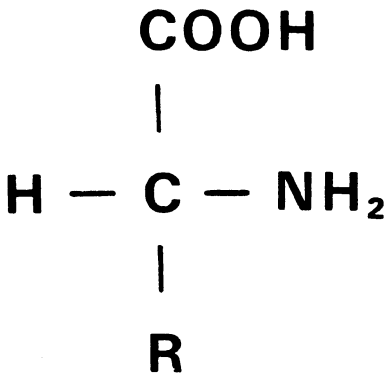


Figure 1.12 Cholesterol

(Fig. 1.15). β-carotene (Fig. 1.16) is a good example of a carotenoid. It is a C_{40} molecule, and oxidative cleavage at the double bond in the middle of the molecule yields two molecules of the alcohol form of Vitamin A.

1.1.1.3 Amino acids and proteins. Proteins are polymers of amino acids, linked together by peptide bonds. Peptide bonds link the $-COOH$ of one amino acid residue to the $-NH_2$ of the next one. Amino acid polymers may also be referred to as *polypeptides.* This term implies a smaller molecule than does *protein,* but may be used to distinguish distinct chains within a single molecule which are bonded together by other than peptide linkages. Smaller polypeptides are sometimes called oligopeptides.

A few more than 20 amino acids occur in proteins in nature. All have the basic structure



where the nature of R determines which amino acid is which. The C to which the H, the COOH and the NH_2 are bonded is called the α-carbon. If the R group contains a carbon chain, its carbons are sometimes designated by sequential Greek letters running from α. Thus, we may refer to the ϵ-NH_2 group of lysine, for example. Table 1.1. shows the naturally occurring amino acids. Each has a more or less standard abbreviation of its name, and these are given in the table as well. The abbreviations are used in writing in sequences of polypeptides, and for the sake of brevity generally. They appear frequently in the remainder of the text of the sourcebook. When R in the generalized amino acid formula above is anything other than hydrogen, the α-carbon is asymmetric and the amino acid exhibits optical isomerism, i.e., it exists in both D- and L-forms. Glycine is the only amino acid which does not show these isomers. The majority of amino acids which does not show these isomers. The majority of amino acids in nature are in the L-configuration. The amino acids, as can be seen in Table 1.1, may be neutral, acidic or basic, and may contain sulfur, hydroxyl groups or aromatic rings. Since amino acids contain a carboxyl and an amino group and in many cases other ionizable groups, their ionic form depends upon the pH. This behavior is illustrated in Fig. 1.17. Each ionizable group is

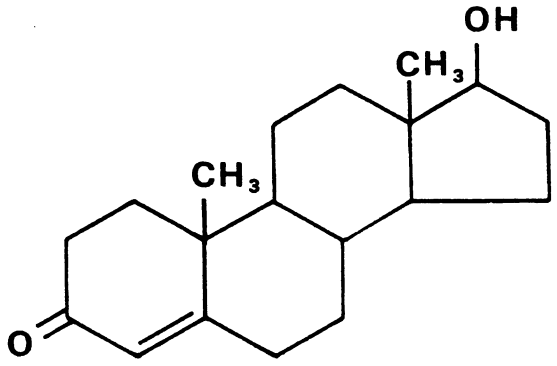


Figure 1.13 Testosterone

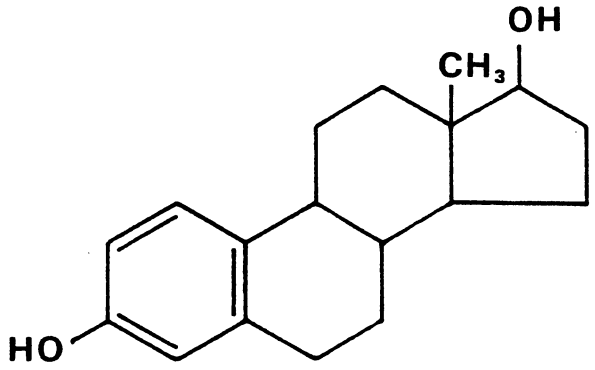


Figure 1.14 Estradiol

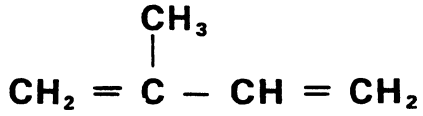


Figure 1.15 Isoprene

characterized by a particular pK_a, the pH at which the protonated and unprotonated forms are present in equal concentrations.

Polymerization of amino acids by means of peptide bonds involves both the α-amino and α-carboxyl group of every residue in the chain except for the ones at the ends. At one end, the amino acid will have a free amino group and the amino acid at the other end will have a free carboxyl group. By convention, the former is written to the left, and is called the N-terminal or amino-terminal end. The latter is called the C-terminal or carboxy-terminal end. Protein structure will be discussed in somewhat more detail in Section 1.1.2.

1.1.1.4 Nucleotides and nucleic acids. These compounds are of interest primarily because of the role they play in biochemical genetics. Their structure is discussed in that context in Section 1.2.2.3.

1.1.2 Proteins

1.1.2.1 Protein structure. Proteins have several levels of structure, which come about because of the large size of these molecules. The *primary* structure is the amino acid sequence. There can be interchain or intrachain disulfide bonds (or disulfide bridges), in which two cysteine residues have their sulfur atoms bonded to one another. Formation of a disulfide bond from the Cys residues is oxidative, in that two hydrogens are removed. In longer protein chains, another level of structure is generated by intrachain hydrogen bonding among those atoms involved in the peptide linkages, and is known as *secondary* structure. The secondary structure may consist of helical or pleated sheet configurations. Finally, proteins generally fold up into more or less complicated three-dimensional structures, and this folded arrangement is known as the *tertiary structure.* The special configuration of every protein is unique. A number of forces are responsible for the maintenance of the three-dimensional conformation of proteins. These forces involve the side chains of the amino acids which constitute the protein. Electrostatic interactions may occur between oppositely charged groups, such as the ϵ-NH_3^+ group of lysine and the side chain $-COO^-$ group of glutamic acid. These interactions are sometimes called "salt bridges". Hydrogen bonding not involving atoms involved in the peptide linkage may occur, such as between the $-OH$ of tyrosine and a carboxyl group of Glu or Asp. Peptide bond hydrogen bonding between different segments of chains of Ala, Val, Leu, Ileu, and Phe are important as well. The sum of all these forces is responsible for maintaining the conformation. There are obviously a large number of different combinations of these interactions which are possible in a particular protein, each of which would lead to a somewhat different conformation. The protein molecule generally assumes the most stable conformation available, maximizing interactions which lend structural stability. In aqueous solution, protein conformation is influenced by the stabilizing effect of having the polar side chains exposed to the aqueous environment, and the nonpolar side chains away from water and more inside the core of the molecule. Under a given set of conditions, a protein will assume a particular conformation, its most stable under those conditions. This structure is determined by the particular amino acid sequence. A specified primary structure gives rise to a unique three-dimensional folded structure. The sum of all the side chain interactions leading to the tertiary structure is a unique function of the sequence. Because of the complex way in which protein molecules do fold up, they assume conformational shapes which have a kind of "inside" and "outside" to them. It is proper to speak

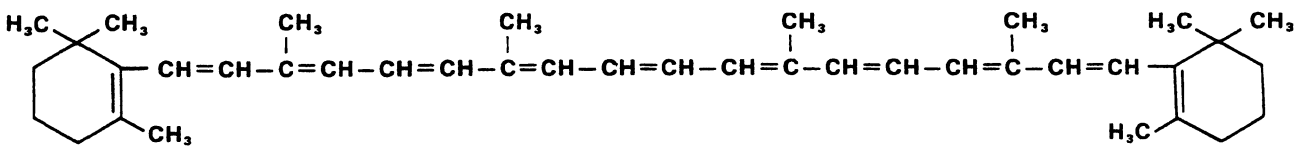
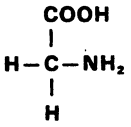
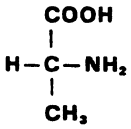


Figure 1.16 β-Carotene

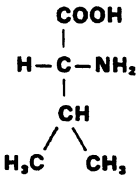
Table 1.1 The Amino Acids



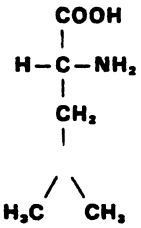
Glycine
(Gly)



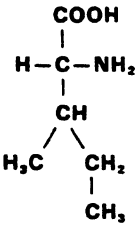
Alanine
(Ala)



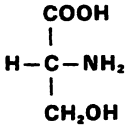
Valine
(Val)



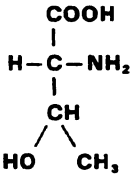
Leucine
(Leu)



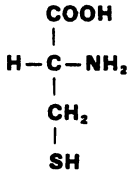
Isoleucine
(Ile or Ileu)



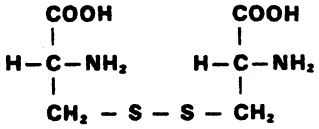
Serine
(Ser)



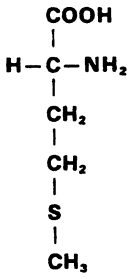
Threonine
(Thr)



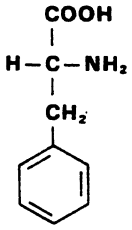
Cysteine
(Cys)



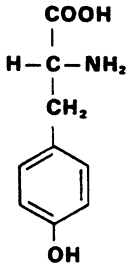
Cystine
(Cy—S—S—Cy)



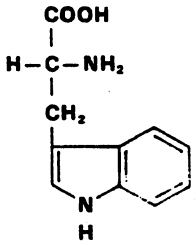
Methionine
(Met)



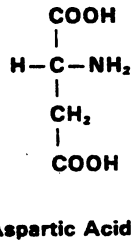
Phenylalanine
(Phe)



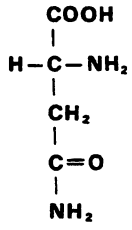
Tyrosine
(Tyr)



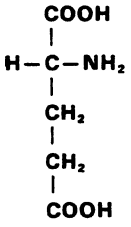
Tryptophan
(Try or Trp)



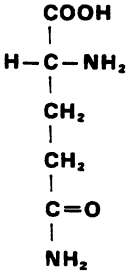
Aspartic Acid
(Asp)



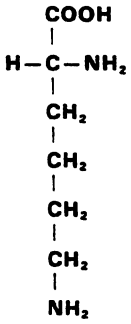
Asparagine
(Asn or Asp-NH_2)



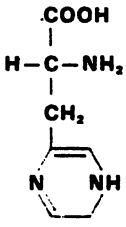
Glutamic Acid
(Glu)



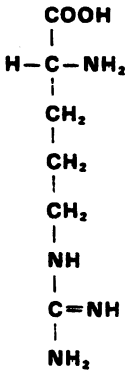
Glutamine
(Gln or Glu-NH_2)



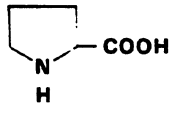
Lysine
(Lys)



Histidine
(His)



Arginine
(Arg)



Proline
(Pro)

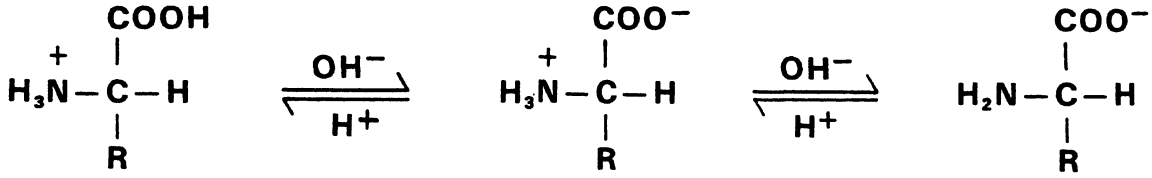


Figure 1.17 Ionic Forms of an Amino Acid

of the "surface of the molecule" with proteins. A fourth level of structure is possible for proteins which consist of two or more distinct polypeptide chain subunits. Called *quaternary* structure, this level refers to the association of two or more individually folded polypeptide chain subunits into aggregates, as the result of non-covalent forces. The number of subunits in such a protein can vary, some proteins consisting of 10 or 12 subunits. Hemoglobin is a good example of a subunit protein. Normal Hemoglobin A consists of four chains, two called α-chains and the other two called β-chains. Each chain complexes a heme group, and the intact molecule is a tetramer, often denoted $\alpha_2\beta_2$, and containing four heme groups (see in section 38).

It should be noted that complete structures are known for very few proteins. Methods of sequence analysis have improved to the extent that sequences are known for a fair number of proteins. It is more difficult and laborious, however, to obtain information about the secondary and tertiary structures. Detailed three-dimensional structures are known for very few proteins. These structures are determined using x-ray crystallography.

Any level of structural analysis of a protein requires that it first be obtained in pure form. Purification of proteins is often fairly involved, yields are often poor, and techniques cannot always be found to purify a particular protein to the desired degree. There is also the nagging problem of the extent to which the purification procedure has modified the molecule with respect to its *in vivo* condition. Proteins which are associated with carbohydrates or lipids in some complicated way, or proteins which are constituents of membranes can present extremely difficult purification problems.

1.1.2.2 Protein purification. A variety of techniques are used for purification of proteins. Cell free extracts are prepared first. These preparations are sometimes called "crude extracts." Any method which gets the protein out of the cell and still retains the biological activity of the protein is suitable for preparations of crude extracts. Sometimes, particular cells or organelles may be prepared, and the crude extract made from them. Thus, one might make beef heart mitochondria first, or a preparation of washed red blood cells. Depending upon the cell or preparation in question, sonic oscillations, osmotic shock, treatment with detergents, or a variety of other techniques could be used to get the protein away from the cells or organelles.

Protein solubility depends on the salt concentration. Different proteins differ in this respect, and variation in salt concentration can be used to precipitate proteins from solution. By collecting the precipitates, pouring off the material still in solution, and then making the solution still more concentrated in salt, a series of fractions can be obtained. Ammonium sulfate is commonly used as the salt because of its high solubility. Certain proteins "salt out" at particular ammonium sulfate concentrations, and the fraction containing the protein of interest can be collected, free of all the proteins that are soluble, and subjected to further purification.

Column chromatography is another popular technique. Two major types of techniques are used, and they differ in principle. In ion exchange chromatography, a support matrix is covalently linked with side chain groups which have either positive or negative charges. Depending upon the acidity (pK_a) of the ionizable group, the over-all charge on the column may depend on the pH. Ion exchange resins are called "cation exchangers" or "anion exchangers" depending on whether their fixed charge is positive or negative. Proteins have net charge at all pH except for their isoelectric point (pI), and can be attached to ion exchange resins. The resins are then eluted with increasing concentrations of salt, which competes for the resin exchanger binding sites, and displaces the different proteins according to their net charge and resultant binding strength. Fractions are collected and searched for the protein of interest. A number of resins are in common use, including various Dowex resins and diethylaminoethyl (DEAE) cellulose or sephadex, carboxy-methyl (CM) cellulose or sephadex and a number of others. Walton (1975a and 1975b) has discussed ion exchange chromatography in detail, and Peterson (1970) has treated cellulosic ion exchangers in detail.

In so-called gel filtration chromatography, or molecular sieving, separation of the proteins is effected on the basis of size. Sephadex (a product of Pharmacia Fine Chemicals in Sweden) is in very wide use for this purpose. Sephadexes are dextrans, cross-linked to varying degrees in such a way as to create pores within the matrix. There are a number of grades of sephadex, having different pore sizes. Cross linked polyacrylamide gels (Bio Gel P-series, Bio-Rad Laboratories), and agarose gels (Bio Gel A-series, Bio-Rad Laboratories and Sepharoses from Pharmacia Fine Chemicals) are also in

use for molecular sieve chromatography. Molecules passing through the matrix, which are small enough to get into the pores, do so, and their passage is thus retarded in comparison to those molecules which are "excluded." Sephadexes and other gels are calibrated by the manufacturer, usually with globular proteins or polysaccharides. Proteins which have peculiar shapes can behave differently on the gels than would be expected on the basis of their molecular weight. For example, a column which contains a gel support calibrated to exclude molecules of 135,000–150,000 in molecular weight will do so if they are globular. But if a molecule were rod-shaped, interaction with the gel pores could occur in spite of the fact that its molecular weight might be greater than 150,000. Additional detailed information on molecular exclusion chromatography may be found in Determann and Brewer (1975) and Fischer (1969).

Ultracentrifugation is widely employed as a purification technique in biochemical studies. Ultracentrifuges produce very intense gravitational fields. Commercial instruments are generally capable of speeds up to 70,000 rpm, depending upon the rotor, and of generating gravitational fields of up to 500,000 × g. Ultracentrifugation techniques may be either sedimentation or density gradient methods. Sedimentation methods are used primarily for characterization and MW studies (see section 1.1.2.4). In sedimentation ultracentrifugation, the medium is of constant density. Density gradient methods are more applicable to isolation and purification. Solutes migrate in these systems in a medium of gradually changing density. In velocity density gradient centrifugation, a solvent medium with a pre-formed linear density gradient is employed, the solute being layered onto the top. Solute materials of differing densities migrate to discrete zones, and are thus separated. In equilibrium density gradient centrifugation, the solute is uniformly mixed with a dense inorganic salt, such as cesium chloride, and a linear density gradient is self-generated under the influence of the gravitational field. Solutes of varying densities will collect in zones corresponding to their own densities and will thus be separated. Fractions from these gradients are readily collected by puncturing the bottom of the centrifuge tube after the run, and collecting fractions in separate tubes. Analytical ultracentrifugation, which is often applied to characterization studies and MW determinations, is briefly discussed in section 1.1.2.4.

At each step of the purification procedure, the material must be assayed for the activity one is trying to purify. Only in this way can it be determined whether a step has worked. One must define units of activity for the protein in some quantitative way in order to have a suitable assay. The assay is based on the protein's function, i.e., whether it is an enzyme, an antibody, etc. After each step of the purification, total protein is determined as well as total units of activity present. The number of units of activity per mg protein is called the *specific activity*. As purification proceeds, the total amount of protein should be decreasing, because unwanted proteins are being purified out, and the preparation should be growing richer in the protein of interest. Specific activity should therefore increase with each step. If it does not do so, the purification step is probably not a good one for that protein.

1.1.2.3 Estimation or protein. There are a number of protein assays in use. The biuret method is based on the fact that compounds containing two or more peptide bonds form a deep blue-purple color with cupric salts in alkaline solution. The reagent is prepared from cupric sulfate, sodium potassium tartrate and NaOH. Optical density is determined in the 540–560 nm region. The method was devised for serum proteins (Robinson & Hogden, 1940; Weichselbaum, 1946; Gornall *et al.*, 1949), but has been modified by many workers and is applicable to protein determination generally (Layne, 1957).

Protein estimation by means of the Folin-Ciocalteu reagent is also called the "Lowry method" (Lowry *et al.* 1951). The color obtained is the result of the biuret reaction, and the reduction of phosphomolybdic-phosphotungstic acid reagent by tyrosine and tryptophane in the protein (see section 10.3.4 for use of Folin-Ciocalteu reagent for estimating phenol). The method is discussed by Layne (1957).

Most proteins absorb in the ultraviolet region at around 280 nm because of the presence of the aromatic amino acids. Nucleic acids, frequent contaminants of protein preparations, absorb maximally at about 260 nm. Warburg and Christian (1942) first described a technique for estimating protein concentration by 280 nm absorption, and putting in a correction for the nucleic acid absorption. In practice, the absorbancy is determined at both 260 and 280 nm and appropriate calculations carried out. Tables, such as the one presented by Layne (1957), simplify the calculations.

Bradford (1976) described a sensitive and rapid protein assay, based on the binding of the protein to Coomassie Brilliant Blue G 250 (see Table 5.3). The assay was usable for quantities of protein varying from 1 to 100 μg. The A_{595} was linear with protein concentration to about 50 μg and only slightly nonlinear at higher amounts. Only detergents in relatively high concentrations interfered with the assay.

All the methods have advantages as well as drawbacks. The Lowry method is somewhat more sensitive than the classical Biuret, although the latter's sensitivity can be increased considerably by modifying the technique and scaling down the volumes employed. The Lowry and 280/260 methods depend upon the aromatic amino acid content of the protein, which is not of course always the same. The protein concentration of a protein which has not been purified is usually obtained by reference to a standard curve. The standard curve has been constructed using some pure protein. Reference to the standard curve, therefore, does not give the absolute protein concentration of a protein other than the one used to make the curve, but all measurements carried out with reference to the same standard curve will be internally consistent, relative to one another. Standard curves with the Lowry method often exhibit non-linearity. The Bradford (1976) dye binding assay is not affected by a number of chemicals which interfere with the Lowry assay, and the color is considerably more stable over the course of

time (maximal development in about 2 min and stable for an hour).

1.1.2.4 Criteria of purity and MW determination. It is always difficult to know when a protein preparation is "pure." As a rule, the preparation is subjected to several separation techniques, such as disc electrophoresis or an ion exchange column, and a single band or peak of activity suggests purity. The analytical ultracentrifuge is sometimes used to check preparations for purity, as well as for determination of the MW. The analytical ultracentrifuge can be used to determine a quantity called the sedimentation coefficient (or s), which is the velocity of sedimentation divided by the centrifugal field strength ($\omega^2 r$), where r is the distance from the center of rotation and ω is the angular velocity of the rotor. The units of s are seconds, and since numbers of the order of 10^{-13} sec are commonly encountered, the quantity 1×10^{-13} is defined as 1 Svedberg unit, denoted S. The MW is related to s by different equations which can be derived from the theory of ultracentrifugation of macromolecules (see van Holde, 1971).

MW can also be determined by molecular sieving techniques. Sephadex can be used for this purpose. A column of sephadex is prepared for the approximate MW range in which one expects the protein to lie. The column can then be "calibrated" with proteins of known MW. The MW of the unknown protein can then be estimated by how it behaves on the column. A similar thing can be done with polyacrylamide gels, since these are synthetic and of controllable pore size (see section 2). If a protein is pure enough, and can be subjected to a complete amino acid analysis, the MW can be calculated from the composition.

1.1.3 Enzymes

1.1.3.1 Introduction. Enzymes are the cellular protein catalysts. Most biological reactions are enzyme-catalyzed, and the intrinsic rates of the reactions *in vivo* are sufficiently slow that the presence of the enzyme is the factor that allows the reaction to occur at a significant rate. This is one of the ways in which genetic control is exercised over specific reactions (see section 1.2.2). Most enzymes exhibit a high degree of specificity, and their activity is subject to regulation in the cell at a number of different levels. Enzymes which exhibit multiple molecular forms, and are polymorphic, are an important class of genetic markers in blood and body fluids, and therefore, of great interest in legal medicine. The first enzyme to be isolated and purified was urease. This work was carried out by Dr. Sumner in 1926.

1.1.3.2 Enzyme Nomenclature. For years, enzyme nomenclature grew up on an *ad hoc* basis. Names were usually, but not always descriptive, and most of them ended in *-ase.* Some trivial names give no information about the enzyme whatever, e.g. pepsin, trypsin. Eventually, the need for a systematic nomenclature became clear, and an international Commission on Enzymes was set up in 1955 in consultation with the International Union of Pure and Applied Chemistry (IUPAC). The final recommendations of the working group were adopted in 1964 as the *Recommendations (1964) of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes.* A numerical system was devised, very like the system which has been used to number the sections and subsections of this book. Six major categories of enzymes were established: 1. Oxidoreductases 2. Transferases 3. Hydrolases 4. Lyases 5. Isomerases 6. Ligases. Subsets of oxidoreductases are denoted 1.1, 1.2, 1.3, etc., of transferases, 2.1, 2.2, etc., and so forth. Subsets of 1.1 are denoted 1.1.1, 1.1.2, and so on, and finally, subsets of 1.1.1 would be denoted 1.1.1.1, 1.1.1.2, etc. At the level where there are four numbers, a specific enzyme is denoted, and the result is that all the enzymes which have been described have a four-number systematic designation. In addition, a number of rules on the systematic nomenclature were issued. The original document should be consulted for the detailed rules. It was recognized that trivial names would still be in use, and that it is not always necessary to use the systematic name. The Commission recommended the preferred trivial name in each case. The systematic designation may be illustrated by means of an example: the systematic name of the well-known polymorphic erythrocyte enzyme phosphoglucomutase (PGM) is α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase. PGM has the designation 2.7.5.1. The 2 denotes transferases; 2.7 refers to transferases which transfer phosphate-containing groups; 2.7.5 are phosphotransferases in which donors are regenerated (catalyzing apparent intramolecular transfers); and 2.7.5.1 is phosphoglucomutase. It is now common in the literature to note the four-number designation of an enzyme. The information is sometimes given parenthetically, sometimes accompanied by the systematic name, and generally appears in the title of the paper or at the point of first reference to the enzyme. One normally prefaces the four-number designation with the letters "EC," for "enzyme commission." Thus, one might write "phosphoglucomutase (PGM; EC 2.7.5.1)." Enzymes discussed in this book are identified by their EC number and their systematic names are given at the point of first reference.

1.1.3.3. Kinetics of enzyme catalyzed reactions Kinetics is concerned with reaction rates. Enzyme kinetics is a complex subject, and is not treated in depth in this section. Simple Michaelis-Menten kinetics is presented because the kinetic parameters derived from this kinetic treatment are often used in describing enzymes, and the terminology comes up in subsequent sections.

Any reaction is characterized at the molecular level by the number of molecules that must interact prior to product formation. This parameter, the molecularity, can be determined only from a knowledge of the reaction mechanism. The dependence of reaction rate on the concentrations of reactants is specified by the kinetic *order* of the reaction. This parameter is determined experimentally by fitting data to rate equations. Suppose a reaction in which A→B. The rate of the reaction, or velocity (v) is equivalent to the rate of disappearance of reactant and to the rate of appearance of product:

$$v = -\frac{d[A]}{dt} = +\frac{d[B]}{dt} = k[A]^n$$

where [ ] represents concentration, t is time, and k is the rate constant. The rate constant is the same for a given reaction under a specified set of conditions. The exponent, n, corresponds to the kinetic order of the reaction. If the rate of the reaction is determined at different values of [A], n can be determined. Then k can be determined as well. If n = O, v = k, the rate is independent of reactant concentration, and the reaction is said to be *zero order.* If n = 1, v = k[A], the rate is directly proportional to [A], and the reaction is said to be *first order.*

Michaelis and Menten studied the kinetics of the enzyme catalyzed hydrolysis of sucrose and, by measuring the initial velocity under different conditions, found that the rate was proportional to the enzyme concentration if substrate was held constant. If enzyme was held constant, the relationship between initial velocity and substrate concentration was found to be hyperbolic. Figure 1.18 illustrates this behavior, which has been found to characterize many enzyme-catalyzed reactions. For various reasons, the rates of enzyme-catalyzed reactions can vary over the course of time, and for this reason it is important to use initial velocities in doing kinetic studies. The Enzyme Commission recommended that enzyme assays be based on the measurement of initial velocities.

In simple terms, an enzyme-catalyzed reaction may be represented:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P$$

where E represents enzyme, S represents substrate, ES is the enzyme-substrate complex, P is product, and the k's are the rate constants. The Michaelis-Menten treatment is based on a rapidly attained steady state, in which the rates of formation and disappearance of ES are equal, i.e.,

$$\frac{d[ES]}{dt} = -\frac{d[ES]}{dt}$$

$$\text{Since } \frac{d[ES]}{dt} = k_1[E][S] \text{ and } -\frac{d[ES]}{dt} = k_2[ES] + k_3[ES]$$

$$\text{then } k_1[E][S] = k_2[ES] + k_3[ES]$$

Assuming that the rate-limiting step is the formation of ES, the initial velocity (v_o) is proportional to [ES] and the maximum v_o (denoted V_{max}) will be proportional to the total enzyme concentration [E_t] because V_{max} occurs when all E is complexed in ES. The above equation may be rewritten:

$$[ES] = \frac{k_2 + k_3}{k_1}[E][S]$$

Since the proportionality constants relating v_o to [ES] and V_{max} to [E_t] must be the same,

$$\frac{v_o}{V_{max}} = \frac{[ES]}{[E_t]}$$

Solving for ES and substituting in the previous equation yields

$$\frac{v_o}{V_{max}} = \frac{k_1}{k_2 + k_3} = \frac{[E][S]}{[E_t]}$$

The term $\frac{k_2 + k_3}{k_1}$ is defined as K_m, the Michaelis constant. Thus,

$$\frac{v_o}{V_{max}} = \frac{1}{K_m}\frac{[E][S]}{[E_t]} = \frac{1}{K_m}\frac{([E_t] - [ES])[S]}{[E_t]}$$

and

$$v_o = \frac{V_{max}}{K_m}\frac{[E_t][S]}{[E_t]} - \frac{[ES][S]}{[E_t]} = \frac{V_{max}}{K_m}\left([S] - \frac{[ES][S]}{[E_t]}\right)$$

Substituting $[ES] = \frac{v_o}{V_{max}}[E_t]$ for [ES], rearranging terms,

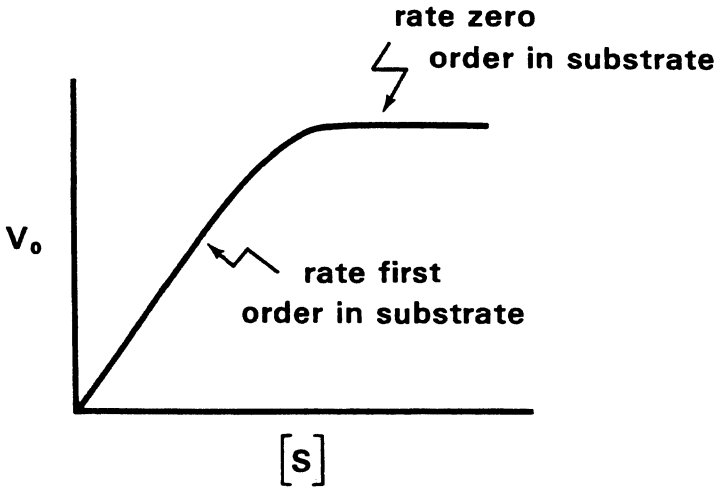


Figure 1.18 Initial Velocity of Enzyme Catalyzed Reaction as a Function of Substrate Concentration

and solving for v_o gives the classical form of the Michaelis Menten equation:

$$v_o = \frac{V_{max}[S]}{K_m + [S]}$$

Two points about this representation are worthy of note. First, if the equation is solved for [S] when $v_o = \frac{1}{2}V_{max}$, it will be found that $[S] = K_m$. In other words, K_m is the substrate concentration when the initial velocity is one half its maximal value. Second, K_m is $\frac{k_2 + k_3}{k_1}$. If k_2 is very much greater than k_3, then K_m will be approximated by k_2/k_1, and will be a measure of the affinity of E for S. Since the ES formation rate constant is in the denominator, larger K_m values are interpreted as being representative of lower affinities of E for S.

K_m may be evaluated in a number of ways. The essential data consists of a series of measurements of initial velocity at various [S] at constant [E]. The Michaelis Menten equation can be rearranged in various ways to give equations of the $y = mx + b$ form, and K_m can be calculated from plotted data. For example, a plot of $1/v_o$ against $1/S$ yields a straight line with a slope of $\frac{K_m}{V_{max}}$ and a y-intercept of $-\frac{1}{K_m}$, and is called a Lineweaver-Burk plot. A plot of v_o/S against v_o also yields a straight line, but with a slope of $-\frac{1}{K_m}$ and a y-intercept of $\frac{V_{max}}{K_m}$, and this is called an Eadie-Hofstee plot. It should perhaps be noted that K_m has units of concentration.

Inhibitors are very important in studying enzymes. Information about the enzyme's catalytic action can be obtained using different inhibitors. In many cases, kinetic studies with inhibitors can be informative. Two important kinds of inhibition are called *competitive* and *non-competitive.* Competitive inhibitors act by competing with the substrate for the binding site on the enzyme. Non-competitive inhibitors may interact with the enzyme at the substrate binding site, or may bind elsewhere to the enzyme, or may bind the ES complex. The kinetic characteristics of the two types of inhibition are different. Equations can be derived from Michaelis-Menten considerations, taking inhibition into account. These can be arranged to yield straight line forms which indicate the changes expected in the presence of different types of inhibitors. Competitive inhibitors increase K_m and leave V_{max} unchanged, while with non-competitive inhibitors, V_{max} is altered but K_m remains unchanged. A Lineweaver-Burk plot illustrating this behavior is shown in Figure 1.19. It may be noted from the figure that the change

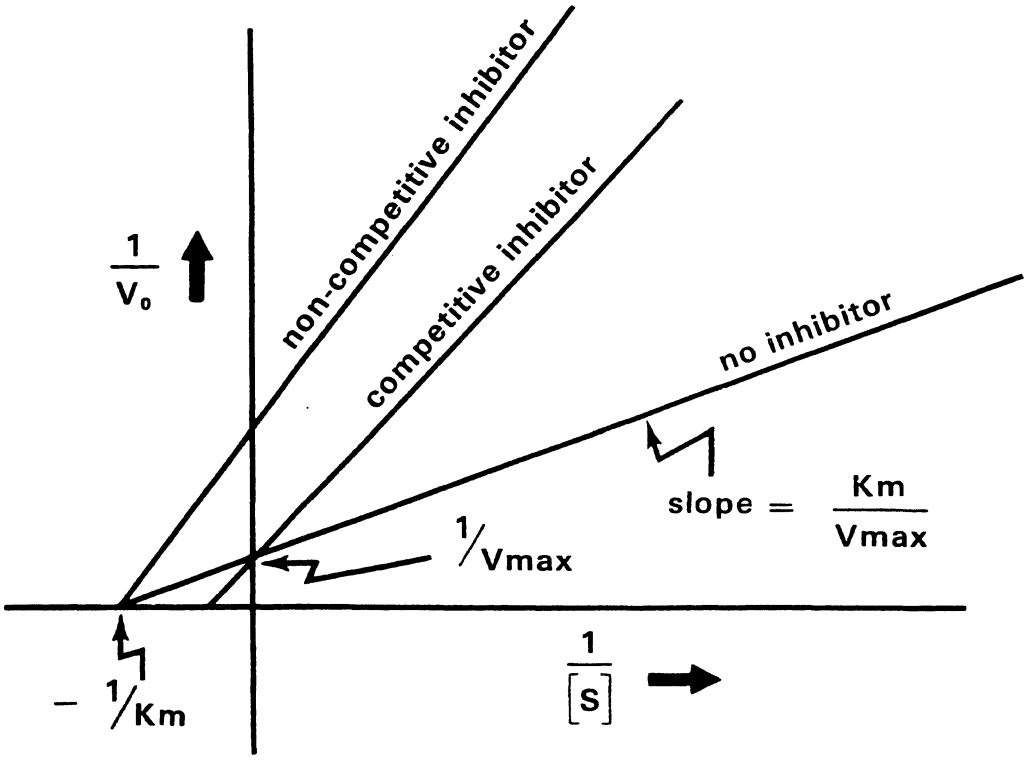


Figure 1.19 Lineweaver-Burk Plot Showing Hypothetical Curves for Enzyme Catalyzed Reaction and Behavior With Competitive and Non-competitive Inhibitors

in K_m or in V_{max} brought about by the inhibitors is equivalent to

$$1 + \frac{[I]}{K_I}$$

where [I] represents inhibitor concentration and K_I is a dissociation constant for the reaction of enzyme with inhibitor: $E + I \rightleftharpoons EI$. Mahler and Cordes (1971) may be consulted for a more detailed treatment of enzyme kinetics.

1.1.3.4 Enzyme-catalyzed reactions and cofactors. The essential feature of enzyme-catalyzed reactions is their specificity. In some cases, the specificity is absolute, but in others, it is broader. Specificity is based on the fact that an enzyme contains a particular constellation of a few amino acid residues in the structure, ordered in space in a particular way. This "active site" recognizes the substrate molecule. Originally, it was thought that the special configuration of the active protein was rigid, and that substrate recognition and binding represented a kind of "lock and key" affair. This explanation suffices well in cases where substrate specificity is absolute, but does not easily explain the cases in which a number of related substrates can be acted upon. Koshland's "induced fit theory" allows for more flexibility in the active site. According to this idea, approach of the substrate may induce subtle conformational changes in the protein, yielding proper orientation of the active site's residues for binding and catalysis.

Most enzymes exhibit maximal catalytic activity over relatively narrow ranges of pH. It is necessary, therefore, to buffer solutions used for enzyme work to pH values near the pH optimum. Extremes of pH can denature enzymes. Similarly, many enzymes do not survive extremes of temperature very well. The behavior of enzymes under various conditions varies widely though. The environment of the enzyme has a good deal to do with its stability. Purified enzymes may be denatured under conditions different from those which would denature the enzyme *in vivo,* and conversely. Many isoenzymes of the red cell are active in lysates that have been frozen. Similarly, dessication alone does not denature many red cell enzymes irreversibly, because these can be determined from dried blood stains. It is true that very little is known about the detailed changes which the proteins undergo in being dessicated, and later reconstituted.

Many enzymes depend on the presence of an additional molecule or atom for their catalytic activity. These additional molecules are called *cofactors.* Sometimes, a simple ion such as Zn^{++} or Cl^- is required for activity, while in other cases the cofactor is an organic molecule. The organic cofactors, many of which are derived from vitamins, are sometimes called *coenzymes.* In the jargon of biochemistry, the enzyme protein without its cofactor is called the apoenzyme, and the intact catalytic unit, protein plus cofactor, is called the holoenzyme. Some cofactors can be quite readily dissociated from the protein, while others are very tightly bound. A few of the more common cofactors, and the types of reactions in which they are involved, are indicated in Table 1.2.

Table 1.2 Coenzymes

Coenzyme	Type of Reaction
Nicotinamide Adenine Dinucleotide (NAD)	Oxidation-Reduction
Nicotinamide Adenine Dinucleotide Phosphate (NADP)	Oxidation-Reduction
Flavin Adenine Dinucleotide (FAD)	Oxidation-Reduction
Coenzyme A	Activation and Transfer of Acyl Groups
Thiamine Pyrophosphate	Acyl Group Transfer
Biotin	Carbon Dioxide Fixation
Pyridoxal Phosphate	Transamination of Amino Acids

1.1.4 Metabolism

Metabolism refers to the sum total of all the reactions participating in the life of an organism in all their particulars. Sometimes, metabolism is subdivided into anabolism, or synthetic reactions, and catabolism, or degradative reactions. Anabolic pathways generally require energy, while catabolic ones generally liberate it.

Metabolic pathways will not be discussed here. Many good standard texts and reviews are available on the subject, which is enormously complex. Some comments on the metabolic roles of enzymes and other compounds discussed in subsequent units are included in those sections.

1.2 Genetics

1.2.1 Introduction

The science of genetics as an independent field of inquiry has developed almost in its entirety in the present century. The first systematic experiments from which constructive conclusions could be drawn on the nature of inheritance were carried out in a monastery in Brünn (now Brno, Czechoslovakia) by Gregor Mendel (1822–1884). The now classical experiments, carried out on garden pea plants, occupied about 8 years, being communicated to the local scientific society in 1865 (Mendel, 1865). The original paper was translated by the Royal Horticultural Society, and was published by Bateson (1909) with modifications and notes. It is reprinted in Peters (1959). For reasons which are not altogether clear, no notice was taken of Mendel's work until 1900, when the European botanists, Correns, Tschermak and deVries, confirmed Mendel's original results. Some authors have marked 1900 as the year in which Mendel's work was "rediscovered." At least a part of the credit for bringing the work, and its confirmation by others, to the attention of the British scientific community must go to Bateson (Carlson, 1966). Bateson became quite interested in Mendel and his work, and Bateson's book, published in 1909, is an excellent source of material on the subject. Mendel was primarily a teacher until 1868, when he became abbot, or "Prälat," of his monastic community. He does not appear to have had much time to devote to his experiments after that time. Among other things, he became embroiled in a dispute with the government over an 1872 law which imposed taxes upon the property of religious houses. During the last decade of his life, he is said to have been bitter and disappointed over a number of matters. He is known to have been deeply disappointed by the fact that the results of his work were largely neglected by scientific colleagues. He wrote a number of letters to Nägeli, a leading naturalist, but was apparently unable to interest him in the discoveries. Bateson (1909) speculated that he may not have made any further efforts. Mendel is known to have done other sets of experiments too, most notably on heredity in bees, but the notes and records he was known to have kept were never found.

The term "genetics" was coined by Bateson in 1905 in a letter he wrote to Sedgwick (Carlson, 1966). The term "gene" to represent the discreet units of heredity, which had been revealed by Mendel's studies, was coined by Johannsen in 1909 (Carlson, 1966). Throughout the first twenty-five years of the 20th century, an understanding of the principles underlying inheritance, and many of their complexities, was gradually reached. The names of William Bateson, R. C. Punnett, G. H. Hardy, W. Weinberg, T. H. Morgan, A. H. Sturtevant, Sewall Wright, H. J. Muller and C. B. Bridges, and many others are associated with this work. Morgan won the Nobel Prize in 1933 "for his discoveries concerning the role played by the chromosomes in heredity"; Muller was likewise honored in 1946 "for his discovery of the production of mutations by means of X-ray irradiation."

1.2.2 Gene action at the biochemical level

1.2.2.1 Development of the one gene-one enzyme hypothesis—the beginning of present day understanding. The behavior of the "genes" in genetic experiments, various types of inheritance, and the chromosomal basis of inheritance, will be considered in later sections. In this section is considered the development of biochemical genetics, which involves the chemical nature of the genetic material and the way in which genes act at the molecular level.

The earliest investigations on the role of genes in metabolism were carried out by an English physician, Archibald E. Garrod. He studied a number of defects, including albinism, cystinuria and alcaptonuria. In 1902, when he directed his attention to the problem, more information was available about alcaptonuria than about the others. Alcaptonuria is a rare condition, harmless for the most part, and characterized by the fact that the urine of affected persons would change to dark colors upon standing. It was clear from the information on marriages which had produced alcaptonuric offspring that the incidence was much higher in cases of first cousin marriages. Garrod was also inclined to believe that the condition was caused by some alteration in normal metabolism. By 1908, when he delivered the Croonian lectures, Garrod was suggesting that a number of these rare anomalies could be grouped together under the heading of "inborn errors of metabolism." They appeared to be inherited as recessive traits according to Mendel's laws and, although little was known about enzymes or metabolism, he appeared to think that metabolism was represented by interdependent, sequential series of reactions, and that specific enzymes played a role in these reactions. It would be several decades before the significance of Garrod's thinking was fully appreciated. Despite the Croonian lectures, and some interest on the part of others, biochemists did not take much interest in genetics until much later. Garrod published a book on "inborn errors of metabolism" in 1923. We know now that alcaptonuria is indeed an "inborn error of metabolism", namely the absence of homogentisic acid oxidase, and that it is controlled by a simple Mendelian recessive gene. Homogentisic acid (2,5-dihydroxyphenylacetic acid) is an intermediate in phenylalanine and tyrosine catabolism. Absence of the enzyme causes an accumulation of the compound, which is excreted in urine. A related inborn error, and one with far more serious consequences, is phenyl-

ketonuria (PKU). Inherited as a Mendelian recessive, this condition is caused by the absence of phenylalanine hydroxylase, the enzyme which converts phenylalanine to tyrosine. Unless this condition is detected at birth, and treated promptly by withholding phenylalanine from the diet, irreversible mental retardation is inevitably the result. PKU was discovered by Fölling in 1934.

It was not until 1941 that the first experimental evidence was put forth that genes have directly to do with specific enzymes in biochemical pathways. Beadle and Tatum (1941), using the mold *Neurospora,* could relate the presence of specific mutant genes in the organism, which affected their ability to synthesize vitamins, to the synthetic pathway involved. Studies on *Neurospora* were continued and biochemical and genetic studies were quickly extended to bacteria by Lederberg and others. These studies led to the development of what has been called the one gene-one enzyme hypothesis. In these terms, genes act by exercising control over specific biochemical reactions, and they do so by controlling the specific enzymes which catalyze those reactions. In 1958, Beadle and Tatum were awarded the Nobel Prize "for their discovery that genes act by regulating definite chemical events"; Lederberg shared the award "for his discoveries concerning genetic recombination and the organization of the genetic material in bacteria".

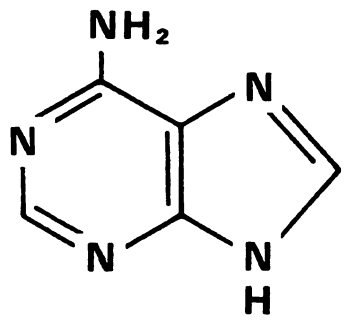
Our current conception of the "gene" is somewhat more well defined in terms of what has been learned about how the genetic material controls protein synthesis. The *gene* may be thought of as a linear segment of DNA, specifying the synthesis of a polypeptide chain. In many cases, more than one such polypeptide chain is required to form a complete, functional product (protein), and thus, more than one gene can be involved in the synthesis of a particular protein. It is known, particularly from studies in bacteria and viruses, that a *gene* may consist of two or more lesser segments of DNA, linearly arranged to form the "gene", but distinguishable in certain kinds of genetic tests. These units are called *cistrons,* because the genetic test used to characterize them is called a "cis-trans" test.

1.2.2.2 Evidence that DNA is the genetic material. Our present understanding of gene action is based on the work of many investigators. Important pieces of information which were necessary to understand the complete mechanism were: (1) DNA is the genetic material; (2) the chemical structure of DNA; (3) the fact that proteins are not synthesized directly from DNA but from a complementary RNA molecule; and (4) the mechanism by which nucleic acid sequences can specify amino acid sequences.

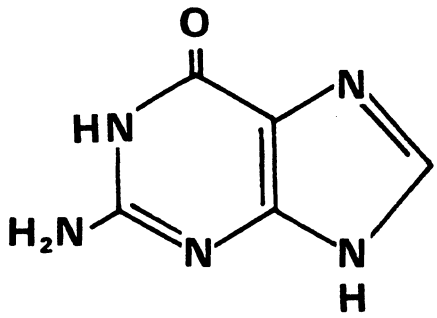
Evidence that deoxyribonucleic acid (DNA) is the genetic material came from several investigators' results. In 1928 Griffith observed that virulent and non-virulent types of *Pneumococcus* could be transformed in some way, one into the other, within an animal. Heat-killed virulent type bacteria (which were no longer virulent) were injected into animals along with living, non-virulent type bacteria. Many of the animals died, and living, virulent type bacteria could be recovered from the survivors. Some principle in the heat-killed virulent type bacteria was thus capable of transforming the living, non-virulent type into virulent type. Later experiments showed that this transformation could be effected *in vitro* as well. The now classical experiments of Avery, McLeod and McCarty (1944) demonstrated that the "transforming principle" was in fact DNA. Another important study was done by Hershey and Chase (1952) with bacteriophages. These viruses infect bacteria, and utilize the bacterial cell's biochemical machinery for their own reproduction. The phage consists of DNA (or sometimes RNA) in a "core," and a protein "coat." By labelling the protein and the DNA with different radioactive tracer atoms, Hershey and Chase could show that it was the nucleic acid which entered the bacterial cell, and that it, and not the protein, was thus the component required for the production of genetically identical viruses.

1.2.2.3 Structure of DNA and RNA. Nucleic acids are polymers of nucleotides. Nucleotides are molecules consisting of one of five nitrogenous bases, a five-carbon sugar, and phosphate. The nitrogenous bases which occur in nucleic acids are of two types, purines and pyrimidines. The purines are adenine and guanine; the pyrimidines are thymine, cytosine and uracil (Fig. 1.20). The names of the bases are sometimes abbreviated to the first letter of the name, A, G, T, C and U. The five carbon sugar may be ribose or deoxyribose (Fig. 1.21). Ribose containing nucleotides make up RNA while deoxyribose containing nucleotides make up DNA. The purines and pyrimidines can be linked to either of the sugars in the absence of phosphate forming molecules called *nucleosides.* The structures of two of the ten possible nucleosides, deoxyadenosine and thymidine, are shown in Fig. 1.22. Note that the purine or pyrimidine ring structures are numbered conventionally, while the sugar ring positions are indicated by primed numbers. Phosphoric acid can be esterified to the nucleosides through the 3′-position in the sugar residue, the resulting nucleoside phosphates being called nucleotides. The nucleotides corresponding to the nucleosides shown in Fig. 1.22 are shown in Fig. 1.23. Esterification at the 5′ position occurs as well. Table 1.3 gives the names of the bases, and the nucleosides and nucleotides formed from each of them for both ribose and deoxyribose forms.

Nucleotide residues may be linked together by sugar-phosphate-sugar bonds to form polynucleotide chains. Long polynucleotide chains are known as nucleic acids. The structure of a deoxyribose polynucleotide chain is shown in Fig. 1.24. Note that the phosphodiester bonds holding the nucleotides together link the 3′ carbon of one sugar residue to the 5′ carbon of the next, and so forth. With some exceptions, which will not be gone into, RNA usually consists of one chain, of the kind shown in Fig. 1.24, except that the sugar is ribose. RNA does not usually contain thymine nucleotides. DNA does not contain uridine nucleotides. The structure of DNA is more complicated than that of RNA because it usually involves two chains, or strands. It consists, as mentioned already, of A, C, T and G nucleotides. Chargaff found that in DNA from mammalian sources, the

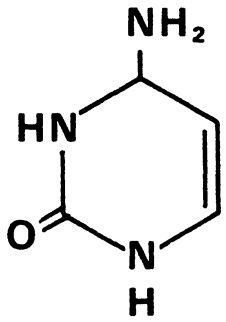


Adenine (A)

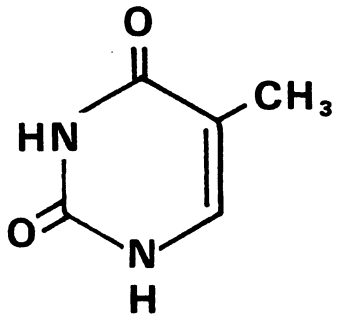


Guanine (G)

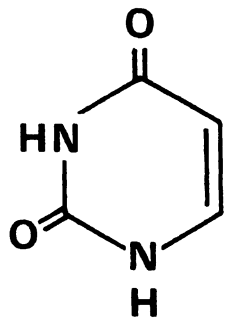
Purines



Cytosine (C)



Thymine (T)



Uracil (U)

Pyrimidines

Figure 1.20 Purine and Pyrimidine Bases

amount of A was the same as that of T, while the amount of C was the same as that of G. The A + T/C + G ratio did vary in DNA from different sources. Data from X-ray diffraction studies by Wilkins and Franklin and the information from chemical studies enabled Watson and Crick to postulate a structure for DNA in 1953. This structure, the now well-known double helix, is the usual structure of DNA. Watson, Crick and Wilkins shared the Nobel Prize in 1962 "for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material". The double helix is constructed from two polynucleotide chains. They are held together by hydrogen bonds which occur between A and T and between C and G. The structure is shown diagramatically in Fig. 1.25. Note that the direction in which the 3′→5′ phosphodiester bonds run is reversed in the two complementary chains, as indi-

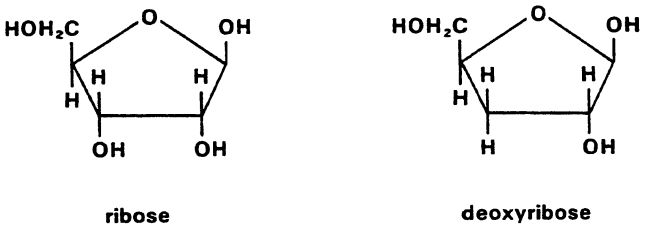


Figure 1.21 Ribose and Deoxyribose Sugars

(3) semiconservative mechanism, in which the double stranded molecule separates into single strands, and each acts as a template for the synthesis of a new complementary strand. In 1958, Meselsohn and Stahl carried out their now classical experiments on DNA replication, and proved that the mechanism was semi-conservative. The process is indicated diagramatically in Fig. 1.27. The cell free synthesis of DNA from single stranded templates and nucleotide precursors in 1956 was completely consistent with what was already known (Kornberg *et al.*, 1956; Kornberg, 1959). He

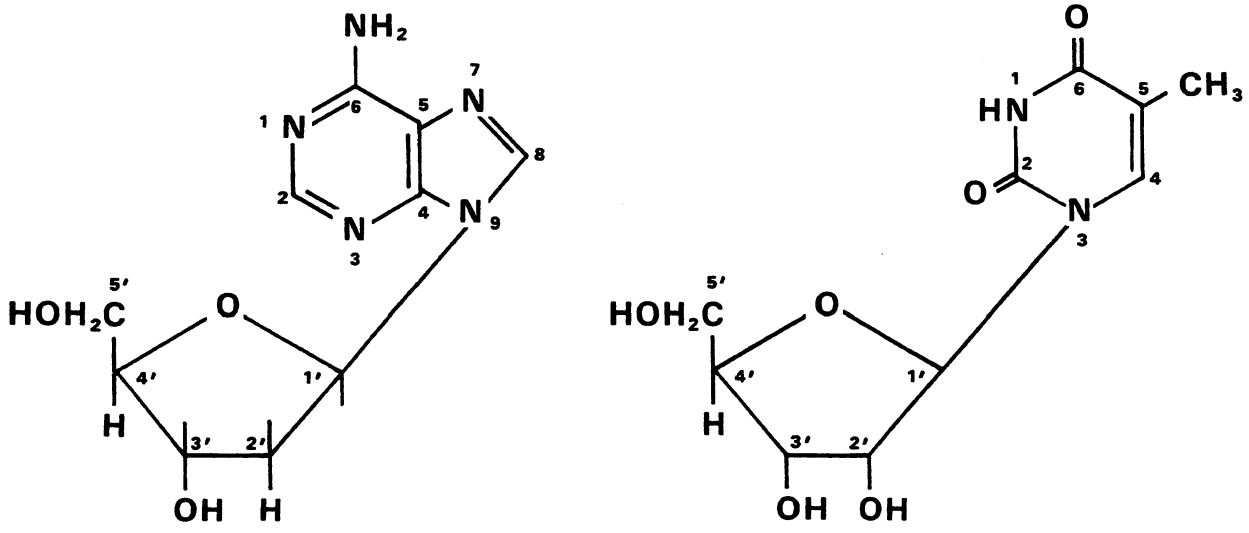


Figure 1.22 Nucleosides

cated by the arrows. Three dimensionally, the molecule is in a helical configuration, one helical chain being wound about the other (Fig. 1.26).

1.2.2.4 Replication of DNA. DNA replicates during cell division, yielding exact copies of the parent molecules. The structure must allow for a mechanism by which this replication can take place. In theory, there are three mechanisms by which the molecule could replicate: (1) dispersive mechanism, in which the molecule was broken up into small fragments, the small fragments replicated, and the larger molecules then somehow reassembled; (2) conservative mechanism, in which the entire double stranded molecule acts as a template for the synthesis of daughter chains; and

received the Nobel Prize in 1959, along with Ochoa, "for their discovery of the mechanisms in the biological synthesis of ribonucleic and deoxyribonucleic acid."

1.2.2.5 RNA, Protein synthesis and the genetic code. There are three types of RNA in cells: messenger RNA (m-RNA), ribosomal RNA (r-RNA), and transfer RNA (t-RNA). The last-mentioned is also called soluble RNA (s-RNA). Messenger RNA serves as the actual template for protein synthesis. It is synthesized enzymatically from one of the strands of DNA, the sequence of the m-RNA being complementary to that of DNA, in accordance with the base pairing rules. In effect, the complementary bases of RNA nucleotides bind to the bases of

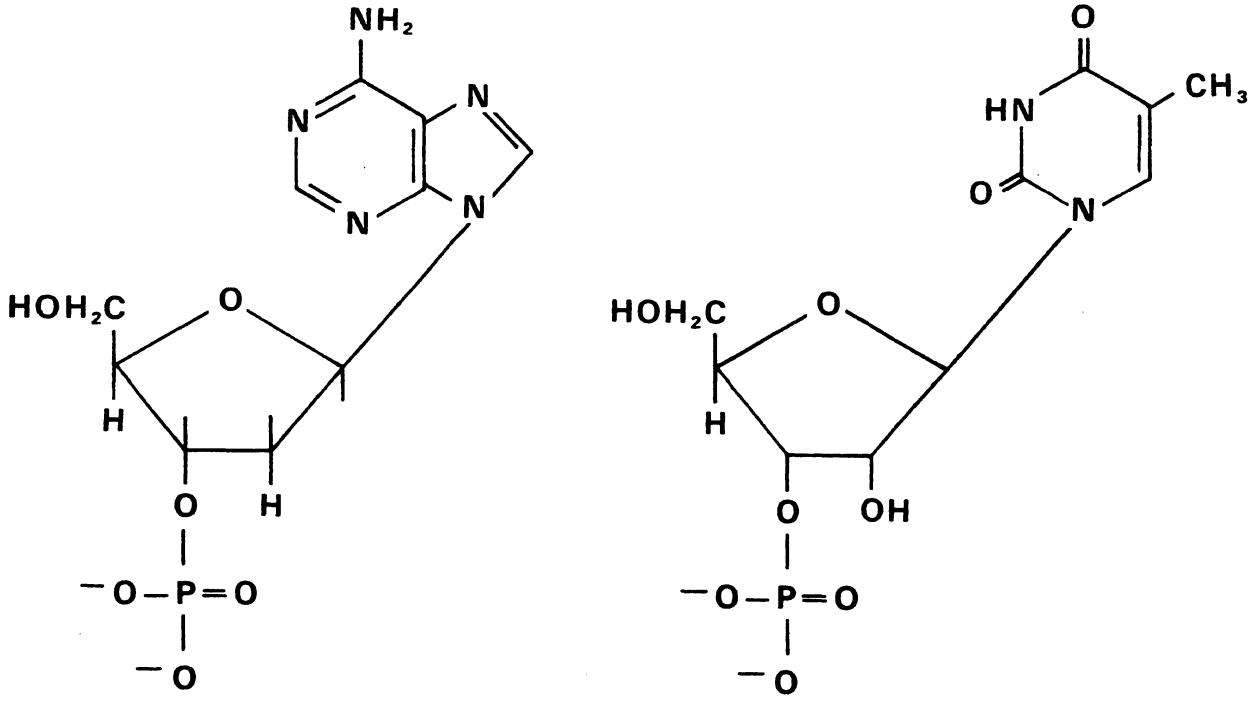


Figure 1.23 Nucleotides

the nucleotides within the DNA strand, and are then enzymatically linked. An A in DNA calls for a U in the complementary RNA, a T in DNA for an A in RNA, a C in DNA for a G in RNA and a G in DNA for a C in RNA. The A-T (A-U) and C-G base pairing serves to align the ribonucleotides on the DNA template. This is illustrated in Fig. 1.29.

Ribosomal RNA is, as the name implies, an integral component of the ribosome, the cytoplasmic site of protein synthesis. Ribosomes are composed of two subunits, and contain protein in addition to the r-RNA. The precise details of ribosome function are not yet fully understood, but in general, the ribosome functions to associate m-RNA, amino acids and the appropriate enzymes into configurations suitable for protein synthesis to take place.

Transfer-RNA molecules are the smallest of the ribonucleic acids. They provide the link between the amino acid and the appropriate nucleotide sequences in m-RNA, serving, in effect, as adapter molecules. There are a number of different t-RNA molecules in the cell, at least one for each of the amino acids which occurs in proteins, and some others which carry out specialized functions. In 1965, Holley *et al.* published the complete sequence of alanine-t-RNA. The structure is shown in Fig. 1.28. There is much evidence to support the "cloverleaf" conformation of the molecule shown in the figure.

During the 1960's, the mechanism of protein synthesis was worked out. It became clear that the amino acid sequence of the protein was specified by the base sequence in the m-RNA which had, in turn, been specified by the base sequence in DNA. There are four bases in RNA, and we now know that a linear sequence of three bases in the chain is required to specify an amino acid. The "code word", or *codon,* therefore, is a triplet. Nirenberg and his collaborators did many of the experiments which resulted in the assignment of the codons. The polynucleotides of known sequence, synthesized by Khorana and his associates, also figured importantly in this work. Nirenberg, Khorana and Holley jointly received the Nobel Prize in 1968 "for their interpretation of the genetic code and its function in protein synthesis."

Using the four bases of RNA to form triplets allows for 64 codons. Since there are only 20-odd amino acids, the *genetic code,* as it is called, is degenerate, i.e., there can be more than one codon for a particular amino acid. It is now known that the code is non-overlapping, and does not contain "punctuation." By non-overlapping is meant that the code is read in strictly linear order, three bases at a time. The se-

Table 1.3 Naming of Bases, Nucleosides and Nucleotides

Base	Ribonucleoside	Ribonucleotide	Deoxyribonucleoside	Deoxyribonucleotide
Adenine	Adenosine	Adenylic acid	Deoxy-adenosine	Deoxyadenylic acid
Guanine	Guanosine	Guanylic acid	Deoxy-guanosine	Deoxyguanylic acid
Cytosine	Cytidine	Cytidylic acid	Deoxy-cytosine	Deoxycytidylic acid
Thymine	Thymidine	Thymidylic acid	Deoxy-thymidine	Deoxythymidylic acid
Uracil	Uridine	Uridylic acid	Deoxy-uridine	Deoxyuridylic acid

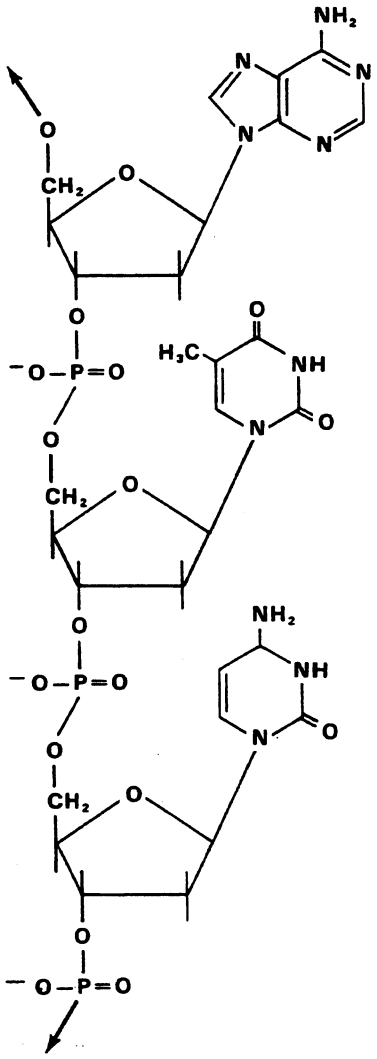


Figure 1.24 A Deoxyribose Polynucleotide Chain

quence AUGUUUGGA, for example, is read AUG UUU GGA, and *not* as AUG UGU GUU, or as AUG GUU UUG, etc. The overall scheme is indicated diagramatically in Fig. 1.29. Transfer-RNA's are able to bind to particular amino acids. In addition, they possess a trinucleotide sequence in their structure which "recognizes" the m-RNA codon by base pairing, i.e., is complementary to the codon. This trinucleotide sequence in the t-RNA is called the *anti-codon.* Therefore, to say that UUU codes for phenylalanine is to say that Phe-t-RNA has the anticodon sequence AAA which can base pair (bind) the UUU codon. The genetic code is shown in Table 1.4. There are codons which call for initiation of protein synthesis as well as those which call for termination.

The process of protein synthesis is quite a bit more complicated than has been outlined here. Many of the details have been omitted for the sake of brevity. Further information on the subject may be found in Levine (1968), Watson (1976), Zubay (1968) and Zubay and Marmur (1973), and in many textbooks.

1.2.3 Chromosomes

The DNA of the cell is organized in the nuclear chromosomes. The behavior of the chromosomes can be observed microscopically. Cell division is the fundamental process by which cells make exact copies of themselves. In unicellular organisms, cell division is equivalent to reproduction, and in multicellular organisms, cell division is required for growth and differentiation. Sexually reproducing organisms carry out a specialized kind of cell division in order to produce gametes, or sex cells. There are, thus, two important kinds of cell division. The first, *mitosis,* is a process in which a cell divides to produce genetically equivalent daughter cells. The second type, *meiosis,* is characteristic of sexually reproducing organisms, and represents the process by which gametes are formed. The gametes contain half the number of chromosomes of other cells in the organism, the original number of chromosomes being restored at fertilization,

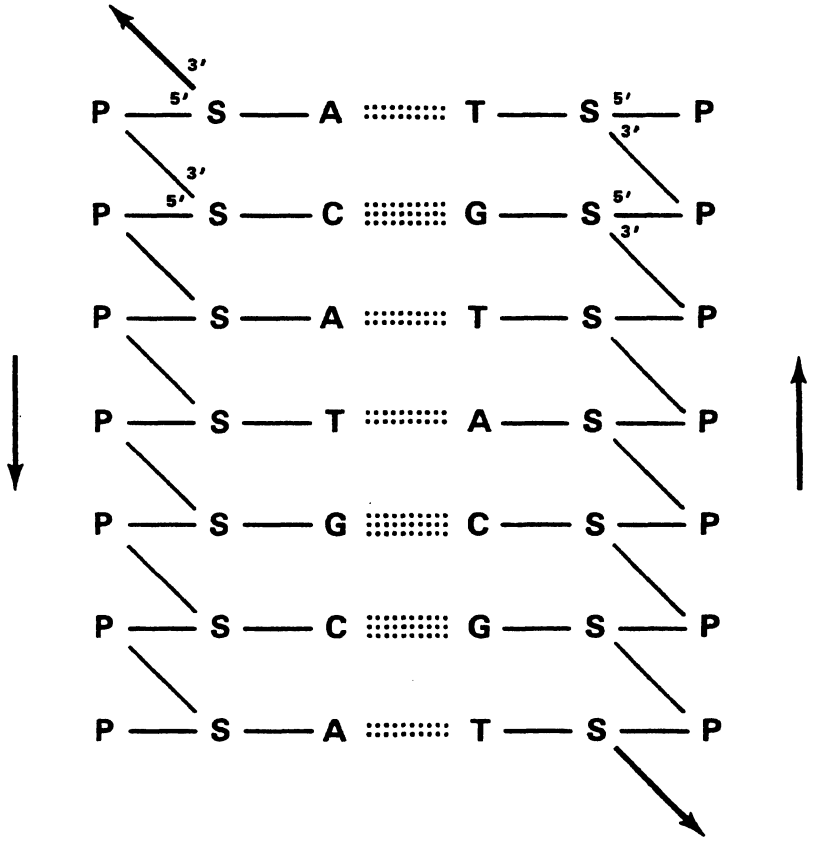


Figure 1.25 Double-stranded DNA. S = deoxyribose sugar P = phosphate A,C,T,G = purine and pyrimidine bases ::::: = hydrogen bonds.

when the gametes combine. The gamete contains in its chromosomes a random half of the genetic information from the parent from whom it is derived. All species contain a characteristic number of chromosomes, and chromosomes occur in pairs. The two members of a pair are said to be *homologous.* The total number of chromosomes is called the diploid (2n) number. Half that number, the number found in gametes, is called the haploid (n) number.

1.2.3.1 Mitosis. Mitosis or cell division is usually divided into a number of phases. The process is represented diagramatically in Fig. 1.30. The terminology used to describe mitotic phases is not important to this discussion. The essential features of the process are the pairing of homologous chromosomes, their replication, and their arrangement in such a way that a complete set of chromosomes is drawn to opposite poles of the dividing cell. Upon division of the cytoplasm, each daughter cell contains a complete set of chromosomes, exact copies of the original set.

1.2.3.2 Meiosis. Meiosis is the process by which sexually reproducing organisms form their gametes. It is similar to mitosis in many respects, but results in four instead of two products, each of which possess a haploid number of chromosomes. The process is illustrated diagramatically in Figure 1.31. Meiosis differs from mitosis in that homologous chromosomes pair and replicate, but there are then two successive cellular divisions, resulting in four cells, each of which possesses one member of each of the original pair of chromosomes. Gametes are therefore haploid cells. At fertilization, the diploid number of chromosomes is restored in a single cell (the fertilized egg, or zygote), and the chromosome number characteristic of the species is maintained and conserved.

1.2.3.3 Human chromosomes and sex determination. For a long time it was believed that human beings had a diploid number of 48 chromosomes. In 1956, Tjio and Levan carefully examined cells in tissue culture, and found

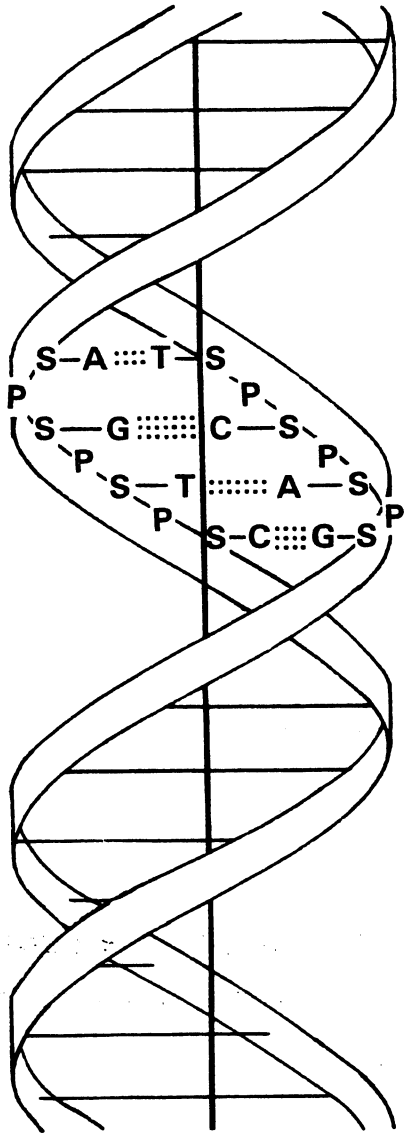


Figure 1.26 Double helical DNA

that the diploid number is, in fact, 46. 44 of these chromosomes consist of 22 homologous pairs, and these are usually called *autosomes*. The remaining two chromosomes in a given person may be either a pair of X-chromosomes, or an X and a Y chromosome. The X and Y are called *sex chromosomes*. XX people are female, while XY people are male. With a few bizarre exceptions in which hormonal or other factors seem to play a major role, sex in human beings is chromosomally determined. Females are XX and ova always contain an X chromosome in addition to 22 autosomes after meiosis. Males are XY, and meiosis gives rise to an equal number of X-bearing and Y-bearing sperm. The sex of the offspring is determined, therefore, by whether an X- or Y-bearing sperm cell fertilizes the ovum.

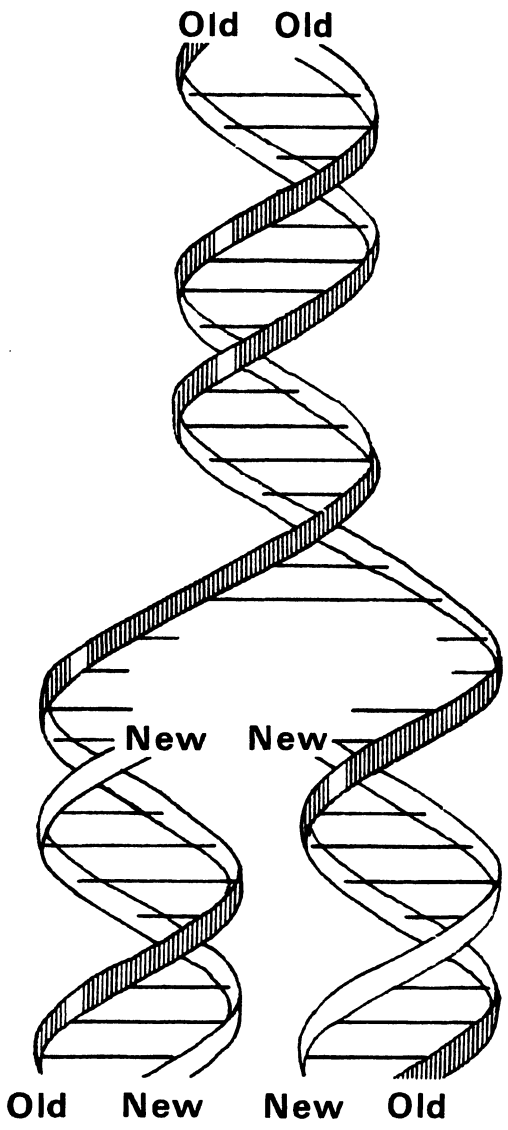
There are a number of chromosomal aberrations in humans. With present-day cytogenetic techniques, a few cells can be removed from an individual, grown in tissue culture, and used to prepare good, stained preparations in which the chromosomes are visible under the microscope. If a photomicrograph is then made and enlarged, the pictures of the chromosomes can be cut out, classified, and arranged in order to see if chromosomal abnormalities are present. This technique is called karyotyping. Fetuses can be karyotyped *in utero* by collecting a few cells from the amnion for the culture, a procedure known as amniocentesis. One of the abnormalities that can occur in human beings is Down's syndrome, or mongolism. This condition, characterized by mental retardation, impaired motor development and decreased life expectancy, is the result of an affected individual having three, instead of two, number 21 chromosomes. The condition of having three chromosomes, where there ought to be two, is called trisomy, and Down's syndrome may be said to be the result of trisomy at chromosome 21. There are abnormalities of the sex chromosomes as well. Persons having 45 chromosomes, and only one X, are said to be XO. They are females and suffer from a kind of gonadal dysgenesis called Turner's syndrome. There are abnormal males who possess 47 chromosomes, with an XXY complement of sex chromosomes. They are said to suffer from Klinefelter's syndrome. There are many other abnormalities as well.

1.2.4 Patterns of inheritance

1.2.4.1 Simple patterns. Mendel's discoveries had to do with simple modes of autosomal inheritance. Individuals have a pair of genes which control a particular, simply inherited trait. Genes are usually represented by letters. Mendel was able to explain the results of his crosses using simple genetic models. The elegance of his findings is difficult to appreciate in view of what is known today, but nothing of the chromosomal basis of inheritance was known at the time when the experiments were done.

The *phenotype* of an organism is the way it looks with respect to a particular trait, i.e. how the gene is expressed. Thus, eye color in *Drosophila* (the fruit fly), flower color in many plants, and blood types in humans are phenotypes. The *genotype* is the gene composition of the organism for the particular trait. To state the genotype is to state which genes are actually present on the chromosomes.

Suppose a hypothetical flowering plant species. Two types are available, one with red flowers, and another with white flowers. Each of these is "breeding true", i.e. crosses among red-flowered plants always produce red flowered offspring, and similarly for the white-flowered plants. Suppose that the red flowered and white flowered plants are crossed, fairly large numbers of off-spring are obtained, and they all have red flowers. This "first filial generation" or F_1 is now crossed with other F_1 plants again yielding large numbers of plants in an F_2 generation. It is now found that there are about 3 red-flowered plants for every white-flowered plant. A simple genetic explanation for these observations is shown in Fig. 1.32. Since red appears exclusively in the F_1, it is called



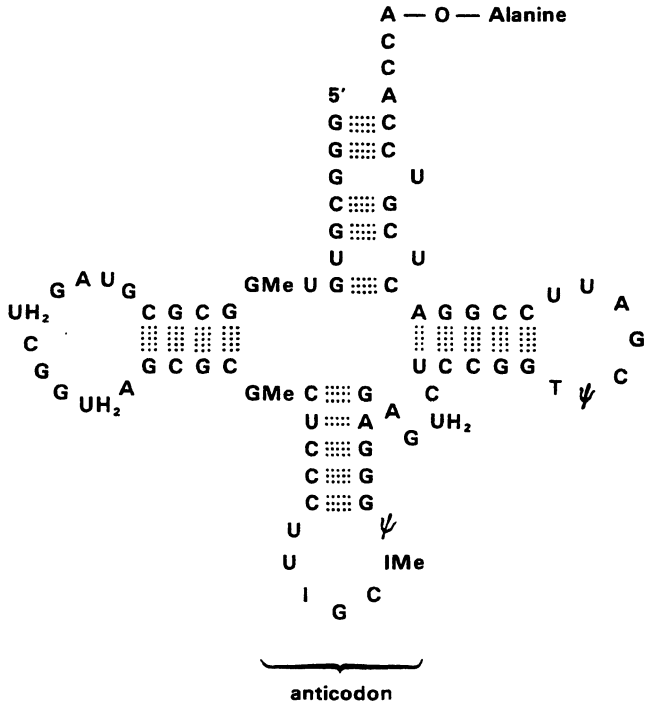


Figure 1.28 Structure of Alanine-t-RNA

Figure 1.27 DNA Replication

dominant over white. White has not disappeared because it reappears in the F_2. White is called *recessive* in this case. Let R stand for the gene determining red flower color and r stand for the gene determining white flower color. True breeding red flowered plants are genotypically RR while the white flowered counterparts are genotypically rr. Red plants which are RR can make only R containing gametes at meiosis and rr white flowered plants can make only r gametes. The F_1 generation must therefore be Rr genotypically, and phenotypically the flowers are red. In the $F_1 \times F_1$ cross, each plant should make an equal number of R and r gametes. Allowing fertilization to occur completely at random, i.e. R gametes fertilize R and r gametes with equal frequency, and r gametes fertilize R and r gametes with equal frequency, one can predict the genotypes of the F_2. An easy way of doing this is to use the so-called Punnett square (Fig. 1.32). Of every four plants, 1 is expected to be RR, two Rr and 1 rr. Phenotypically, three out of four should be red flowered. This simple situation, which is quite similar to the experiment Mendel carried out, illustrates Mendel's so-called first law, the law of segregation. Simply put, it says that the members of a gene pair segregate from one another at meiosis and are distributed evenly in gametes. The members of a gene pair are often called *alleles.* In the example, R is the dominant allele, r, the recessive allele. When the genotype consists of two of the same allele, as in RR or rr in the

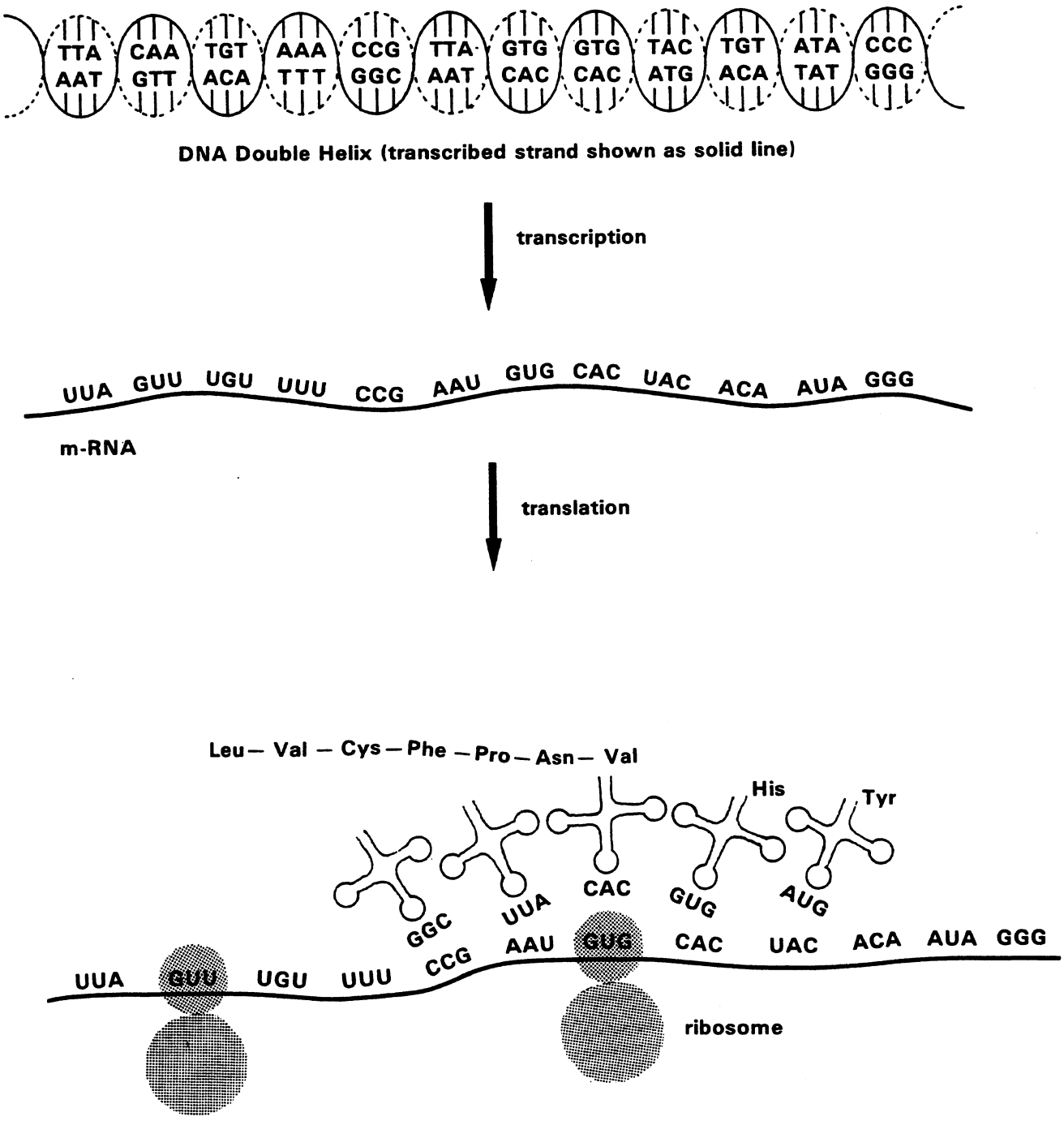


Figure 1.29 Overall Scheme of Protein Synthesis

example, the individuals are said to be *homozygous.* The red-flowered plant is homozygous dominant, the white-flowered one is homozygous recessive. The Rr genotype individual is referred to as being *heterozygous.*

Mendel's second law has to do with the segregation of the genes for two independent characteristics. Let us suppose that our hypothetical plants have another characteristic, tallness, which is under simple genetic control. If we crossed true breeding tall plants with true breeding short plants, the F_1 would all be tall. If the members of the F_1 were self-crossed, the F_2 would have approximately 3 tall plants for every 1 short plant. Suppose the same experiment is done,

Table 1.4 The Genetic Code

UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
UUC		UCC		UAC		UGC	
		UCA					
UUA	Leu	UCG		UAA	Terminate	UGA	Terminate
UUG				UAG			
		CCU				UGG	Try
CUU		CCC	Pro	CAU	His		
CUC	Leu	CCA		CAC		CGU	
CUA		CCG				CGC	Arg
CUG				CAA	Gln	CGA	
		ACU		CAG		CGG	
AUU		ACC	Thr				
AUC	Ileu	ACA		AAU	Asn	AGU	Ser
AUA		ACG		AAC		AGC	
AUG	Met	GCU		AAA	Lys	AGA	Arg
		GCC	Ala	AAG		AGG	
GUU		GCA					
GUC	Val	GCG		GAU	Asp	GGU	
GUA				GAC		GGC	Gly
GUG						GGA	
				GAA	Glu	GGG	
				GAG			

but that both traits are followed at the same time. Tall, red-flowered plants are crossed with short, white-flowered ones to yield an F_1 which is all tall, red-flowered (Fig. 1.33). The F_1 members are now self-crossed, and it is found that the phenotypic ratio is 9 tall, red:3 tall, white:3 short, red: 1 short, white. The cross, and its genetic explanation, are shown in Fig. 1.33. This experiment illustrates the so-called law of independent assortment (Mendel's second law). This says that the segregation of the flower color genes is in no way influenced by the segregation of the tallness-shortness genes, and conversely. The Punnett square in Fig. 1.33 predicts the outcome of the $F_1 \times F_1$ cross by assuming independent assortment, and the observation of the predicted phenotypes experimentally serves as verification that the genetic hypothesis is correct.

1.2.4.2 Variable expressivity, codominance and multiple allelic systems. A substantial number of traits in a large number of organisms are inherited according to the simple patterns discussed in 1.2.4.1. In tribute to Mendel, the patterns are often referred to as "Mendelian." We can say, in the example in Fig. 1.32 that red flower color is inherited as a simple Mendelian dominant, and that white flower color is inherited as a simple Mendelian recessive.

There are many examples of exceptions to the patterns though. The term "variable expressivity" refers to a situation in which a gene is not fully expressed in every individual having it. If, in our flower color example above, the heterozygotes (Rr) had had flowers which were not uniformly red in the F_1, but varying degrees of pink depending upon the individual, we would say that R showed variable expressivity. Another possibility would be that RR plants have red flowers, but that Rr plants have pink flowers. In the F_2 we would have been able to distinguish the RR from the Rr plants in that case. Variable expressivity can occur in homozygotes as well, and there are numerous examples of it. A closely related concept is that of "penetrance." In the example of Rr plants exhibiting pink flower color, it would be said that in the heterozygous condition, the R gene is "incompletely penetrant."

Not all alleles show dominance-recessivity relationships. Sometimes both members of the allelic pair are expressed if present. Such genes are said to exhibit *codominance.* Very

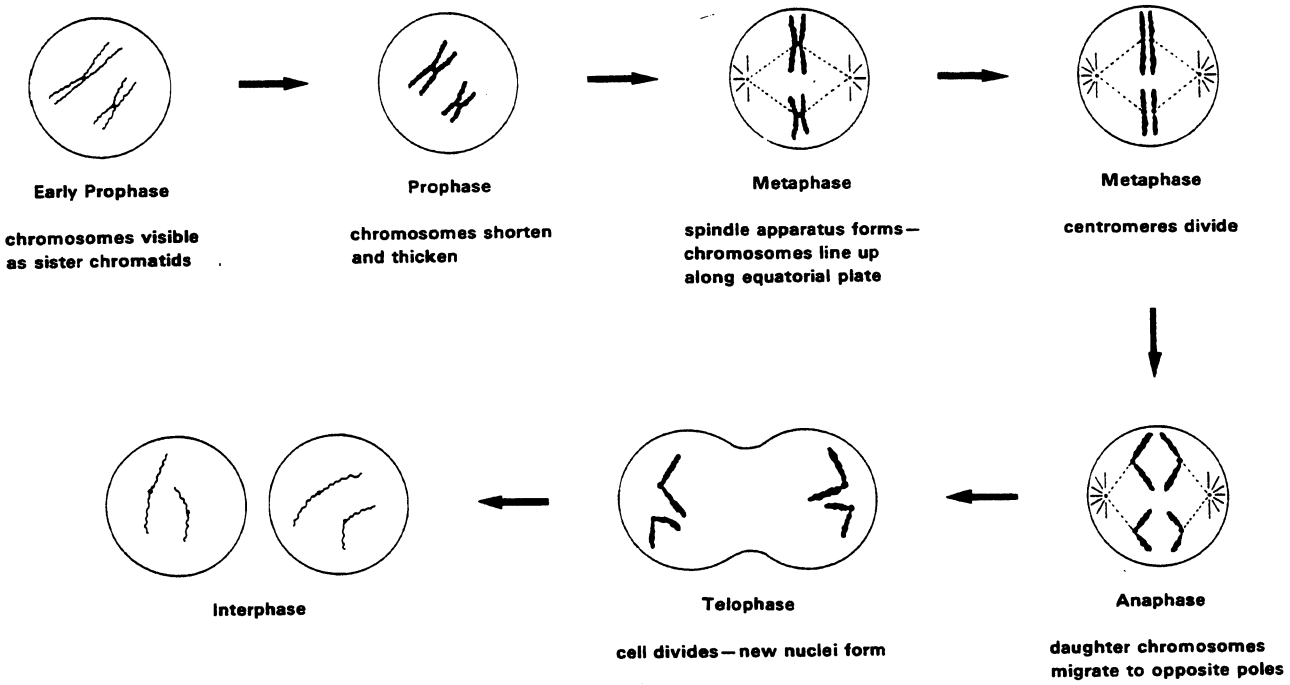


Figure 1.30 Mitosis

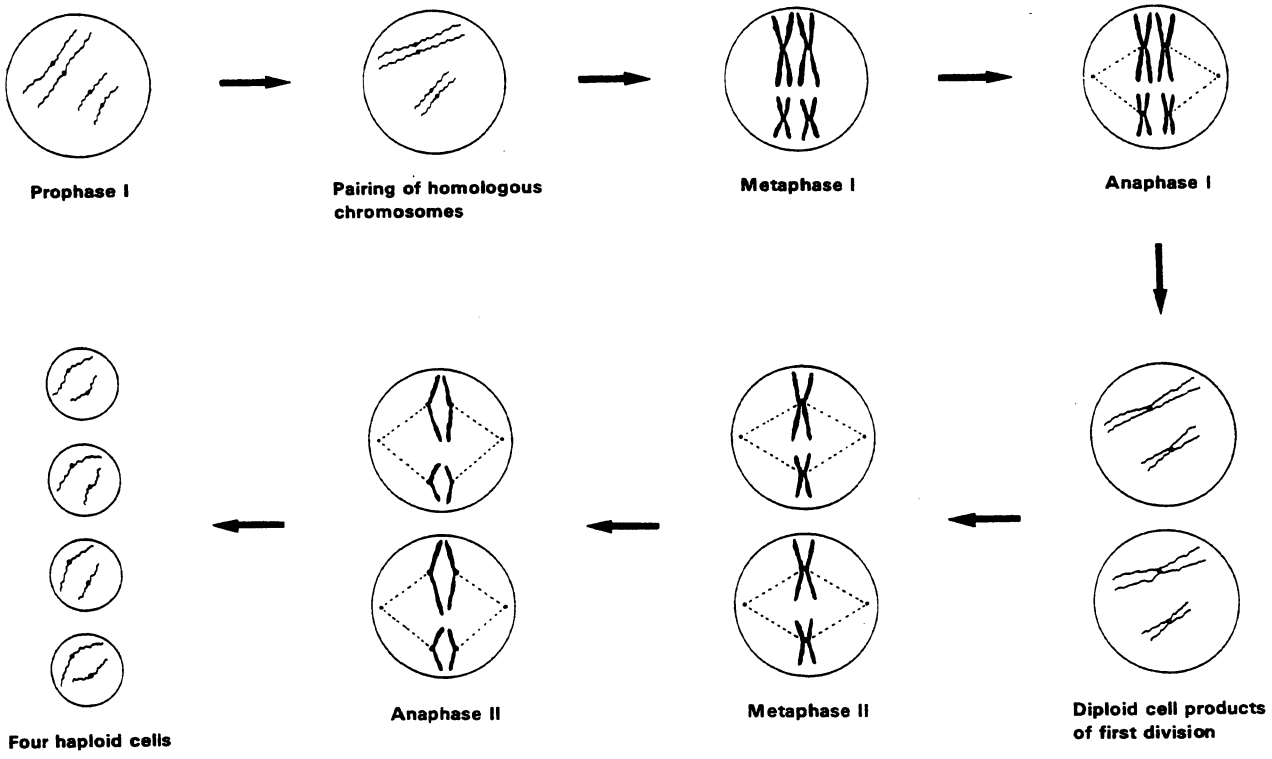


Figure 1.31 Meiosis

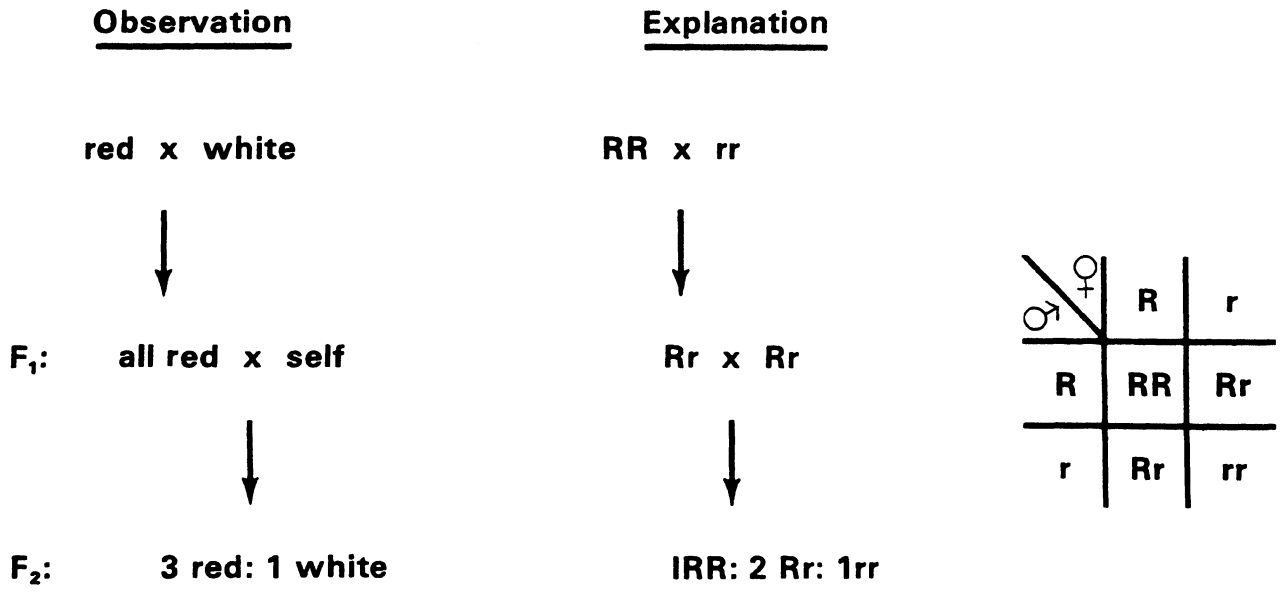


Figure 1.32 Illustration of Segregation

many of the blood group systems, isoenzyme and serum protein markers which are of great interest in legal medicine exhibit codominance. An example is the MN system. Without getting into all the complexities of the system (which will be done in Unit V), MN may be considered as a simple Mendelian codominant system. Genotypically, people can be MM, MN or NN. The heterozygotes, the MN persons, can be detected since both genes are expressed, and their red cells will be agglutinated by anti-M as well as by anti-N sera. In codominant inheritance, the genotype may be determined from the phenotype, whereas if one gene is dominant, heterozygotes cannot be distinguished from homozygous dominants. In the ABO blood group system, for example, if we call the genes for A, B and O blood groups I^A, I^B, and I^O, respectively, we cannot tell from the blood group (the phenotype) whether a person who groups as A is genotypically I^AI^A or I^AI^O.

Another important aspect of inheritance patterns is that of *multiple alleles.* The ABO blood group system provides an example. Genes are located upon the chromosomes, and it is said that the alleles controlling a particular trait are at a particular *locus* on the chromosome. In many cases, there are only two genes operating at a locus. But in many other cases, a locus can be occupied by one of several possible alleles. Such loci are said to be *multiple allelic.* In the ABO blood group system, there are three alleles. The possible genotypes are, therefore, I^AI^A, I^BI^B, I^AI^O, I^OI^O, I^BI^O, and I^AI^B. Most of the isoenzymes which will be considered in Unit VI have more than two possible alleles. In a number of cases, there are two common alleles and a number of rarer ones.

1.2.4.3 Linkage, crossing over and genetic mapping. The term *linkage* refers to the situation in which the gene loci controlling different traits are on the same chromosome. As an illustration, let us return to our hypothetical plant with tall-short and red or white colored flowers. Suppose the same experiment is done as was done to illustrate the law of independent assortment. Red flowered, tall plants are crossed with white-flowered, short ones, and a red-flowered, tall F_1 is obtained. Upon self crossing the F_1, the F_2 shows not the 9:3:3:1 obtained previously, but 3 red flowered, tall plants to 1 white flowered, short one. This result can be explained if the flower color locus and the tall-short locus are on the same chromosome (Fig. 1.34).

The results in Fig. 1.34 are highly idealized for the sake of illustration. A much more likely outcome for the $F_1 \times F_1$ self cross in this example would be that 80% of the F_2 progeny would be red, tall or white, short while the remaining 20% would be red, short and white, tall. The reason for the appearance of the latter two phenotypic classes brings up the subject of crossing over. Homologous chromosomes can and do exchange genetic material with one another while they are paired during meiosis. A kind of "break" occurs at a particular point along the chromosome, and the "loose ends" exchange places between the homologues. Such exchanges can be observed cytologically, and are called *chiasmata.* Fig. 1.35 illustrates crossing over in the $F_1 \times$ self cross for the example in Fig. 1.34. New combinations of loci on a

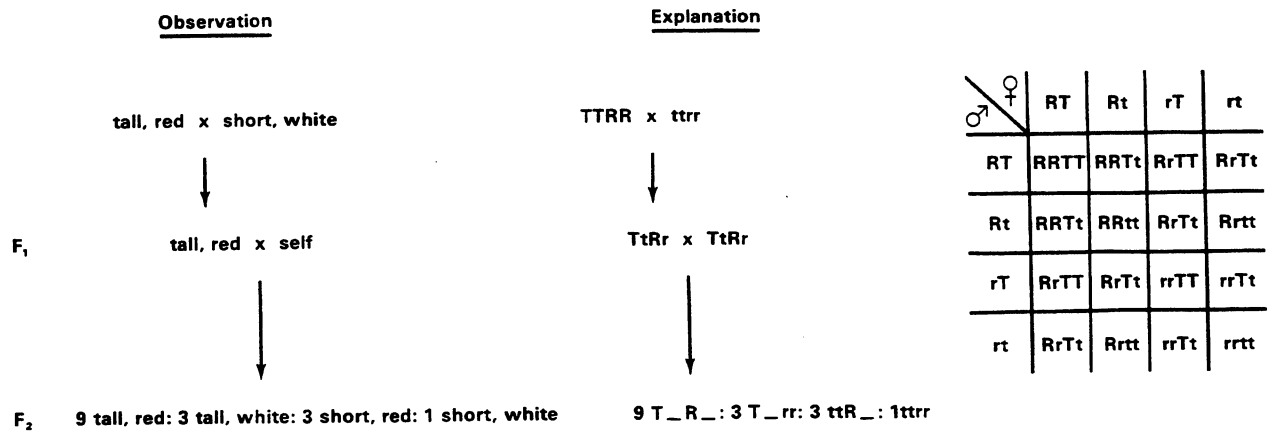


Figure 1.33 Illustration of Independent Assortment

chromosome, not present in the parents, and resulting from crossovers are called *recombinants.* Crossing over can only be detected if its occurrence gives rise to non-parental combinations. Thus crossing over in an organism with RRTT genotype would not be detected. If the parental organism is a double heterozygote, as in Fig. 1.35, it should be noted that two genotypic configurations are possible, $\frac{R\ T}{r\ t}$ and $\frac{R\ t}{r\ T}$. The former is called "cis" or "coupling" phase, the latter, "trans" or "repulsion" phase. The terms may be applied even to codominant genes, if one of the alleles is regarded as being abnormal. The crossover frequency between loci is usually the same regardless of the phase. The argument will not be developed here, but it can be shown that the theoretical maximum probability of crossing over between loci is 50% (see Levitan and Montagu, 1971). With a few known restrictions, crossing over takes place along the chromosome essentially at random. Therefore, the more distance there is between loci, the greater the probability of a crossover taking place between them. It is this correlation which forms the basis of genetic mapping. More than one crossover may occur between the same loci, and it should be clear that an even number of crossovers between loci leaves the gene positions unchanged, while an odd number of crossovers yields new, recombinant types.

Gene mapping is carried out by studying crossover frequencies between loci. A "map unit" was originally taken to be the distance between loci which exhibited a 1% crossover frequency. Because of multiple crossovers, the theoretical 50% limitation on crossover frequency, and the fact that a crossover at a certain point may influence the probability of a subsequent one, the "map distance" does not always correlate exactly with the crossover frequency. For loci which lie fairly close together, the correlation is linear, but begins to deviate from linearity as distances become greater. Haldane in 1919 suggested that the unit of map distance be given its own name, and he suggested the term "morgan" to honor the Nobel laureate geneticist Thomas Hunt Morgan. One "morgan" is divided up into 100 "centimorgans", a centimorgan corresponding to the map distance between two loci showing 1% crossover frequency. For loci fairly close together, crossover frequency can be directly translated into centimorgans.

It should be noted that distant loci, with crossover frequencies approaching 50%, will give results in crosses which are indistinguishable from the results if independent assortment is obeyed. It is impossible from such data, therefore, to know that the loci are linked.

Linkage in humans is more difficult to assess than in organisms more well suited to genetic studies. Geneticists must look around the population for the types of marriages which have produced children that will yield useful information. These are called "informative matings". Since family sizes are small in humans, data from large numbers of informative matings are pooled and analyzed in the aggregate. With relatively rare characteristics, informative matings are rare, and progress in mapping is correspondingly slow. Details of the methods used for estimating linkage in humans will not be discussed. Further information may be found in Levitan and Montagu (1971), Stern (1973), Emery (1976) and Morton (1962). A diagrammatic summary of the gene map of human chromosomes, as presented by McKusick & Ruddle (1977), is given in Fig. 1.36.

1.2.4.4 Sex-related inheritance. Characteristics controlled by genes situated on the autosomes are inherited in the same way, regardless of the sex of the parents or the offspring. A number of characteristics have been found, however, where inheritance pattern does depend on the sex of the parents and offspring. These patterns can be explained by the fact that the genes for the characteristics are located on the X chromosome. Such genes were originally said to be "sex linked", and are also called "X linked". Females have homologous X chromosomes, while males have an X and a

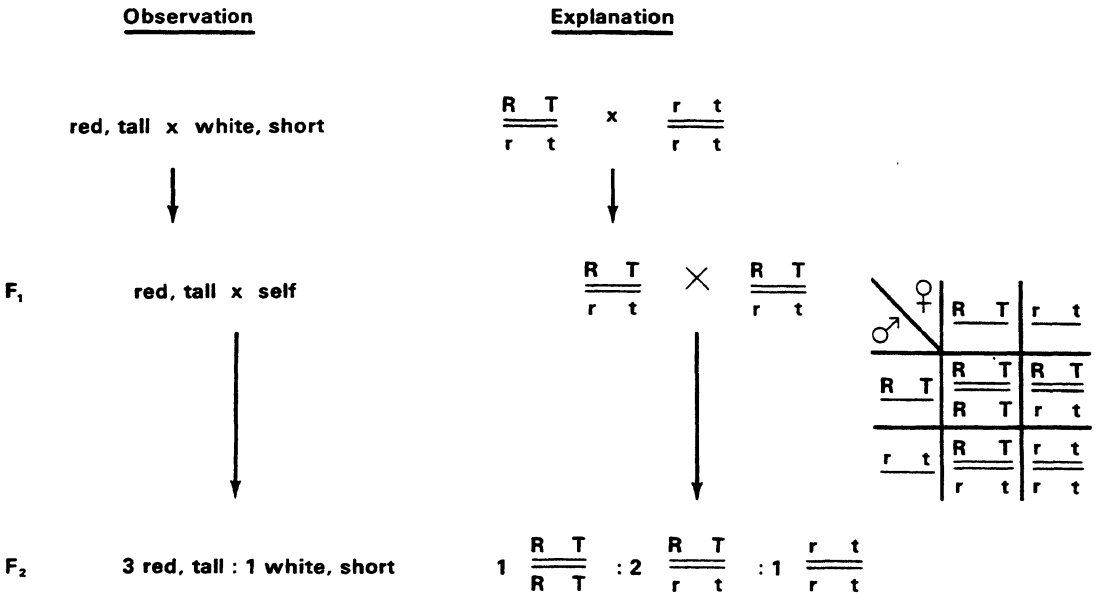


Figure 1.34 Illustration of Linkage

Y, the Y not being homologous to the X. Genes, even recessives, are therefore usually expressed in males, and are inherited from the mother. Females have to be homozygous for a recessive X-linked gene before it is expressed. Males are said to be *hemizygous* for X-linked genes.

There are genes whose expression is different in the two sexes, because of differing degree of penetrance. These are called "sex-influenced". Some genes manifest themselves only in one sex or the other, and these are called "sex-limited".

Of interest in the context of medico-legal examinations are the enzyme G6PD, the Xg blood group system and the Xm serum group system. All the evidence suggests that these are under the control of X-linked genes. McKusick (1964) should be consulted for further information about X-linked inheritance. McKusick has, in addition, compiled an extraordinary reference catalogue of genetically controlled characteristics in humans, according to whether the genes are dominant, recessive or X-linked. Each trait is assigned a catalog number, and a brief description of the characteristic is given in every case with original references. This catalog is now in its 4th edition (McKusick, 1975).

It might be supposed that the X-linked characteristics should be more intensely expressed in females who possess, in effect, a "double dose" of the gene, than in hemizygous males. In a number of cases which have been carefully examined, however, this has not turned out to be the case. Women who are homozygous for G6PD deficiency, for example, have enzyme levels closely resembling those of affected males. This phenomenon has been called "dosage compensation", compensation, as it were, for the double gene dose. This effect is generally not seen in the case of autosomal characteristics, in which homozygotes express characteristics to about twice the extent of heterozygotes. In 1961, Lyon in England put forth an hypothesis which said that only one of the X chromosomes in each cell is actually active (Lyon, 1961, 1962a, 1962b). The decision point, at which one of the X chromosomes becomes inactivated, occurs early in embryogenesis. The descendants of a particular cell abide by the original decision, and the decision is based on chance. Thus, the same X chromosome is not inactivated in every cell. The tissues of heterozygotes would be expected on this basis to exhibit mosaicism, some cells having an active paternal X, while others would have an active maternal X. Studies on several X-linked markers have indicated the kind of somatic cell mosaicism predicted by the Lyon hypothesis. Beutler (1969) and Linder and Gartler (1965) have studied G6PD in this regard. Davidson (1968) found that markers governed by autosomal loci do not show the inactivation characteristics. Deys *et al.* (1965) studied phosphoglycerate kinase, and found the data consistent with X-chromosome inactivation. Heterozygous female subjects with Lesch-Nyhan syndrome due to a genetic absence of hypoxanthine-guanine phosphoribosyl transferase usually show two red cell populations, although in at least one case, a heterozygote for normal and mutant enzyme, the mosaicism could not be demonstrated (McDonald & Kelley, 1972). Another observation can be explained by the Lyon hypothesis, namely, the presence of so-called "Barr bodies". In 1949, Barr and Bertram noted that interphase (non-dividing) nuclei of cells from female cats contained a well-defined mass of chromatin material which is not present in males. This variation was

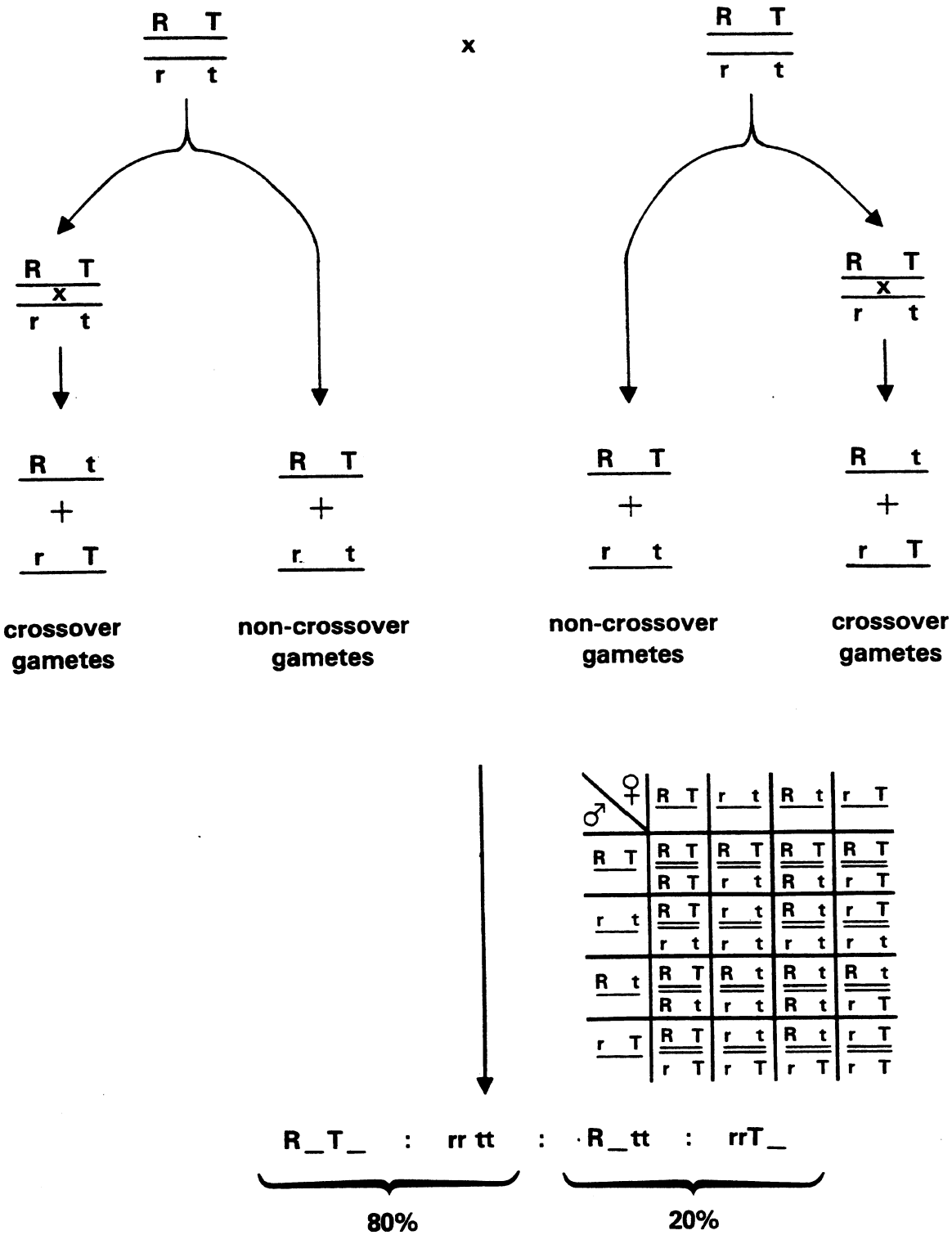


Figure 1.35 Illustration of Crossing Over

Key to Abbreviations in Figure 1.36

ABO	ABO blood group (chr. 9)
ACO	Aconitase, mitochondrial (chr. 3)
ACO-S	Aconitase, soluble (chr. 9)
ACP-1	Acid phosphatase-1 (chr. 2)
ACP-2	Acid phosphatase-2 (chr. 11)
ADA	Adenosine deaminase (chr. 20)
adeB	FGAR amidotransferase (chr. 4 or 5)
ADK	Adenosine kinase (chr. 10)
AdV12-CMS-1p	Adenovirus-12 chromosome modification site-1p (chr. 1)
AdV12-CMS-1q	Adenovirus-12 chromosome modification site-1q (chr. 1)
AdV12-CMS-17	Adenovirus-12 chromosome modification site-17 (chr. 17)
AHH	Aryl hydrocarbon hydroxylase (chr. 2)
AK-1	Adenylate kinase-1 (chr. 9)
AK-2	Adenylate kinase-2 (chr. 1)
AK-3	Adenylate kinase-3 (chr. 9)
AL	Lethal antigen: 3 loci (a1, a2, a3) (chr. 11)
Amy-1	Amylase, salivary (chr. 1)
Amy-2	Amylase, pancreatic (chr. 1)
ASS	Argininosuccinate synthetase (chr. 9)
APRT	Adenine phosphoribosyltransferase (chr. 16)
AVP	Antiviral protein (chr. 21)
Bf	Properdin factor B (chr. 6)
β2M	β2-Microglobulin (chr. 15)
C2	Complement component-2 (chr. 6)
C4	Complement component-4 (chr. 6)
C8	Complement component-8 (chr. 6)
Cae	Cataract, zonular pulverulent (chr. 1)
CB	Color blindness (deutan and protan) (X chr.)
Ch	Chido blood group (chr. 6)
CS	Citrate synthase, mitochondrial (chr. 12)
DCE	Desmosterol-to-cholesterol enzyme (chr. 20)
DTS	Diphtheria toxin sensitivity (chr. 5)
El-1	Elliptocytosis-1 (chr. 1)
E11S	Echo 11 sensitivity (chr. 19)
ENO-1	Enolase-1 (chr. 1)
ENO-2	Enolase-2 (chr. 12)
Es-Act	Esterase activator (chr. 4 or 5)
EsA4	Esterase-A4 (chr. 11)
ESD	Esterase D (chr. 13)
FH-1 & 2	Fumarate hydratase-1 and 2 (S and M) (chr. 1)
αFUC	Alpha-L-fucosidase (chr. 1)
Fy	Duffy blood group (chr. 1)
Gal$^+$-Act	Galactose + activator (chr. 2)
αGAL	α-Galactosidase (Fabry disease) (X chr.)
βGAL	β-Galactosidase (chr. 22)
GALT	Galactose-1-phosphate uridyltransferase (chr. 3)
GAPD	Glyceraldehyde-3-phosphate dehydrogenase (chr. 12)
GAPS	Phosphoribosyl glycineamide synthetase (chr. 21)
Gc	Group-specific component (chr. 4)
GK	Galactokinase (chr. 17)
GLO-1	Glyoxylase I (chr. 6)
GOT-1	Glutamate oxaloacetic transaminase-1 (chr. 10)
G6PD	Glucose-6-phosphate dehydrogenase (X chr.)
GSR	Glutathione reductase (chr. 8)
GSS	Glutamate-γ-semialdehyde synthetase (chr. 10)
GUK-1 & 2	Guanylate kinase-1 & 2 (S & M) (chr. 1)
GUS	Beta-glucuronidase (chr. 7)
HADH	Hydroxyacyl-CoA dehydrogenase (chr. 7)
HaF	Hageman factor (chr. 7)
HEM_A	Classic hemophilia (X chr.)
Hex A	Hexosaminidase A (chr. 15)
Hex B	Hexosaminidase B (chr. 5)
HGPRT	Hypoxanthine-guanine phosphoribosyl-transferase (X chr.)
HK-1	Hexokinase-1 (chr. 10)
HLA	Major histocompatibility complex (chr. 6)
Hpα	Haptoglobin, alpha (chr. 16)
HVS	Herpes virus sensitivity (chr. 3)
H-Y	Y histocompatibility antigen (Y chr.)
If-1	Interferon-1 (chr. 2)
If-2	Interferon-2 (chr. 5)
IDH-1	Isocitrate dehydrogenase-1 (chr. 2)
IDH_m	Isocitrate dehydrogenase, mitochondrial (chr. 15)
ITP	Inosine triphosphatase (chr. 20)
LCAT	Lecithin-cholesterol acyltransferase (chr. 16)
LDH-A	Lactate dehydrogenase A (chr. 11)
LDH-B	Lactate dehydrogenase B (chr. 12)
αMAN	Lysosomal α-D-mannosidase
MDH-1	Malate dehydrogenase-1 (chr. 2)
MDH-2	Malate dehydrogenase, mitochondrial (chr. 7)
ME-1	Malic enzyme-1 (chr. 6)
MHC	Major histocompatibility complex (chr. 6)
MPI	Mannosephosphate isomerase (chr. 15)
MRBC	B-cell receptor for monkey red cells (chr. 6)
NP	Nucleoside phosphorylase (chr. 14)
NPa	Nail-patella syndrome (chr. 9)
OPCA-I	Olivopontocerebellar atrophy I (chr. 6)
P	P blood group (chr. 6)
PepA	Peptidase A (chr. 18)
PepB	Peptidase B (chr. 12)
PepC	Peptidase C (chr. 1)
PepD	Peptidase D (chr. 19)
Pg	Pepsinogen (chr. 6)
PGK	Phosphoglycerate kinase (X chr.)
PGM-1	Phosphoglucomutase-1 (chr. 1)
PGM-2	Phosphoglucomutase-2 (chr. 4)
PGM-3	Phosphoglucomutase-3 (chr. 6)
6PGD	6-Phosphogluconate dehydrogenase (chr. 1)
PHI	Phosphohexose isomerase (chr. 19)
PK3	Pyruvate kinase-3 (chr. 15)
PP	Inorganic pyrophosphatase (chr. 10)
PVS	Polio sensitivity (chr. 19)
Rg	Rodgers blood group (chr. 6)
Rh	Rhesus blood group (chr. 1)
rRNA	Ribosomal RNA (chr. 13, 14, 15, 21, 22)
rC3b	Receptor for C3b (chr. 6)
rC3d	Receptor for C3d (chr. 6)
RN5S	5S RNA gene(s) (chr. 1)
SA7	Species antigen 7 (chr. 7)
SAX	X-linked species (or surface) antigen (X chr.)
Sc	Scianna blood group (chr. 1)
SHMT	Serine hydroxymethyltransferase (chr. 12)
SOD-1	Superoxide dismutase-1 (chr. 21)
SOD-2	Superoxide dismutase-2 (chr. 6)
SV40-T	SV40-T antigen (chr. 7)
TDF	Testis determining factor (Y chr.)
TK_m	Thymidine kinase, mitochondrial (chr. 16)
TK_s	Thymidine kinase, soluble (chr. 17)
TPI	Triosephosphate isomerase (chr. 12)
TRPRS	Tryptophanyl-tRNA synthetase (chr. 14)
tsAF8	Temperature-sensitive (AF8) complementing (chr. 3)
UGPP	Uridyl diphosphate glucose pyrophosphorylase (chr. 1)
UMPK	Uridine monophosphate kinase (chr. 1)

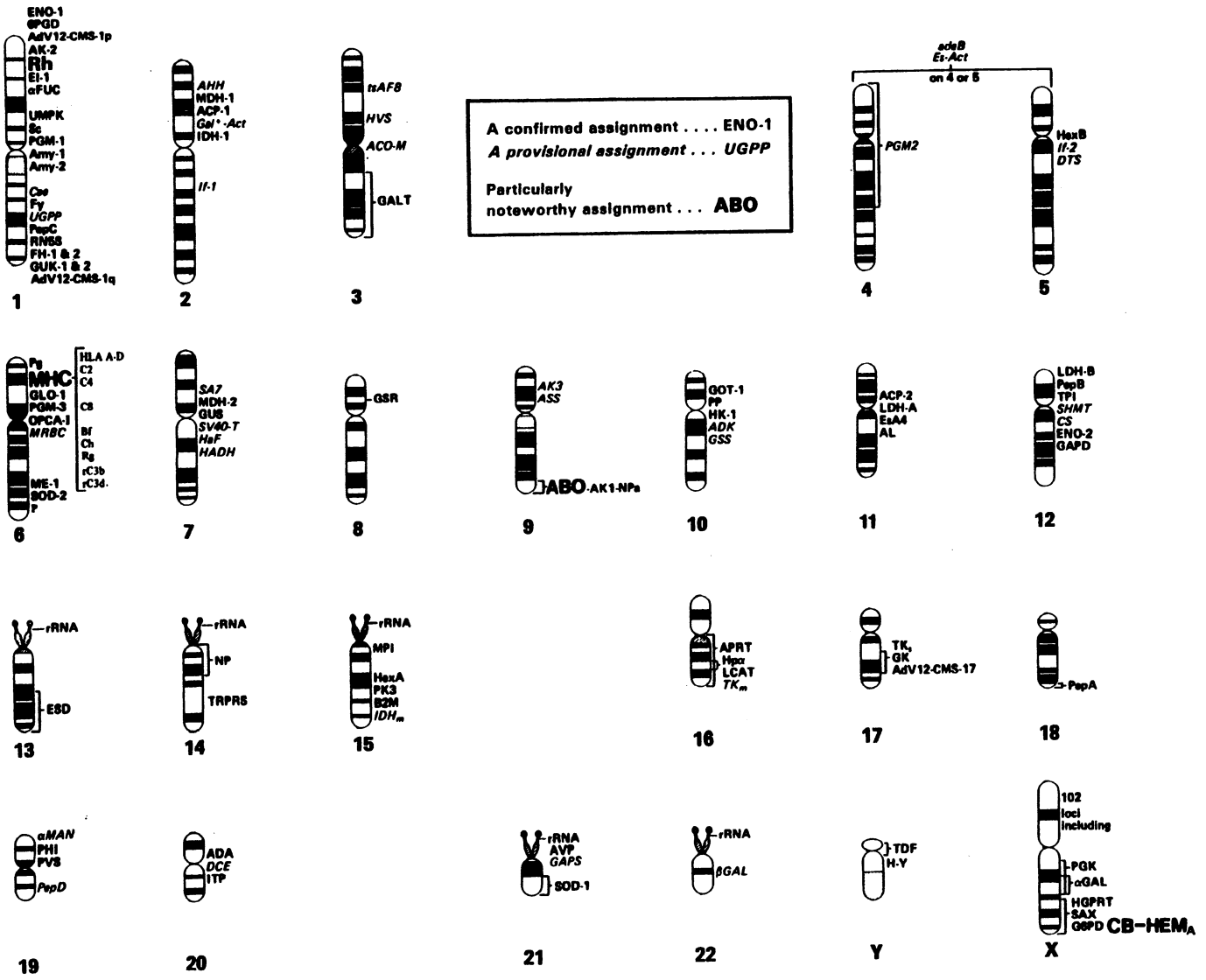


Figure 1.36 Diagrammatic Summary of the Gene Map of Human Chromosomes. An assignment is considered confirmed if it is found in two or more laboratories or several families, and provisional if based on evidence from one laboratory or family.

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soon found in humans as well (Barr & Bertram, 1949; Barr, 1957 and 1960). Barr bodies are quite easily determined in buccal or vaginal epithelial cell smears. A significant number of cells must be scored, since the structure is not present in every cell. In examining the correlation between the number of Barr bodies in cells and the sex chromosome composition of persons with various peculiar sex chromosome abnormalities, it was noted that there is always one less Barr body than the number of X chromosomes in the cell. Thus, XY (normal males), XO (Turner females), XYY and XYYY males have no Barr body, XX (normal females), XXY (Klinefelter males) and XXYY people have one, persons with three X have two Barr bodies and so forth. According to the Lyon hypothesis, the Barr body represents the inactivated X chromosome. The Lyon hypothesis is not universally accepted, other explanations for the observations being possible. One of the puzzling aspects of the notion is imagining a mechanism for the inactivation of an entire chromosome, an event which has virtually no precedent in other organisms. X-chromosome inactivation applies only to the somatic cells. Germ cells do not have Barr bodies.

1.2.5 Mutation

It has been known for some time that the genetic material can undergo spontaneous changes in structure or composition without losing its ability of self-replication. Such changes are often reflected in changes in gene action. Structural changes in the genetic material are called *mutations.* Mutations can be understood in terms of present day biochemical genetics (section 1.2.2). Mutations represent changes in the DNA base sequence which lead to changes in the amino acid sequences of the proteins being coded for. The degree to which the protein is affected depends on which amino acid is affected and its position in the protein. Some amino acids in the protein play key roles in the maintenance of the active conformation, some are involved in the active site, and some can be changed without affecting the catalytic activity very much at all. A single base change in DNA can account for the difference between normal and sickle-cell hemoglobin, a matter which will be discussed in more detail in Unit VII. Hb-S differs from Hb-A in a single amino acid residue, the former having Val where the latter, normal Hb, contains Glu in the beta chain. The codons for Glu are GAA and GAG, but GUA and GUG code for valine. On the other hand, a single base change in DNA could result in no change at all in the protein, and would never be detected. CUA, for example, codes for leucine, but so does CUU. Only in those cases where the amino acid sequence of proteins is known, and some information is available about the three-dimensional structure, can mutations be so clearly understood. It is widely agreed though that the biochemical explanations of mutation based on the well studied cases are universal. There are other ways in which the genetic material can undergo changes, such as chromosomal aberrations. The term mutation tends to be restricted to changes in a single gene.

The mechanism of mutation is not precisely known. Even in those cases where nucleotide base changes can account for the observed results, the exact way in which the changes come about is not clear. Spontaneous mutation rates can be measured. In suitable organisms which have relatively short life cycles, and produce fairly large numbers of offspring, the rates can be measured quite accurately. In humans, the rates are far more difficult to determine, but estimates are possible for some loci. Mutation is a rare event. Different loci have different rates of mutation, and not every allele at the same locus necessarily undergoes mutation at the same rate. Ordinarily, mutation rate for a "normal" to an "abnormal" allele is considered to be the forward direction. The reverse can occur as well, and is called "back mutation". The back mutation rate is generally less than the forward rate.

Mutation can be induced by external factors. Much of what is known about genetics has resulted from the ability to induce mutations artificially in organisms suitable for genetic study. Muller made the discovery that X-radiation can induce mutations in *Drosophila* in 1927, and received the Nobel Prize for this work. Mutation can also be induced by other types of radiation, by a variety of chemicals, and in some organisms by temperature shocks.

1.2.6 Polymorphism

Genetic polymorphism is a type of variation in which members of a population with two or more different characteristics controlled at a particular locus coexist normally, and in such proportions that the rarest of them cannot be accounted for on the basis of recurrent mutation. The concept was first devised by E. B. Ford in 1940, and its implications have been explored in great detail (see Ford, 1965). Loci having alleles which are consistently deleterious to an organism are not considered polymorphic, because the characteristics are being selected against. In some cases, such as that of sickle-cell hemoglobin and several of the other structural variants of hemoglobin, the condition itself, especially in the homozygote, is quite deleterious. But heterozygotes show a considerable advantage in coping with malaria. Many loci exhibiting polymorphism control characteristics whose selective advantage to the organism is not at all clear. Much thought has been given to the mechanisms by which polymorphism is maintained in populations. Several major factors are probably involved. One is selective forces, whether they are recognized and understood or not. Another is recurrent mutation. A third is so-called "genetic drift". Genetic drift refers to changes in the proportions of genes in a population due to chance deviations from the most probable outcome in going from one generation to the next. Said another way, the most probable outcomes in the distribution of alleles into gametes, fertilization and subsequent survival, are not always the real outcomes. There is some tendency on the part of geneticists to regard selective forces as the major factor in maintaining polymorphism, but it is rarely possible to establish with certainty that such is the case, and to understand the forces at work. A great deal of attention has been paid to blood groups in this regard, as well to the

isoenzyme and serum protein polymorphisms. The selective forces in most cases are not that clear.

Genetic polymorphism provides the basis for using genetically determined characteristics as markers in populations. The frequencies of the various markers are known to vary within human populations, a fact which has been of interest to geneticists as well as to physical anthropologists. The determination of a number of markers in an individual along with a knowledge of the frequencies of the markers in a population allows for determination of the expected frequency with which such a person is expected to occur. Medico-legal applications are based in part on this aspect of polymorphic markers. In theory, if enough marker systems could be used, it would be possible to individualize a blood sample, but in practice such a potential is some ways away. Discrimination in populations improves, however, as more and more systems are added to the list of those which are practical and useful in medico-legal inquiries.

1.2.7 Methods in human genetics

Some of the methods used in human genetics will be mentioned briefly. Many good references are available if further information is wanted (e.g. Levitan and Montagu, 1971; Stern, 1973; Emery, 1976; Cavalli-Sforza and Bodmer, 1971; Dahlberg, 1948).

One of the major ways of getting information is through the study of families, and the classical method is pedigree analysis. Pedigrees are simple, diagrammatic representations of the occurrence of a characteristic in a family, often covering several generations. There is not universal agreement on pedigree symbols, but it is common to find circles representing females and squares representing males. Generations are usually designated by Roman numerals, individuals being specified by arabic numbers from left to right throughout the pedigree. Individuals can then be referred to by giving the Roman numeral and the arabic number. Fig. 1.37 shows a hypothetical pedigree for a simple Mendelian recessive trait, and Fig. 1.38 shows a hypothetical pedigree for an X-linked recessive trait. Affected individuals are indicated by blackening the sex symbols. Persons of unknown sex are represented by diamonds. Illegitimate unions are denoted by dotted lines, and consanguineous unions of whatever degree are usually denoted by double connecting lines. Pedigrees should contain an arrow indicating the index case, the first person examined, and the one responsible for initiation of the study. The person is also called the proband, or propositus (fem. proposita). Persons who are dead may be indicated by a cross (†) within or near the sex symbol. Aborted, or stillborn infants are indicated by smaller symbols or by a diagonal line through the symbol.

Studies of twins are used to get information as well. Monozygotic twins develop from the same fertilized egg and are expected to be concordant on all traits. Genetic markers can be used to establish monozygosity or dizygosity in many cases. A number of characteristics are examined, and the probability of the particular combination of traits can be calculated under the assumptions of monozygosity and of dizygosity. The probabilities of the twins being MZ or DZ can then be calculated (see, for example, Wilson, 1970; Selvin, 1970).

1.2.8 Population genetics

Population genetics is based on considerations of all the genes in a population for a characteristic, rather than the genes of any individual or particular mating. Familiarity with statistics and probability is necessary for an understanding of the methods used in population genetics. Only a few aspects of the area will be considered in this section. Further information may be found in Cavalli-Sforza and Bodmer (1971), Dahlberg (1948), Burdette (1962), Emery (1976), Mather (1973), Jacquard (1974), and Crow and Kimura (1970).

1.2.8.1 Hardy Weinberg equilibrium. One of the basic principles of population genetics was derived by Hardy in England and Weinberg in Germany in 1908. Known as the *Hardy-Weinberg Law*, this principle states that subject to certain conditions each genotype in a population will exist in a proportion which remains constant from generation to generation. A population with this characteristic is said to be in "Hardy-Weinberg equilibrium". The following conditions are sufficient for a population to be in Hardy-Weinberg equilibrium.

a. The population is randomly mating. This means that the probability of a mating between two particular genotypes is simply the product of the proportions of those genotypes in the population (i.e., matings are independent of genotype).
b. There is no natural selection.
c. There is no effect from mutation or migration.
d. The population is very "large" (essentially infinite).
e. There is no sex difference in proportions of alleles.

The following example illustrates the principle: Suppose a population satisfies conditions a, b, c, d and e, above with respect to a certain trait controlled by a simple Mendelian pair of alleles A and a. Let our population start with 50% AA and 50% aa individuals. Then, under the random mating, offspring will come from the four types of matings, AA♂ × AA♀, aa♂ × AA♀, AA♂ × aa♀, and aa♂ × aa♀, with equal frequencies of 0.5 × 0.5 = 0.25 (25%) each. It is easily seen (Fig. 1.39) that these matings yield AA, Aa and aa individuals in the proportion 1:2:1, i.e. 25% AA, 50% Aa and 25% aa. If we go through the same exercise with the population of offspring (see Figure 1.40), we find that the following generation is still made up of AA, Aa and aa individuals in the proportion 1:2:1. Every succeeding generation will have this same ratio of genotypes.

The *gene frequency* of an allele is the proportion of all genes at the locus where the allele occurs represented by that allele. In the example of above, where the steady state population consisted of ¼aa:½Aa:¼AA, ½ of all genes are A and ½ are a. The gene frequency of A is 0.5. In this case the gene frequency of a is identical to that of A. In general, in a system consisting of two alleles, A and a, we might designate the gene frequencies as p and q, respectively, where $p + q = 1$.

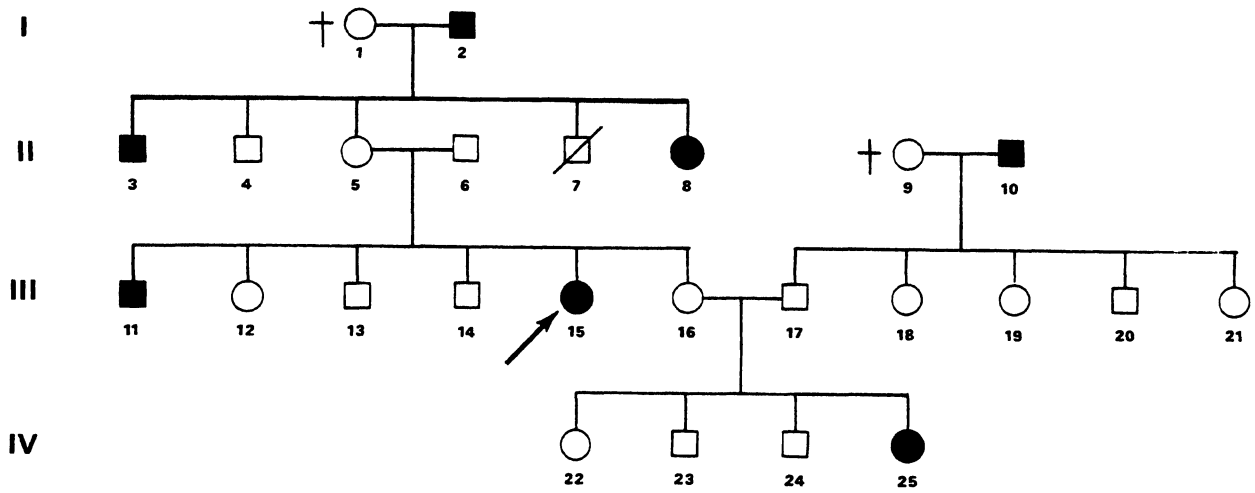

Figure 1.37 Hypothetical Pedigree for a Simple Mendelian Recessive Trait

Then the population is in approximate Hardy-Weinberg equilibrium (at least with respect to random mating) we expect the following proportions: p^2 AA individuals, 2pq Aa individuals and q^2 aa individuals. However, a population exhibiting these proportions exactly (even if p and q could be determined) would be extremely rare. Furthermore, we can examine only a small sample from such a population, and because of statistical sampling error, the observed sample may not be representative of the population. Therefore, statistical techniques are needed to see if the population is behaving according to Hardy-Weinberg equilibrium with variations due to random sampling errors. The main technique used is the Chi-square goodness of fit test.

When performing a Chi-square (χ^2) test we partition the population into several (k) different cells. For each cell we count the observed number of individuals in that cell and the number which would be expected if the population was in Hardy-Weinberg equilibrium and denote these numbers by O and E, respectively. The chi-square statistic is defined by the sum

$$\chi^2 = \Sigma \frac{(O - E)^2}{E}$$

over all cells in the partition. If χ^2 is "large", we take this as evidence that the population is not in Hardy-Weinberg equilibrium; if it is "small", that the population is probably in Hardy-Weinberg equilibrium. To decide what is "large" we must determine the number of *degrees of freedom* of χ^2 and then consult a chi-square table appearing in most statistics or genetics texts (e.g. Fisher, 1970). The table gives us the approximate probabilities of χ^2 from a Hardy-Weinberg population exceeding the tabled values by chance alone. The following examples will illustrate the point. The number of degrees of freedom equals the number of cells minus 1 minus the number of independent parameters which must be estimated from the sample to get the E's, assuming the Hardy-Weinberg conditions hold.

For example, suppose we have a codominant system, i.e., one in which p and q can be calculated since heterozygotes are distinguishable. Moreover, suppose that we "expect" the offspring to be in the ratio 1:2:1 (¼AA,½Aa,¼aa). Note that we are assuming that p=q=½. In a random sample of 100 of the offspring, suppose that our sample exhibited 20 AA, 58Aa and 22 aa individuals. Then, in summary:

Cell	*AA*	*Aa*	*aa*
observed	20	58	22
expected	25	50	25

$$\chi^2 = \frac{(20-25)^2}{25} + \frac{(58-50)^2}{50} + \frac{(22-25)^2}{25} = 2.64$$

There are 2 degrees of freedom (d.f.), computed by subtracting one from the number of cells, i.e. 3. Nothing was estimated from the sample. Using the chi-square table we see that there is a slightly less than 30% chance of getting a sample deviating at least this much from expectation by chance alone.

Another example: Suppose we have the above problem but do not know the population p value. We expect offspring to be in the ratio p^2:2pq:q^2 (AA,Aa,aa). However, we must estimate p (or q) from the sample. Since our system is codominant we can use the phenotypic frequencies to determine the sample gene frequencies. We then use these sample frequencies as our estimates of p and q to determine expected values. In the sample from the above example, there are (2 × 20 + 58) or 98 A genes and there are (200−98) or 102 a genes. Thus we estimate p = 98/200 = 0.49 and q = 0.51, and using these values, we expect offspring to be

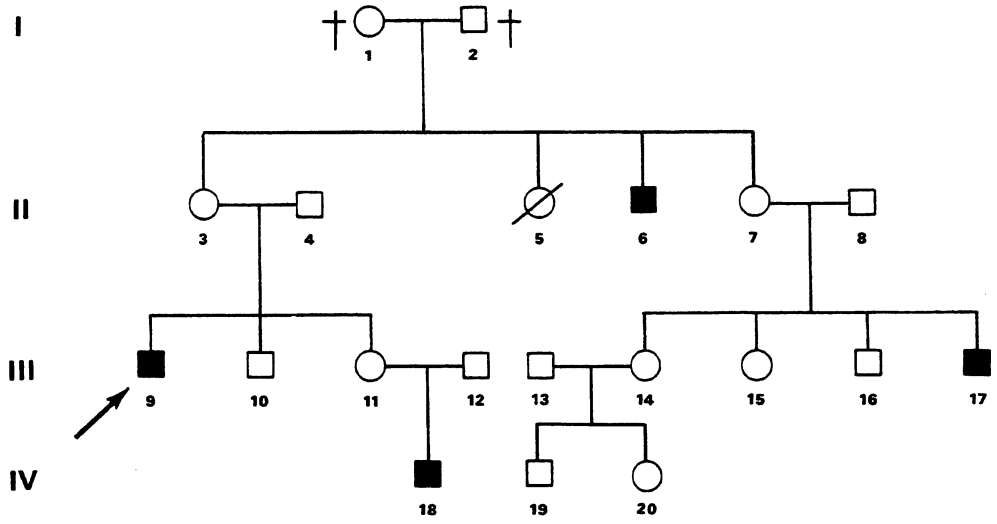

Figure 1.38 Hypothetical Pedigree for an X-linked Recessive Trait

in the proportion $(0.49)^2$ AA:2(0.49) (0.51) Aa:$(0.51)^2$ aa. In summary:

Cell	*AA*	*Aa*	*aa*
observed	20	58	22
expected	24.01	49.98	26.01

$$\chi^2 = \frac{(20-24.01)^2}{24.01} + \frac{(58-49.98)^2}{49.98} + \frac{(22-26.01)^2}{26.01} = 2.57$$

In this case, there is one degree of freedom. We lose one degree of freedom as compared with the previous example due to the fact that one independent parameter had to be estimated from the sample (p and q are related as $p+q=1$). Using the chi-square table we see that there is just about a 10% chance of getting a sample deviating this much from expectation by chance alone.

Many workers say that deviations from expected values are significant if there is less than a 5% chance of getting deviations as large as those observed by chance alone. If the chance is less than 1%, the workers say the results are highly significant. A highly significant result indicates that the population is probably not in Hardy-Weinberg equilibrium.

The Chi-square method may be used in more complex situations, e.g., in cases of multiple alleles. In cases where one gene is dominant or where phenotype does not indicate genotype rather sophisticated statistical procedures must be used to estimate the gene frequencies. Readers interested in this subject should consult mathematical or statistical genetics texts (e.g. Kempthorne, 1969).

There are many possible reasons for deviations from Hardy-Weinberg equilibrium. If any of the sufficient conditions mentioned at the beginning of the section do not hold, then deviations may occur. Selection, heterogeneity of the population, inbreeding and nonrandom mating patterns may all cause deviations. With regard to sampling, in addition to statistical sampling variation, we may not be taking a truly random sample of the population under consideration and we may be making errors in classifying the phenotypes. The work of Brinkman *et al.* (1971) on erythrocyte acid phosphatase types in European Caucasians illustrates, for example, how Hardy-Weinberg equilibrium analysis of data can indicate possible misdiagnosis of phenotypes. Data from a large number of people were pooled and analyzed. Deviations of observed values from those expected in CB and C phenotypes were quite significant, leading to speculation that some laboratories might be misdiagnosing CC phenotypes as CB. It must also be remembered that, since we are usually limited to small samples, our estimates of population characteristics may not be very accurate. If we wish to estimate a gene frequency for a population, then, in general, a large sample will give a more accurate estimate than will a small sample. However, data for a large sample may be too expensive to obtain or may simply be unavailable. This point should be remembered when reading papers that claim 5 place accuracy in a gene frequency estimate based upon a sample of 100 observations.

1.2.8.2 Significance of marker systems. A major objective of the use of genetic markers in blood or body fluids in medicolegal investigations is the establishment of identity or non-identity between samples which are typed and compared. In addition, however, judgments must sometimes be made as to which systems are likely to be most informative. These judgments may involve what is known about the sample's age and history in order to avoid using up samples of limited size in attempts to type markers which may no

♂ \ ♀	½AA	½aa
½AA	¼AA	¼Aa
½aa	¼Aa	¼aa

Figure 1.39 Prediction of outcome of crosses in a population with 50% AA and 50% aa individuals under random mating

♂ \ ♀	¼AA	½Aa	¼aa
¼AA	AA 1/16	1/16 AA, 1/16 Aa } 1/8	Aa 1/16
½Aa	1/16 AA, 1/16 Aa } 1/8	1/16 AA, 1/8 Aa, 1/16 aa } 1/4	1/16 Aa, 1/16 aa } 1/8
¼aa	Aa 1/16	1/16 Aa, 1/16 aa } 1/8	aa 1/16

1/16 + 1/16 + 1/16 + 1/16 AA = 1/4 AA

1/16 + 1/16 + 1/16 + 1/8 + 1/16 + 1/16 + 1/16 Aa = 1/2 Aa

1/16 + 1/16 + 1/16 + 1/16 aa = 1/4 aa

Figure 1.40 Prediction of outcome of crosses in a population with 25% AA : 50%Aa : 25%aa individuals under random mating.

longer be detectable. Such decisions are based on knowledge of the rate at which the various markers become undetectable as a function of time under various conditions.

Another issue, though, is the ability of a system to discriminate among members of a population. It is intuitively clear that the more phenotypes a system exhibits, and the more evenly distributed they are in the population, the greater the chance that two randomly chosen individuals from that population are different (i.e., exhibit different phenotype). Jones (1972) treated this problem quantitatively. In summary, the results given are that for each system, if we have a large enough population and if at least one phenotypic frequency is large compared to population size, then the probability of failing to distinguish between two randomly chosen individuals from that population (probability of identity) is approximated by the sum of the squares of the different phenotypic frequencies. For example, Race and Sanger give the following frequency of occurrence of ABO blood types from a large sample in Great Britain:

Phenotype	A	B	AB	O
Observed Frequency	0.417	0.086	0.030	0.467

The probability of nondiscrimination is given approximately by: $(0.417)^2 + (0.086)^2 + (0.030)^2 + (0.467)^2 = 0.400$. The probability of being able to distinguish between two individuals randomly chosen from the population, i.e., the probability of discrimination, is given by (1 − probability of identity), that is, (1.0 − 0.4) or 0.6 in the above example. This probability is sometimes called the *discrimination index.*

If there are several independent tests on a population for discrimination purposes, then the probability of identity on all of the tests is equal to the product of the probabilities of identity on the individual tests. If, for example, in addition to the 0.4 probability of identity in ABO typing above, we estimated a 0.38 probability of identity in the MN blood group system, then under independence, the probability of identity when using both groupings would be (0.4) (0.38) = 0.152. The discrimination index, therefore, becomes 0.848. Frequently, instead of considering the probability of identity (say P), workers consider the logarithm of the reciprocal of P, -log(P). This value is large when P is small, and small when P is large (Fisher 1951). It has the advantage of being

additive with respect to different independent tests when being used as a discrimination index. This additivity property makes it easier to compare the relative values of different systems when used together for discrimination. For examples of the use of this measure of discrimination, see Fisher (1951).

1.3 Immunology and Serology

1.3.1 Introduction

Since the earliest investigations into the basis of resistance to infectious diseases, and into ways of producing immunity by artificial means, immunology has become a complex and rapidly developing discipline unto itself. It encompasses the subjects of immunity, serology, immunochemistry and immunobiology. As with any rationally developed intellectual discipline, a number of unifying concepts have emerged over the course of time. From the point of view of forensic science, immunological methods and techniques are primary tools for identification and individualization of biological materials. Many aspects of immunology are discussed in subsequent units in the context of their medico-legal applications. Section 1.3 consists of a general discussion of some of the principles of immunology, with emphasis on those that have a bearing on medico-legal tests and methods. Many important topics have been omitted from the discussion because they do not have much direct relationship to forensic immunology. Readers interested in immunology will find considerable information in the books and reviews cited in the reference list.

1.3.2 Antigens

1.3.2.1 The nature of antigens. Antigens are ordinarily defined in terms of their actions and effects. There is no entirely satisfactory definition of an antigen. They have been defined in terms of the antibodies which they elicit; if, however, antibodies are then defined in terms of the antigens which elicited them, a kind of meaningless circularity is introduced. An antigen is a substance which (1) will cause the production of specific antibodies when introduced into an animal and/or (2) reacts in some visible way with antibodies. More generally, it can be said that an antigen is any substance which can induce a specific immunological response in an animal, or which can be involved in a specific immunological reaction, either *in vivo* or *in vitro*. The term *immunogen* has been used to denote a substance which can bring about a specific immunological response *de novo*, as against those substances which can react with preformed antibodies, or evoke specific immunological responses in previously immunized animals, but which cannot elicit *de novo* immunological responses. The latter are often referred to as *haptens*, a term introduced by Landsteiner. Some authors have used the term *hapten* to refer to the specific chemical groupings on antigens with which the antibody interacts, but these groupings are now more often called *determinant groups*.

1.3.2.2 Conditions of antigenicity. A complete understanding of all the properties that render a particular material antigenic in a particular animal has not yet been attained, but some of the criteria are known: (1) Antigens must contain chemical groupings which are foreign to the immunologically competent cells of the test animal; (2) Antigens must have access to the antibody-forming machinery of the test animal; (3) Antigens must possess a certain minimal degree of molecular complexity and size. There are probably other criteria as well, which are not well understood. Proteins and higher MW carbohydrates are immunogenic, but lipids and nucleic acids ordinarily are not, unless they are combined with a protein.

It is an accepted principle of immunology that animals do not ordinarily make antibodies against constituents of their own bodies. This notion was first enunciated by Ehrlich and Morgenroth in 1901:

> In the third communication on isolysins, we pointed out that the organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements, and so giving rise to autotoxins. Further investigations made by us have confirmed this view, so that one might be justified in speaking of a *horror autotoxicus* of the organism.

The "foreignness" of the material does not have to reside in its consisting of an entirely different chemical substance, but can be a function of configuration or of conformation. Generally speaking, the more "foreign" a material is to the animal (i.e. the more taxonomically remote the source of the antigen), the more antigenic it is likely to be. There are apparent exceptions to this generalization, in that constituent molecules of organisms, especially proteins, have evolved at different rates. Thus, certain molecules may be very similar in structure and conformation, even though they come from taxonomically remote animals. Such similarities tend to be found in molecules which are involved in biochemical activities common to most animals, and which are at the same time vital to the maintenance of life. It is as though evolution, proceeding blindly by trial and error, has conserved those structures which were well adapted and which were vital to survival. Organisms which possessed deviant molecules, not as well suited to their function, were selected against, and probably perished. Horse hemoglobin, for example, is poorly antigenic in the rabbit, and insulin, even from remote animals, is not normally antigenic in humans. Immunological reactions can, in fact, be used to assess taxonomic relationships between the source animal and the test animal, a subject which is discussed in more detail in sections 16.8 and 16.9.

Antibodies are not elaborated to an antigen unless the antibody-forming system "sees" them. Antigens must therefore be soluble or solubilizable. Some synthetic polymers are not very antigenic, and the fact that they are neither very soluble, nor very effectively degraded, may account for this observation. The form in which the antigen is presented, and its route of administration are also important factors. It is

usually said that antigens are substances which elicit an immunological response when administered parenterally. Strictly speaking, it does not matter how the antigen is administered, but the fact is that in most instances, the digestive system destroys or greatly alters substances which are ingested. Hypersensitivity to food allergens, however, is clearly the result of antigenic materials which have been ingested. Hypersensitivity is discussed in section 1.3.6. The use of adjuvants is related to the necessity for exposure of the animal's antibody-forming system to antigen. Adjuvants prolong the period during which the antigenic stimulus is operative, and they may also have a role in causing the antigen to come into contact with the right kind of cells.

Low molecular weight compounds are not antigenic. Very few substances with molecular weights of less than 5000 are antigenic. Generally, the higher the MW of an antigen, the more antigenic it is. The reason for this correlation probably resides in the fact that all high MW biological molecules are polymers of smaller molecular weight compounds, whose composition and three-dimensional structure can vary in an enormously large number of different ways. It is likely that larger, and correspondingly more complex molecules, therefore, present the animal with a much larger array of antigenic determinants. Size alone, however, does not determine antigenicity. The composition, and corresponding molecular complexity is important as well. Carbohydrates are generally less antigenic, for example, than proteins of about the same MW and size. Low MW polysaccharides are not particularly antigenic. Dextrans, ranging in MW from 50,000 to 100,000 are antigenic in some species, but not very much so in others. Poorly antigenic molecules may sometimes be rendered more antigenic by absorbing them on particulate materials, such as colloidon or kaolin. Large synthetic polymers, such as polystyrene and nylon, are not antigenic presumably because they are made up of only a few monomeric components, which do not offer the opportunity for very much internal molecular complexity in the finished molecule.

Another criterion for antigenicity may be maintenance of rigidity of structure within the antigenic determinant groups. Thus, there is some relationship between the antigenicity of a protein and its aromatic amino acid content. Aromatic diazo compounds make far better haptens than do long chain fatty acids, presumably because of the rigidity of the aromatic ring and the non-rigidity of paraffin chains.

1.3.2.3 Types of antigens. The terms *autologous, heterologous, homologous,* or *heterophile* are sometimes used in describing antigens. An autologous antigen is one from the same organism, which under appropriate circumstances, would induce antibody formation. Heterologous antigen is a different one from that which was used for the immunization. It may cross react with the antiserum or it may not. Homologous antigens refer to those which were used to prepare the antiserum. So-called "heterophile antigens" are those which exist in very different animals and plants, but which are so closely related structurally that antibodies to one cross-react with the others. In many instances, heterophile antigens are polysaccharides. Human blood group A antigen, for example, is cross reactive with antibodies to pneumococcus capsular polysaccharide type XIV, and human blood group B antigen reacts with antibodies to certain strains of E. coli. Perhaps the best known example of a heterophile antigen is the Forssman antigen. As originally described, Forssman antigen is one present in most guinea pig tissues which would elicit the production of sheep red blood cell lysins in rabbits. The "antigen" is, in fact, a hapten and not a complete antigen, but it is widely distributed in animals and in plants. The nature of the Forssman hapten from sheep erythrocytes was investigated by Diehl and Mallette (1964) among many others.

1.3.2.4 Antigen specificity and the nature of the antigenic determinant. The evidence concerning the relationship between antigenic structure and antigenic specificity indicates that specificity resides in structure. It is fairly clear as well that only a part or parts of the antigen molecule actually induce formation of the antibody. The parts which do so are called antigenic determinants, and they comprise the structural constellation which also reacts with the antibody. When antibodies are obtained to simple chemical compounds, or haptens, by coupling them in some way to larger molecules, the hapten is then a kind of "artificial antigenic determinant". Approaches to the study of the nature of antigenic determinants have consisted of studies on degraded antigens, synthesis of antigens, and alterations of antigens. This subject was discussed by Sela (1971). It has been known for quite some time that antibodies are capable of distinguishing between very subtle differences in antigenic structure. If, for example, L-, D- and meso-tartaric acids are conjugated to proteins by a diazo reaction with the corresponding aminotartranilic acids, and employed as antigens, Landsteiner showed that antibodies prepared with a different carrier protein conjugated with the various isomers of tartaric acid would readily distinguish these stereoisomeric determinants. Degradation studies on "artificial" antigens have indicated that a relatively small fragment of the protein, polypeptides of about 8–12 residues, could inhibit the reaction of antibody with undegraded antigen. The presence of aromatic amino acids in the fragment can also have a disproportionately large effect on the ability of the fragment to inhibit the reaction. The minimum number of residues required to constitute an antigenic determinant in a polysaccharide antigen is also small.

A number of studies on specific protein antigens whose complete structure is known have yielded considerable information about the particular amino acid constellations which constitute the antigenic determinant. These include studies on TMV protein, RNase and lysozyme. Antigens may be said to have "valence". In some cases, the number of antibody molecules which combine with a molecule of antigen can be determined, and the value varies considerably from one antigen to another. It is probable that the value obtained, when the measurement can be made, is a minimal

one, because the antibody molecules are large, and may well sterically hinder one another from combining with every available site on the antigen.

1.3.3 Antibodies

1.3.3.1 Formation of antibodies—The immune response. Antibody formation is induced by administration of an antigen to an animal. The antibody molecules appear in serum, and their formation period is accompanied by the elimination of the antigen from the animal. Characteristically, intravenously injected antigen is cleared from the body in three stages. The first is very rapid (minutes), assuming that the serum concentration is being followed as a measure of clearance, and represents the equilibration between circulating antigen and that in tissues and other body fluids. Phagocytosis in liver and lung is involved in the first stage in the case of particulate antigens. Soluble antigens are removed somewhat more slowly. The second stage is slower and involves gradual catabolic degradation of the antigen. This stage lasts for several days. The persistence of antigen in this stage is, by and large, a function of the enzymatic degradation capability of the host organism. The third stage of antigen removal is again rapid. In this, the immune-assisted stage, antibodies have formed and elimination of the antigen is being enhanced by the combining of antibody with antigen. The amount of detectable antibody in serum with time follows a fairly reproducible course as well. It may be several hours to several days before detectable antibody appears, this depending upon the kind of antigen administered, the route of administration, the species of the host animal and the state of its health. Antibody usually appears within 5–10 days. The latent period reflects the time required for the concentration of antibody to build up to detectable levels within serum, and not an absence of antibody production at the cellular level, for it can be shown that antibody production by isolated antibody-forming cells begins very quickly (within minutes) of exposure to the antigenic stimulus. Antibody titer in serum then increases over the course of a few days to a few weeks, plateaus, and then decays slowly. The exact time course of antibody production varies with test animal, the antigen given, the dosage, the route of administration, and a number of other things. Synthesis of antibody in an animal in response to an antigen never before encountered by the animal is sometimes called the primary response. If a subsequent dose of antigen is administered to the same animal, a so-called secondary immunological response, which differs substantially from the primary response, is seen. Circulating antibody levels decrease briefly in the secondary response because of their combination with the newly administered antigen. Very shortly thereafter, within 2–3 days certainly, antibody titer in serum increases markedly, the increase continuing for several days. The final titer is ultimately much greater than the maximal titer obtained in the primary response. The secondary response is also sometimes called the *memory, anamnestic* or *booster* response. Secondary response time characteristics indicate that the antibody-forming system of the test animal has been in some way "primed" for rapid response to previously encountered antigen. The secondary response represents a rapid synthesis of new antibody, and not a rapid release of prefabricated molecules. It can be induced at almost any time after the primary response has occurred, even after very long time intervals, although the booster response after lengthy time intervals is somewhat less dramatic than that which occurs after shorter ones. The booster response can be repeated a number of times, and cross-reacting antigens will induce it as well, its intensity being directly proportional to the degree of cross reactivity with the primary antigen. The nature of the antibodies is different in the secondary response as well, the serum titer of IgG being very much higher, whereas the primary response tends to give rise primarily to IgM antibodies. IgG and IgM and other immunoglobulin types are discussed below.

The cellular mechanism through which immune phenomena become possible in an animal, and antibodies are thus synthesized, is quite complicated, and not yet fully understood. The spleen, the lymphatic tissues, and bone marrow, as well as the thymus, are involved in immunological responses. The principal cells of the immunological system are lymphocytes, derived from stem cells of bone marrow. They migrate via circulation to various sites and take up residence there. Those which develop in the thymus become so-called T-lymphocytes, or T-cells, while those that develop in the bursa of Fabricius in birds or in its mammalian equivalent become B-lymphocytes, or B-cells. Lymphocytes are resting cells until the cell surface is perturbed by an outside agency, such as so-called mitogenic agents (e.g. concanavalin A or milkweed extract), or an extrinsic antigenic material. Lymphocyte response to the latter is specific; to the former, nonspecific. Response to antigenic material varies considerably with different animals, antigens, and so forth, and the combination of factors ultimately determines what sort of immunological response will be observed. Lymphocytes active in cell-mediated immunity and delayed hypersensitivity responses are T-cells, which do not secrete antibody. Humoral immunity and antibody production are functions of activated B-cells, and it is known that T-cells play some role in this process as well.

There is considerable variation in the immunological response of different animals of the same species. Responsiveness is a function of the genetic makeup of the animal, and of its age. Some animals are not responsive to immunization regardless of the adjuvant or route of inoculation employed. Older animals are generally less immunologically responsive than younger ones.

1.3.3.2 Types and structure of antibody molecules. Antibodies are immunoglobulins. Immunoglobulin molecules consist of two light and two heavy polypeptide chains held together by disulfide bridges through the half-cystine residues in the chains. The polypeptide chain arrangement in rabbit IgG is shown in Fig. 1.41(a), and the diagrammatic structure of a human IgG in Fig. 1.41(b).

Immunoglobulins are distributed in five classes, according to differences in their structure, which, in turn, lead to differences in the character of their own antigenic determinants. Some properties of the five classes of immunoglobulins, IgG, IgA, IgM, IgD and IgE are shown in Table 1.5.

The structure of IgG was solved first, and it is the most well studied of the immunoglobulins. The decisive studies were done by G. M. Edelman in this country, and by R. R. Porter in England, for which they shared the 1972 Nobel Prize for Physiology or Medicine. Edelman found that the purified molecule was resistant to reductive cleavage by sulfhydryl reagents, such as mercaptoethanol, unless it were first treated with high concentrations of urea or guanidine. These compounds interrupt the hydrogen bonds which maintain the unique folded structures of globular proteins, and expose groups within the molecule that are inaccessible when the protein is in its native conformation. In the case of IgG, urea denaturation allowed the sulfhydryl reagent to reduce the -S-S- bonds to free -SH groups. When the molecule was examined after this treatment, it became clear that the original molecule had been split into two pieces, one heavier than the other, and comparison of the MW of the fragments with that of the native protein indicated that the molecule consisted of two heavy and two light fragments. The heavy fragments are usually called heavy chains, or H-chains, the light fragments, light chains, or L-chains. The structure of IgG may be simply represented, therefore, as H_2L_2.

Porter's observations were based on treatment of the native molecule with proteolytic enzymes in a mildly reducing environment. Such treatment results in cleavage of the molecule into fragments of two types, but which are quite different from those obtained by reductive cleavage in urea. One of the fragments crystallizes at 4° and is termed the Fc fragment (fragment crystallizable). It does not bind antigen, and represents the carboxy-terminal ends of the two H chains. The remaining fragment, which still possesses antigen-binding activity, is known as the Fab fragment (fragment antigen binding). Based on the fact that intact IgG molecules can bind two antigen equivalents, and on consideration of the molecular weights, it became clear that the intact protein consisted of 1 Fc and 2 Fab fragments. The Fab fragment consists of an amino-terminal half of an H chain and an L chain. The molecule can be cleaved with either papain or by pepsin, the latter yielding a fragment which is almost identical to Fc (obtained with papain) which is known as Fc′. The papain cleavage point in the molecule is indicated diagramatically in Fig. 1.42. For further details on these studies, the brief but interesting accounts by Porter (1976) and by Edelman (1976) may be consulted. Largely through the availability of relatively large amounts of particular types of molecules from patients suffering from various neoplastic diseases, it has been possible to carry out amino acid sequence studies of some immunoglobulin molecules, greatly increasing our understanding of their structure.

The IgG molecule may have either of two types of light chains, these being designated κ or λ. The H chains of IgG are designated γ chains, to distinguish them from the H chains of the other immunoglobulins. An IgG molecules may, therefore, have the structure $\gamma_2\kappa_2$ or $\gamma_2\lambda_2$. κ and λ chain containing IgG molecules are found in the same individual, but κ and λ chains do not occur in the same molecule. It has been found that certain segments of both the L and H chains tend to vary in amino acid sequence in molecules from different sources while other segments are relatively constant in sequence regardless of source. The former type of segment is referred to as a variable region, while the latter type is known as a constant region. In κ and λ chains, the constant regions, sometimes designated C_κ and C_λ, respectively, consist essentially of the carboxy-terminal halves of the chains. The amino-terminal halves of the chains are variable and are sometimes designated V_κ or V_λ. γ-chains show similar properties in this regard, the amino-terminal segment of about 120 amino acid residues, and residing in the Fab fragment, being variable. This segment may be designated V_γ. The carboxy-terminal segment of the γ-chain, about 325 amino acid residues, may be subdivided into three subregions, each of whose sequences are found to be relatively constant in molecules from different sources. These constant regions are sometimes called $C_\gamma1$, $C_\gamma2$ and $C_\gamma3$ (see Fig. 1.41(b)).

The remaining immunoglobulin classes are distinguishable on the basis of the nature of their H chains. The heavy chains of an IgA molecule are desiganted α, of an IgM molecule, μ, of an IgD molecule, δ, and of an IgE molecule, ϵ. Thus, just as the structure of IgG may be represented by $\gamma_2\kappa_2$ or $\gamma_2\lambda_2$, the structure of IgA is $\alpha_2\kappa_2$ or $\alpha_2\lambda_2$, that of IgD is $\delta_2\kappa_2$ or $\delta_2\lambda_2$ and that of IgE is $\epsilon_2\kappa_2$ or $\epsilon_2\lambda_2$. IgM is a very large molecule with a MW of 800,000–900,000. Its heavy chains are designated μ, and it has been found to consist of five subunits. Its structure is written $(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$.

Edelman (1971) reviewed antibody structure in detail. Detailed studies on the structure of IgA have been carried out by Grez *et al.* (1971) and on IgE by Kochwa *et al.* (1971). Lie *et al.* (1976) reported the complete covalent structure of a human myeloma IgAl immunoglobulin. Kabat (1970) discussed the approaches used to elucidate the nature of antibody combining sites.

It may be noted that the nomenclature of immunoglobulins and their components can become quite cumbersome, since there are a number of different terms and symbols. The nomenclature has been standardized for the most part by international agreement, and the recommendations have been incorporated into the literature rather quickly. For detailed presentations of the recommended nomenclature, see Ceppellini and many others (1964), Kunkel and others (1966), Bennich and others (1968), Asofski and others (1969) and Subcommittee for Human Immunoglobulins of the IUIS Nomenclature Committee (1972). The recommended standardized rules for nomenclature have been followed in the above discussion. None of the older nomenclature has been used. It was formerly acceptable to designate immunoglobulins either by Ig or by γ. Although

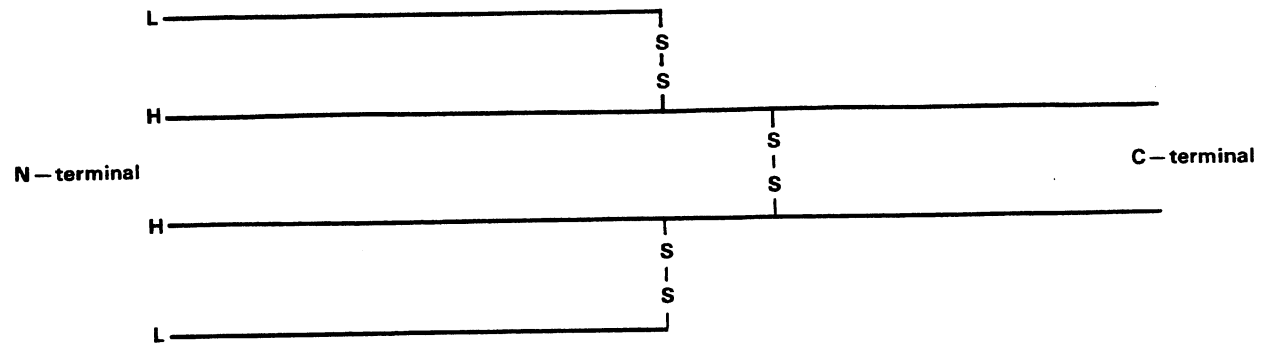

Figure 1.41 (a) Diagrammatic Structure of Arrangement of Polypeptide Chains in Rabbit Immunoglobulin Molecule

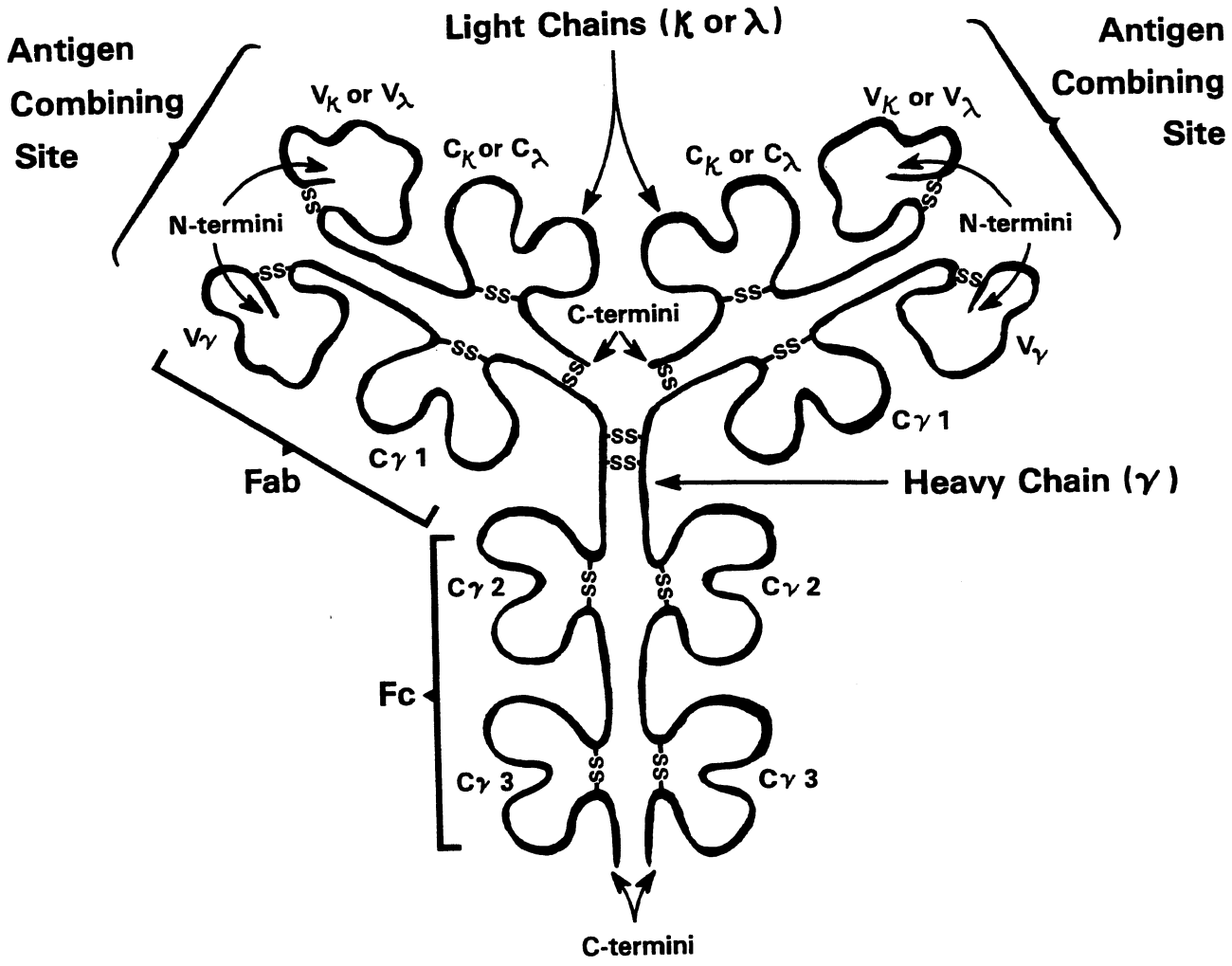

Figure 1.41(b) Diagrammatic Structure of Human IgG Molecule

Table 1.5

Comparison of some properties of the classes of immunoglobulin molecules

Characteristic	Immunoglobulin				
	IgG	IgA	IgM	IgD	IgE
Percent of total immunoglobulin in serum	75-85	5-10	5-10	1	1
Sedimentation coefficient	6-7S	7-11S	19S	6.1S	8.2S
Molecular weight	143,000-149,000	158,000-162,000	800,000-950,000	175,000-180,000	185,000-190,000
Carbohydrate content (o/o)	2.5	5-10	5-10	10	12
Half-life (days)	25-35	6-8	9-11	2-3	2
Stable at 56-60°	Yes	Yes	Yes	Yes	No
Immunologic valence	2	2	5-10	—	—

the nomenclature groups have recommended that the usage of γ as a synonym for Ig be discontinued, one still encounters it. Thus γG would be identical to IgG, γM to IgM, and so forth.

It is worthy of mention here that immunoglobulins are known to exhibit hereditary differences in structure, which can be readily detected using antibodies to specific antigenic determinants within the immunoglobulin molecule. This phenomenon has been termed *allotypy*, and the various subclasses of chains which can be distinguished on the basis of these variations are known as *allotypes*. The most important examples of allotypy from a medico-legal point of view are those occurring in the γ chain of IgG and in the κ light chains. The system of genetic variants which can be detected in the γ chain is known as the Gm system. It is quite complex, and a large number of Gm allotypes have been discovered. The κ chain allotypes constitute what is known as the Inv or Km system. These immunologically detectable variations constitute an important category of genetic markers in human beings, and will be discussed fully in Unit VII, section 44.

The blood group antibodies may belong to several different immunoglobulin classes. Early studies indicated that the isoagglutinin activity of human serum was associated with what are now called IgG and IgM (Pederson, 1945; Deutsch *et al.*, 1947). This activity may also be associated with IgA antibodies (Rawson and Abelson, 1964). Most IgG molecules can cross the placenta, and are associated with hemolytic disease of the newborn (see Fahey, 1970). IgG antibodies are usually poorly agglutinating in saline, but can be detected with anti-human globulin (or Coombs) sera, which are strongly anti-IgG (see section 1.3.4.1), by the use of high protein media, or by the use of proteolytic enzyme treatment of red cells.

1.3.4 Antigen-antibody reactions

It has been customary to regard antigen-antibody reactions as taking place in two stages. It should not be concluded from this statement that there is no overlapping between the stages; indeed, thinking of the reaction as a two stage event is primarily a convenient way of looking at it. It is clear that certain events must take place before others, and the two stage concept helps in understanding what takes place. The first stage of the reaction is the combination of specific groups of the antibody with specific groups of the antigen or hapten. This stage is not accompanied by any visible evidence that any reaction has taken place. In the second stage, observable reactions may take place, such as agglutination, precipitation, lysis, complement fixation, and so on. Bordet was the first to propose this sort of two-stage mechanism for the reaction. The first stage proceeds quickly, and is detectable only indirectly. In the case of small haptens, the first stage may occur without a second stage following at all. The first stage does not require electrolytes, whereas the second stage does, and the second stage can be prevented from occurring by excluding electrolytes. The second stage is slow in comparison to the first. It is thought that the major change in free energy takes place in the first stage. The forces involved in antigen-antibody binding are believed to be relatively weak ones, most likely consisting of a combination of electrostatic, hydrophobic and van der Waals interactions. Antigen-antibody reactions are reversible, and can be treated thermodynamically just like other chemical reactions. The second stage of the reaction is the

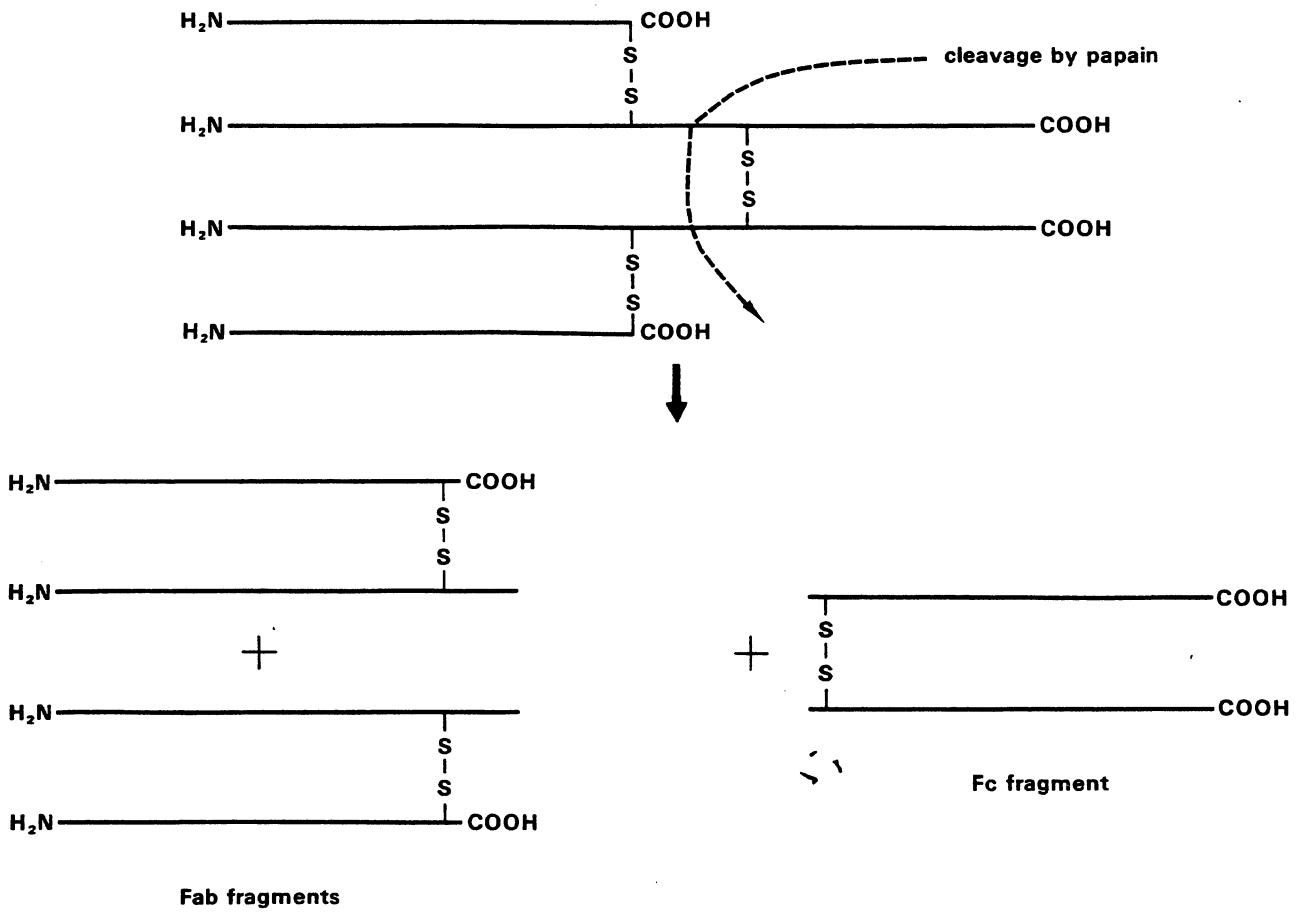

Figure 1.42 Proteolytic Enzyme Cleavage of IgG.

visible, detectable one. Particular antigen-antibody reactions are identified by the kind of second stage reaction which occurs, e.g. agglutination, precipitation, lysis, etc. Antibodies which bring about particular second stage reactions are often similarly identified, e.g. agglutinins, precipitins, hemolysins, etc. Although these descriptive names for antibodies grew out of the period in the development of immunology when it was not yet clear that the same antibody could exhibit many of these functions, the terminology has persisted, and is still useful. The second stage reaction manifestations that are of particular interest in immunological and serological tests in legal medicine are agglutination, precipitation and complement fixation and cell lysis. Reactions involving bacterial cells, or neutralization of toxins by antitoxins are not of major concern in medico-legal techniques. An interesting series of papers in the older literature by Huntoon (1921), Huntoon and Estris (1921) and Huntoon *et al.* (1921) gives a good perspective on the state of knowledge about antigen-antibody reactions and about the nature of antibodies at that time.

1.3.4.1 Agglutination. Agglutinin-containing sera can be prepared against both bacterial and red cell antigens. Other cells, such as yeast, or fungal cells, spermatozoa and leucocytes, can be agglutinated by specific antisera as well, and artificial insoluble particles, such as antigen-coated latex particles, can be used for agglutination tests.

Agglutination is believed to be the result of di- or multivalent antibody molecules being combined with the antigenic determinants of two or more cells, forming a kind of network of links which holds the cells together in an array.

It is well to keep in mind the great differences in size between cells and antibody molecules. Van Oss (1973) has noted that if a red cell were imagined to be about the size of an opened book, then the largest immunoglobulin molecule, the decivalent IgM, would fit comfortably within a lower case "o"; and divalent IgG molecules would be roughly the size of a comma. It is not surprising, therefore, that on the basis of size alone IgM is a much better agglutinin than is IgG. Depending upon the spacing of the antigenic determinants, their total number, and avidity, IgG antibodies

may be incapable of effecting agglutination. Such antibodies are often referred to as "incomplete antibodies", because they do not alone bring about agglutination in saline, and because it was once erroneously supposed that they were monovalent. The IgG antibodies do bind the antigenic determinant on the cells, but may attach with both valences to the same cell. With a concentrated antiserum, containing a high proportion of such IgG molecules, the cell's antigenic receptors may become saturated with them, such that available IgM antibodies cannot bring about agglutination for want of an available binding site. Antibodies which fail to bring about agglutination in saline may be detected by the use of anti-human globulin (AHG) antisera. In this case, the AHG serves as the linking agent to bring about the agglutination of cells sensitized with incomplete antibody in a prior step. Cells which have a specific antibody bound to the antigenic determinant, but which are not agglutinated, are said to be "sensitized". In addition, antibodies which fail to bring about agglutination in saline may do so in high protein media, or if the cells have been treated with proteolytic enzymes. The "incomplete" antibodies are most frequently IgG. A number of studies have focused on the mechanism of agglutination, in an effort to arrive at a satisfactory explanation for these various phenomena.

The antigen-antibody interaction involving red cell antigens may be treated physico-chemically. The material to be discussed here may be found in Mollison (1972), and in the papers by N. C. Hughes-Jones (1974 and 1975). The antigen-antibody reaction may be represented as

$$Ab + Ag \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AbAg$$

where Ab represents antibody, Ag antigen, AbAg the complex, and k_a and k_d are association and dissociation constants. According to the mass action law, the velocity of the forward reaction (to the right as written) is given by

$$v_f = k_a [Ag][Ab]$$

and that of the reverse reaction by

$$v_r = k_d [AgAb]$$

where the terms in square brackets represent the concentrations of the reacting species.

At equilibrium, $v_f = v_r$, and $k_a [Ab][Ag] = k_d [AbAg]$. The equilibrium constant is

$$K = \frac{k_a}{k_d} = \frac{[AbAg]}{[Ab][Ag]}$$

Equilibrium constants may be estimated by putting various red cell concentrations with radiolabelled antibody, and determining the concentration of free antigen when half the Ab is combined with Ag. Under this circumstance,

$$\frac{[AbAg]}{[Ab]} = 1$$

and since

$$\frac{[AbAg]}{[Ab]} = K [Ag], \text{ then } K = \frac{1}{[Ag]}$$

In determining the equilibrium constant for a particular reaction, it is usually assumed that each Ab molecule combines with a single site, and that the total number of available antigenic sites is equal to the total number of Ab molecules of a particular specificity that can combine with a red cell. The equilibrium constants for the reactions of blood group antibodies investigated have K values of the order of 10^8 ℓ/mole, with a range from 10^4 to 10^{10} ℓ/mole. Variation is seen not only between different examples of antisera, but between different antibody molecules in the same antiserum. The measured value is thus a weighted average for the different antibodies within an antiserum. Antibodies with higher K values are more effective in binding to antigen at lower concentrations than those with lower values, as may be appreciated by considering that

$$\frac{[AbAg]}{[Ag]} = K [Ab]$$

Pollack *et al.* (1965) studied the second stage of the agglutination reaction rather extensively from a physico-chemical point of view. Their findings support an hypothesis which emphasizes the importance of the surface potential of the cells in the agglutination reaction, and provides a framework for the explanation of the effect of adding high MW polymers to the medium, and of treating the cells with proteolytic enzymes.

Under ordinary circumstances, the cells in suspension have a net negative surface charge, and are therefore repelled by one another to an extent governed by the surface charge density. When cells are suspended in electrolyte solutions, the ions present orient themselves around the cell surface, the orientation being more orderly if they are closer to the cell. The result is a kind of diffuse electrical double layer about the cell surface, across which exists a so-called zeta-potential (ζ-potential). The stability of a cell suspension is related to the zeta potential. Pollack *et al.* (1965) derived some theoretical relationships between the ζ-potential, the electrophoretic mobility of the cell, the surface charge density, the dielectric constant of the medium, and the "thickness" of the double layer. In this way, a number of measurements could be carried out to determine the effects of changes in the medium, or treatment of the cells with proteolytic enzymes, on the ζ-potential.

As noted above, the addition of certain high MW polymeric colloids to suspensions of red cells, along with specific antisera to a cell antigen, can bring about agglutination which would not occur in their absence, or can greatly enhance agglutination in comparison to what it would have been in their absence. Among the materials that have been employed in this way are serum albumin, ficoll, polyvinylpyrolidone (PVP) and dextran. The results of Pollack *et al.* (1965) indicated that these compounds act by increasing the dielectric constant of the medium, thereby reducing the ζ-potential. The surface charge on the cells was not affected by these materials. There is, however, more to it. Goldsmith (1974) reported his studies on the induction and enhancement of agglutination by bovine serum albumin. In an effort to understand the variable effects of different lots of albumin, it was discovered that different lots contain different amounts of polymerized albumin. The optimal conditions for

agglutination with incomplete anti-D occurred when the preparations contained 85% monomer and 15% polymer. The effect did not appear to be due to an increased uptake of antibody in the sensitization step. As for the effect on the second stage of agglutination, it turned out that some albumin preparations containing relatively large amounts of polymer could induce agglutination in unsensitized cells (false positives). And it could be calculated that the change in dielectric constant with albumin solutions containing different amounts of polymer was not great enough to account for the effect. Studies by Brooks and his collaborators are mentioned which suggested that neutral polymers (such as dextran) did not affect the dielectric constant as Pollack *et al.* had claimed, and that enhancement of agglutination might be the result of intercellular polymer bridging. There is no doubt that albumin does affect the dielectric constant of a solution, but the studies do indicate that the mechanism of enhancement by charged and neutral polymers might not be the same. Reckel and Harris (1978) have recently studied the enhancement of agglutination by polymeric bovine albumin, as compared with the monomeric form. The polymeric form was more effective in promoting agglutination.

Pollack *et al.* (1965) also carried out measurements to determine the effect of treating cells with various proteolytic enzymes. Treatment of cells with papain, ficin, bromelin or trypsin often renders them agglutinable by a specific antiserum containing incomplete antibodies which does not bring about agglutination in untreated cells. The data showed that enzyme treatment of cells brought about a significant, and fairly reproducible, reduction in the net surface charge, and thus in the ζ-potential. The probable mechanism for the change was suggested to be the removal of ionogenic surface groups from the cell membrane, most probably sialic acid. In this regard, the proteolytic enzymes mimic the action of neuraminidase (sialic acid is N-acetylneuraminic acid), and their role in the enhancement of erythrocyte agglutination by specific antibodies was believed to be attributable to their esterase activity rather than to their proteolytic activity. In the case of anti-D agglutination of D cells, enzyme treatment is known to increase the value of the equilibrium constant by two- or three-fold, and this is entirely due to an increase in k_a (Hughes-Jones, 1975). One explanation is that removal of sialic acid-containing peptide from the cell improves the accessibility of antigen sites, because the number of available sites is not substantially increased by the enzyme treatment. The increased uptake of antibody, however, does not explain the enhancement, since, at least with IgG anti-D, the amount of antibody required for agglutination of enzyme treated cells was much less than the amount that could be bound to untreated cells without any agglutination taking place. Gunson (1974) has discussed the mechanism of enhancement by enzyme treatment. He noted that the role of the ζ-potential, as put forward by Pollack *et al.* (1965) has been questioned. Studies in which the electrophoretic mobility of cells is used as a measure of the ζ-potential, and cells are separately treated with neuraminidase and then proteases indicate that neuraminidase treatment alone alters the zeta-potential to about the same extent as papain, but does not enhance agglutination. It is, therefore, apparently not enough to reduce the ζ-potential, or only to remove surface sialic acid residues. It has been suggested that the polypeptide stems may cause steric hindrance to antibody approach in cells treated by neuraminidase alone. Papain and other proteolytic enzymes remove mucopeptide fragments, that is, they remove the polypeptide stems along with the associated sialic acid residues.

Ionic strength is an important factor in the rate of association of antigen and antibody as well. The presence of electrolytes which interact with the charged groups on the reacting species have the effect of reducing the attractive forces between oppositely charged groups on the antigen and antibody. Since the electrostatic interactions contribute to antigen-antibody binding, the association constant k_a is increased as the ionic strength of the medium is lowered. This has been shown to be the case for a number of Rh, Kell, Duffy and Kidd system antibodies (Hughes-Jones, 1975).

Van Oss *et al.* (1978) carried out extensive studies on the mechanism of enhancement of agglutination by a variety of factors, including cell shape, cell distance, cell ζ-potential and degree of hydration of the cell surface. All of these are changed to varying degrees by the action of various soluble polymers, ionic strength, the action of enzymes and interaction with different kinds of antibody molecules. The influence of ζ-potential was said to be fairly minor, compared with cell distance and cell shape.

In addition to being brought about directly by mixing cells with specific antibodies to determinants on those cells, agglutination may be brought about in various indirect ways. Agglutination can thus be used as a tool in the measurement of antigen-antibody reactions. A number of terms are in use to describe the various situations. Agglutination may be active or passive, and either of these types may be direct or indirect. Active hemagglutination refers simply to the agglutination of red cells by antibodies directed specifically against antigens which comprise part of the cell surface. A great many serological tests are done in this way. In direct active hemagglutination, it is necessary only to mix the cells with the antibody reagent under proper conditions. In some cases, involving antisera containing large amounts of IgG antibodies, the antibodies bind the antigenic receptors of the cells, but do not bring about agglutination directly, as mentioned above. In these situations, agglutination may be brought about using specific antibodies against the IgG molecules. Known as indirect active hemagglutination, this process demonstrates the presence of the "incomplete" or "blocking" IgG antibodies on the cell's antigenic receptors. It is often called a Coombs test, after Dr. R. R. A. Coombs, who, with Mourant and Race in 1945, first described it for the detection of incomplete Rh antibodies. The antihuman globulin serum is often called Coombs serum (see Fig. 16.1). Passive hemagglutination refers to a situation in which cells are agglutinated by specific antibodies directed against soluble antigens which have been absorbed onto the cell surface.

Many polysaccharide antigens spontaneously absorb onto the red cells. Protein antigens do not readily absorb onto red cells, but will do so if the cells have been treated with dilute solutions of tannic acid at 37°. These so-called "tanned red cells" can be treated with a soluble antigen, washed, and will then be agglutinated by specific antisera against the antigen. Protein antigens can also be chemically coupled to red cells by diazotization reactions. Passive hemagglutination, like active hemagglutination, may be direct or indirect, depending upon whether Coombs serum must be employed to bring about the agglutination. Tanned red cell techniques are discussed in more detail in section 16.3. Anti-human globulin inhibition tests for the determination of the human origin of bloodstains are discussed in sections 16.2, and represented diagramatically in Figures 16.1 and 16.2.

Studies on the temperature dependence of agglutination have resulted in the antibodies being divided into two classes, those whose reaction is not greatly affected by temperature (the "warm" antibodies), and those in which agglutination strength increases as temperature is lowered ("cold" antibodies). Present evidence suggests that the effect is primarily on the first stage of the reaction (Hughes-Jones, 1975). On thermodynamic grounds, the phenomenon may be understood on the basis of the fact that exothermic reactions proceed further to completion as temperature is lowered. The "cold" antibody reactions would be expected to be exothermic, while "warm" antibody reactions would not be expected to be exothermic (i.e., primarily entropy driven). Such data as there is indicates that those expectations are true. Williams (1971) discussed various aspects of the structure and reactivity of cold antibodies.

Zone phenomena may occasionally be observed with agglutinating antisera, depending upon the technique being used and the composition of the antiserum. If a series of successive dilutions of antisera is prepared, and tested with a constant concentration of cells, three "zones" of reactivity may be observed. At high antiserum concentrations (low dilutions), no agglutination is observed. This part of the series is often called the "prozone" or the "prezone". As the dilution of antiserum becomes greater, agglutination is observed, this segment of the series being called the "equivalence zone". At sufficiently high dilutions of the antiserum, agglutination is once again not observed, this part of the series being known as the "postzone". Agglutination is thought to take place by antigenic determinants entering into specific combination with antibody binding sites in a relatively strong, but dissociable combination. A dynamic process of dissociation and recombination then occurs until a stable network of alternating antigen and antibody molecules is formed. At the equivalence point, all the antigen and antibody are consumed in the lattice array. In the prozone, where there is excess antibody, agglutination is not observed because, when an antibody dissociates from an antigenic determinant, there is a higher probability of the vacated determinant being re-occupied by a free antibody molecule than by one which is already bound to another cell. At the other end of the titration series, in the postzone, there is too little antibody present to bring about a complete reaction.

The term "mixed agglutination" is used in different senses. In general, it refers to the demonstration of the presence of antibodies on the surface of a cell (or other surface) which were raised in species X by noting that red cells, coated with an incomplete antibody raised to the cells in species X, are agglutinated by a linking antibody to species X immunoglobulins, raised in species Y. The antibodies one wishes to detect in this way may be bound to receptors on a red cell, a tissue surface or the surface of an object, such as a bloodstained thread. The presence of rabbit antibodies on sheep red cells could be demonstrated, for example, using sheep red cells coated with rabbit anti-sheep red cell antibodies as indicator cells, and goat anti-rabbit antibodies as linking agents. A mixed agglutination technique applied to species-specific tissue antigens is discussed in section 17. The term mixed agglutination can have a slightly more restrictive meaning as well. It refers to a general technique in which specific antibodies are used to detect the presence of antigen on a cell or other surface by virtue of cross-linking the antigen-containing cell or material to red cells containing the same antigen. Mixed agglutination may be used for determination of the ABO groups in bloodstains (Section 19.10.3.5), body fluid stains (Section 19.10.5.2) and tissue cells (Section 19.10.5.3). A mixed agglutination test for species of origin can be done as well. The technique employs anti-human globulin (AHG or Coombs) serum, and is based on the presence of human globulin in a bloodstain of human origin. The method, called "mixed antiglobulin technique" by its originators, is discussed in Section 16.4 and diagramatically represented in Fig. 16.3. The term "mixed hemagglutinative system" was used in the past in an entirely different context. Hooker & Boyd (1937) employed the term to describe a system in which a mixture of human and chicken red cells were agglutinated simultaneously by anti-human and anti-chicken agglutinin sera. The experiments were based on earlier studies by Topley *et al.* (1935) who had studied a similar system using different strains of bacteria and their corresponding agglutinins. Wiener and Herman (1939) extended these studies. Because of this earlier meaning of the term "mixed agglutination" in the literature, Akaishi (1965) argued that the method for determining the ABO group in bloodstains by cross-linking the indicator cells with the antigenic determinants on the stained material, as first elaborated by Coombs and Dodd (1961), should be called "group specific double combination method". The Japanese workers have tended to use Akaishi's term rather than "mixed agglutination" in the literature. Other terms, which are introduced in the sections devoted to the particular application, have been used in the literature as well.

Agglutination of inert particles coated with antigen is also possible with specific antibodies. The particles serve as inert carriers of antigen in these cases. Latex particles are used for a number of tests, and this subject is discussed in connection with its medico-legal applications in Section 16.5.

If the conditions for carrying out a serological test are to be optimized, the concentrations of antigen and antibody must be adjusted so that maximal antigen-antibody interaction takes place. It is customary to test the strength of an antiserum by preparing a series of doubling dilutions of it, and testing these with a constant concentration of cell suspension (antigen), the last tube in the dilution series showing agglutination being called the *titer.* With red cell antigens, this technique is about the only one available for estimating the strength of an agglutinating antiserum. It should be recognized, though, that it is fairly crude. Antisera will vary in their content of various immunoglobulin types, which in turn vary in their valencies. The proportions of complete and incomplete antibodies may vary from one antiserum to another. And antibodies vary in their binding potency toward an antigenic determinant. Antisera consist of a population of antibodies which, although they possess the same immunological specificity, vary in their reactivity with the antigen. This property has been called the "antibody spectrum". A closely related concept is that of so-called "avidity" of antibodies. Avidity refers to the strength with which antibody binds to its antigenic receptor. The speed with which an agglutinin serum brings about agglutination has been taken as a measure of its avidity. The notion of avidity was first applied to antitoxic sera, based upon the observation that the curative effect of antitoxic sera did not always parallel their antibody content. Jerne (1951) carried out extensive studies on this subject. The term "avidity" is still used to describe the affinity of antibodies for their antigenic receptors. The titer obtained with an agglutinin serum depends upon the conditions employed, because many antisera contain mixtures of antibodies with different characteristics. An antiserum will frequently yield different titration values, depending upon whether the tests were carried out in saline, in the presence of protein media or other colloids, by the Coombs technique, or with enzyme treated red cells. These factors must be taken into account in evaluating an antiserum for any particular kind of test. Depending upon the conditions under which the tests are carried out, terms such as "saline titer", "albumin titer" or "papain titer" are sometimes used. In medico-legal work, evaluation of an antiserum will be based on the intended application, e.g. whether it is to be used for grouping whole cells or dried bloodstains, which blood group system is under consideration, which technique is to be used, and so forth. Evaluation of antisera for particular medico-legal applications will be discussed further in connection with the applications and systems themselves as they come up in subsequent units.

1.3.4.2 Precipitation. Precipitating antibodies are of importance in medico-legal investigations, the use of precipitin sera against species-specific proteins being the most widely employed method for determination of the species of origin of blood or body fluid stains (see section 16.1). Precipitin sera directed against specific body fluid proteins may be employed to identify the body fluids as well (see sections 7.1, 10.3.1, 11.4 and 12.7).

Precipitation may be regarded as a second stage in an antigen-antibody reaction in the same way as was agglutination, the first stage consisting of the combination of specific antigen with specific antibody. Assuming that the antigen/antibody ratio is within the optimal range, precipitation then occurs due to extensive cross linking between antigen-antibody complexes. Precipitation occurs in part because of the large size of these lattice structures, and also probably because the combination of antigen with antibody masks some of the polar groups which are important in maintaining macromolecules in solution, and thus allows aggregation through the interaction of apolar groups.

Precipitin systems exhibit zone phenomena. Because precipitin sera very quickly become ineffective upon dilution, it is the usual practice to carry out successive dilutions of antigen and test them with constant, relatively high concentrations of antisera. In a titration series, therefore, antigen is ordinarily in excess in the first few tubes, and antibody is in excess at the end of the series, this in contrast to the situation in titrating agglutinin sera where the antibody is diluted, and where antibody excess thus occurs at the beginning of the series. Therefore, what would be called a "prozone" in the terminology of agglutinin titrations where antibody is in excess, would occur at the end of a precipitin titration series. For this reason, a prozone on a precipitin titration, so named because it occurs at the beginning of the series, may correspond to a postzone in an agglutination titration.

Classically, the amounts of precipitate obtained were determined quantitatively by measuring the total antibody-nitrogen in the washed precipitate by means of the Kjeldahl method. With polysaccharide antigens, total-N was a direct measure of antibody, since the polysaccharides contained no nitrogen. In the case of protein antigens, the N-contribution of the antigen had to be subtracted from the total. It is now more common to carry out quantitative precipitin tests by turbidimetric techniques. One of these, the method of Boyden and his collaborators, is discussed in section 16.8. Another method which has been employed for determining the optimal proportion of reagents is determination of the velocity of precipitate formation. This procedure has been carried out in two ways, the first employing constant concentration of antiserum and varying antigen, and the second, varying the amount of antiserum while holding antigen constant.The former is sometimes referred to as the Dean and Webb procedure (Dean and Webb, 1926), while the latter is called the Ramon technique (Ramon, 1922a, 1922b, 1922c). Marrack (1938) referred to Dean and Webb's technique as the α procedure, and to Ramon's as the β procedure, this terminology being occasionally encountered in the literature. Ordinarily, it is found that the optimum obtained by the α-procedure is not the same as that obtained by the β-procedure using the same serum. Boyd (1941) pointed out that most precipitin antisera could be categorized into one of two classes, R or H, based on their behavior in α- or β-procedure titrations. R-type antisera give only one optimum, this with the α-procedure. H-type antisera give optima with both procedures. H-type antisera were originally

designated as such, because they were typified by a horse antitoxin serum, while R type behavior characterized many antisera prepared in rabbits. It is not universally true, however, that all rabbit antisera are of the R type, nor that all horse antisera are of the H type. Boyd (1966) discussed this matter in his text as well. Because H type antisera tend to inhibit precipitation when present in excess, it has been common to prepare antisera to species-specific serum proteins in rabbits for medico-legal testing. This matter is taken up in section 16.1.1. It may be noted also that the optima obtained with the α- and β-procedures, which are based on the *velocity* of the reaction, do not necessarily correspond to the equivalence zone as determined by the *amount* of precipitate obtained with varying concentrations of antigen and antibody.

It is common today to conduct precipitin tests in gel media. The development of these techniques is discussed in section 16.1.2. Immunoelectrophoresis and immunodiffusion are also discussed in section 2.

1.3.4.3 Radioimmunoassay. Radioimmunoassay (RIA) has become an important technique in the assay of a variety of biological materials. The method combines the specificity of antigen-antibody reactions with the sensitivity afforded by the use of radioactively labelled tracers. A properly constructed radioimmunoassay can be extraordinarily sensitive. The method is discussed briefly in this section, because RIA assays of steroid hormones have been applied to the medico-legal determination of the sex of origin of bloodstains.

In theory and in practice, RIA may be carried out using either radiolabelled antigen or radiolabelled antibody. Assays in which radiolabels have been introduced into antibodies have been described, but those employing radiolabelled antigen or hapten have been more common. In applying the method to low MW molecules, it is generally easier to introduce the label into the hapten than it is into the antibody.

Determination of the concentration of some material by RIA (assuming radioactive antigen or hapten is to be used) requires a specific antibody to the antigen or hapten, radioactively labelled antigen or hapten, and unlabelled antigen or hapten. A method for separating antibody-bound antigen or hapten from unbound material is also required. The assay is based on the ability of unlabelled antigen to inhibit competitively the binding of radioactively labelled antigen by the antibody. Labelled and unlabelled antigen are incubated under proper conditions with antibody, and the antibody-bound antigen is separated and counted. By carrying out a series of measurements in which the amount of unlabelled antigen is varied, a standard curve can be constructed. The standard curve relates the counts observed to the amount of antigen present in the sample over a range of antigen concentration. There are a number of different ways of handling the data to plot the standard curve. The amount of antigen or hapten in an unknown sample may then be determined by reference to the standard curve, or, by suitable computational manipulation of the data, by calculation from the standard curve data.

There are many practical details and problems associated with radioimmunoassay procedures. A good general reference to the technique is Parker (1976). Details of more specific applications may be found in Luft and Yalow (1974). Steroid and steroid hormone RIA methods are discussed in detail in Abraham (1974) and in Jaffe and Behrman (1974).

1.3.5 Complement and complement-mediated reactions

1.3.5.1 Introduction. Complement is a collection of proteins in serum which, acting in concert, participate in a variety of immunological reactions. The complement system may be activated under suitable conditions, and will combine with antigen-antibody complexes to give irreversible structural and functional changes in membranes and cell death, or to activate various specialized cell functions. The components of complement react in a particular order, the overall reaction sometimes being referred to as the complement sequence, or the complement cascade. The reaction of the complement components with an antigen-antibody complex is also referred to as "complement fixation". One of the more striking activities in which complement participates is red cell lysis, or hemolysis. It has been common, therefore, to use red cells, sensitized with an antibody to a membrane antigen, as a hemolytic indicator system. If an antigen-antibody reaction has been allowed to take place in the presence of complement (many vertebrate sera contain complement, but guinea pig serum is a particularly rich source), the mixture can then be tested to see whether complement is still present. A common indicator system is sheep red cells, sensitized with rabbit antibody. If hemolysis occurs upon addition of the indicator cells, complement is still present, but the absence of hemolysis indicates that complement has been "fixed" in the original antigen-antibody reaction. This kind of procedure is known as a complement fixation test. Complement fixation tests have been used in medico-legal tests for species of origin, and this subject is discussed in greater detail in section 16.6.1.

Complement has had a number of different names. Buchner (1899 and 1900) had first referred to it as "schutzstoff", but introduced the name "alexine". The latter term was adopted by Bordet and his collaborators (see, for example, Bordet and Gay, 1906). Ehrlich and Morgenroth (1899a and 1899b) had first referred to complement as "addiment", but introduced the term "complement", which has persisted to the present time, soon afterward.

1.3.5.2 Nature and properties of complement. There are now known to be two operationally distinct complement pathways, each composed of a number of different proteins. The classical pathway, activated by IgM or IgG complexes, consists of 11 proteins which have been grouped into three functional units. The recognition unit is composed of components C1q, C1r and C1s, the activation unit consists of C2, C3 and C4, and the membrane attack unit consists of C5 through C9 (Müller-Eberhard, 1975). The alternative, or properdin pathway, activated by IgA aggregates and by

polysaccharides and lipopolysaccharides, consists of 5 proteins, one of which is C3. The alternative pathway bypasses C1, C2 and C4 and acts on C5–C9 in the same manner as the classical pathway. In addition to the proteins already mentioned, there are several others involved in the system as well. The remainder of the discussion deals with the classical pathway.

The components of the complement system are designated C1, C2, C3, etc., through C9. They were formerly designated C'1, C'2, C'3, etc., through C'9. The three subcomponents of C1 are designated C1q, C1r and C1s. Physiological fragments of components, which result from cleavage by enzymes within the complement system, are distinguished by lower case arabic letters, e.g. C3a, C3b. If a component, or composite product consisting of a number of components, possess enzymatic activity, a bar may be written above the component(s) in which the enzymatic activity resides. The complement cascade sequence is indicated in Fig. 1.43. This scheme gives but a brief notion of the sequence of events, and is a great oversimplification of the molecular complexity of the system. C1 is a Ca^{++}-dependent complex of C1q, C1r and C1s, and reversibly combines with IgG or IgM. Recognition of the immunoglobulin resides in C1q. The C1q binding site resides in the Fc fragments of IgG or IgM. C1q binding initiates a change in $\overline{C1r}$, which acquires enzymatic activity and converts C1s to $\overline{C1s}$. $\overline{C1s}$ can act upon C4 and C2 yielding C4a, C4b, C2a and C2b fragments. C4b can bind $\overline{EAC1}$ and C2a can bind EAC14b to form EAC14b2a. The $\overline{C42}$ complex is a C3 convertase, which cleaves C3 into C3a and C3b. C3b can bind EAC14b2a. The interaction of C3 with C3 convertase gives rise to a $\overline{C423}$ complex which is a C5 convertase, yielding C5a and C5b from C5. The attack on C5 by its convertase initiates a self-assembling process which gives rise to a stable C5b–C9 complex without further enzymatic intervention. The complete complex on the membrane gives rise to the ultrastructural lesions which are the underlying cause of cell lysis. It is to be noted that the various complement components and fragments are known to be involved in other immunological reactions in which the complement system participates. C5a and C3a, for example, participate in anaphylactic release of histamine from mast cells, leading to an increase in capillary permeability. A brief, highly readable review of the complement system was given by Meyer (1976) and a somewhat more involved review was done by Müller-Eberhard (1975).

1.3.5.3. Complement fixation. The major reason for discussing complement fixation is that it can be used as an indicator of antigen-antibody reactions. In general, it is exceedingly sensitive, and is often the method of choice in particular tests. It is common to use sheep red blood cells which have been sensitized with rabbit antibody to them. The antibody, or hemolysin, was called "sensibilisatrice", or "sensitizer" by Bordet and his collaborators (Gengou, 1902; Bordet and Gay, 1906). Ehrlich and Morgenroth (1899a) referred to the hemolysin as the "immunkörper", or immune body, at first, following Pfeiffer's terminology. They later called it the "interbody", and in 1901, introduced the term "amboceptor" which persisted in the literature for quite some time. Complement is very labile, and complement fixation tests must be carried out with careful attention to the proportions of reagents used.

A *unit* of complement is usually taken as the smallest amount which will produce complete hemolysis at 37° in a specified amount of time with specified quantities of cells and amboceptor. Amboceptor concentration must be adjusted so that it is not present in excess. Guinea pig serum is the best source of complement for the sheep red cell hemolytic system. Complement must be titrated each day. Complement fixation tests may be qualitative or quantitative. The quantitative methods are based on the measurement of the Hb released from a fixed amount of red cells spectrophotometrically. Such techniques can be used to carry out titrations of unknown antigens for which pure antibody is available, and vice-versa, since there is a direct relationship between the extent of antigen-antibody reaction and the amount of complement fixed. Complement may be fixed *in vivo* in circumstances where immunological reactions are taking place, whether these be in defense against infectious agents, or in pathological conditions.

1.3.6. Hypersensitivity

Although hypersensitivity is not directly related to most current medico-legal applications of immunology, it constitutes an important area. Hypersensitivity tests have been proposed for medico-legal applications in the past, and although they are impractical and were never widely employed, the are of some historical importance (sections 10.3.2. and 16.6.2).

Hypersensitivity refers to a state of increased reactivity to a foreign agent based on prior exposure to the same or a chemically similar agent. The term *allergy* is often used synonymously, although originally it had a broader meaning. A wide variety of substances can induce hypersensitivity, and the generic term *allergen* is sometimes employed to designate these materials. Sensitization requires one or more exposures to the allergen, a latent period and a subsequent exposure which elicits the reaction. Originally, hypersensitivity was classified as *immediate* or *delayed,* depending upon the speed with which a sensitized individual displayed a reaction to the inciting agent. It is now clear that the mechanisms of the immediate and delayed types of hypersensitivity are different. Immediate hypersensitivity is antibody mediated, while delayed hypersensitivity is cell mediated. Reaction in the former case is the result of the specific interaction of specific antibodies with the allergen, and sensitivity can be transferred passively to a normal individual by transferring serum from a sensitized one. In delayed hypersensitivity, the triggering event involves the interaction of allergen with antibody-like receptors on the surfaces of sensitized lymphocytes. The transfer of serum from a sensitized individual to a normal one does not bring about the passive transfer of delayed type hypersensitivity.

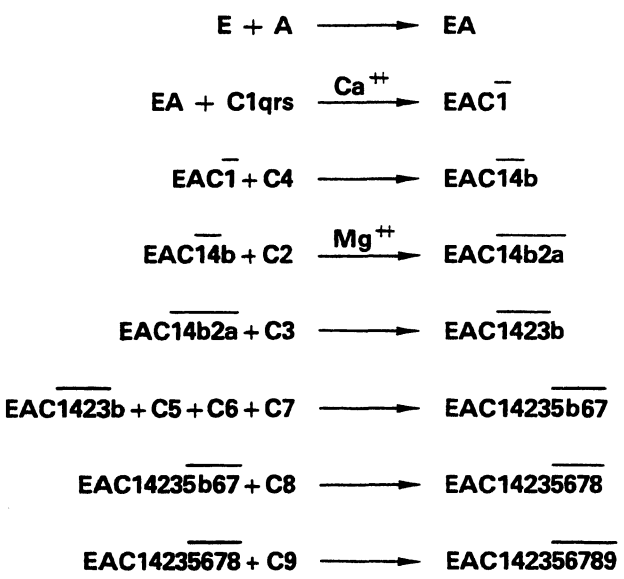

Figure 1.43 The Complement Cascade. E = red cell A = antibody (Igm or IgG) C1 through C9 = complement components

1.3.6.1. Immediate hypersensitivity. Immediate hypersensitivity reactions are due to humoral antibodies combining with a specific antigen, and resulting in the production of cell injury and inflammation. Examples of this type of hypersensitivity include anaphylaxis, atopic disease and serum sickness.

Anaphylaxis is a term which originated with Portier and Richet in 1902. They attempted to immunize dogs against toxic extracts of eel serum or sea anemone tentacles. Reinjection of the substance some weeks later, even with doses sublethal to normal animals, resulted in violent illness and death. Since this phenomenon represented the reverse of prophylaxis, or protection, they termed it "anaphylactique". It is now clear that a variety of antigens can bring about anaphylactic reactions. The first, or sensitizing, dose is given intradermally, intraperitoneally or intravenously. Antibodies develop over the course of the ensuing 2 to 21 days, and are present in serum as well as being fixed to cells in some tissues. The second injection, called the shocking, or challenging, injection, is given intravenously or intracardially, or may be given by aerosol, and the dose is usually several times greater than was the sensitizing dose. Antigen reacts with the cell-fixed antibodies, causing mediators to be released which then give rise to the pathological symptoms. The symptoms can be characteristic primarily of one or a few organs, or can be virtually systemic, depending upon the species of animal, the mediators and so forth. Anaphylaxis in human beings is mediated by IgE immunoglobulins with rare exceptions.

Atopic diseases are mainly common allergies, such as hay fever, asthma and eczema, and represent another kind of immediate hypersensitivity reaction. The antibody involved in these syndromes, IgE, was classically termed "reagin". Human reaginic antibody does not appear to be artificially inducible with antigen. It develops as the result of natural exposure to the antigen in certain persons, presumably because of their genetic constitution. A rare, but illustrative example of immediate hypersensitivity is provided in the cases described by Reunala *et al.* (1977) in which two young women exhibited severe, IgE-mediated allergic reactions to human seminal plasma protein.

Other examples of immediate hypersensitivity include the so-called Arthus reaction and serum sickness. The Arthus reaction was first described in rabbits after a series of injections of antigen. The reaction is localized around the site of the injection and involves vascular injury. Serum sickness is the systemic counterpart of the Arthus reaction, and results from the administration of heterologous serum. The illness has a characteristic set of symptoms, generally runs its course in about a week, and is ordinarily not fatal.

1.3.6.2 Delayed hypersensitivity. The delayed hypersensitivity reaction is, like the immediate type one, mediated by non-antibody substances, but the initial event is the interaction of the specific antigen with antibody like receptors on the surface of sensitized lymphocytes, rather than with humoral antibodies. The reaction is characterized by a slowly evolving inflammatory response at the site of injection in a previously sensitized individual. A variety of antigenic and haptenic materials can induce this type of hypersensitivity, and intradermal injections are much more effective in promoting sensitivity than are intravenous ones. Incorporation of the antigen into an adjuvant enhances production of the hypersensitive state. The reaction is mediated by the T-cells. The lymphocyte population mediating the delayed hypersensitivity response is capable of producing a variety of mediator substances which are not themseleves antibodies. They bring about a variety of biological effects, and are collectively called *lymphokines.*

It should be noted that the reactions described in the foregoing sections do not always occur in isolation from other reactions. Complex reactions, involving both types of hypersensitivity response, are possible, and it is not always easy to determine exactly what the observed symptoms mean in terms of the underlying immunological mechanisms.

SECTION 2. SURVEY OF SELECTED METHODS

2.1 Introduction

Any of scores of chemical, biochemical or immunological methods may be applied in various circumstances to particular medico-legal case studies. A number of these, particularly the electrophoretic and immunoelectrophoretic ones, tend to be especially applicable in forensic biology, and the focus of this section is on them. As with the background material generally, the treatment of the methods is not comprehensive nor fully documented. Discussion of specific techniques has generally been placed in subsequent sections in the context of their application.

2.2 Immunodiffusion

The development of immunodiffusion techniques is discussed in section 16.1.2, in connection with the "precipitin test" for species of origin. Immunodiffusion is, in effect, any technique in which a precipitin antibody and its precipitinogen are allowed to diffuse together, forming a precipitate and thereby indicating that antigen has reacted with antibody. Immunodiffusion may be done in solution or on a variety of support matrices. If one of the reactants is stationary while the other diffuses, the technique is called single diffusion. If both reactants are diffusing, the technique is called double diffusion.

2.2.1. Single immunodiffusion

Single diffusion techniques were developed first. These tests are often carried out in gels, and may be linear or circular. Classically, the linear test involved allowing antigen to diffuse into an agar gel containing antiserum. Antigen concentration is usually in great excess. The diffusion of antigen into the antibody-containing gel causes a precipitin band to form and, as diffusion progresses, the precipitin band will be dissolved by excess antigen but will re-form further into the gel. The precipitin band will thus appear to be moving through the gel. If the antigen concentration is balanced with respect to the antibody concentration, the band will form and remain at or near the interface. The rate of precipitin band movement in the system described, with antigen diffusing, is directly proportional to the antigen concentration and inversely proportional to the antibody concentration. If antigen concentration is in excess, the distance of the precipitin band movement is linearly proportional to $\sqrt{\text{time}}$. The slope of the line at any particular antigen concentration is linearly related to antibody concentration. These relationships hold only within certain concentration ranges, however. Many factors influence the formation and apparent migration of the precipitin band. Further details of this technique are discussed by Crowle (1973).

The radial technique differs somewhat from the linear one. Diffusion of excess antigen is usually allowed to proceed radially into an antibody containing gel. A disc of precipitate forms and appears to expand outwardly until the front of the diffusing reactant has reached equilibrium with the antibody. Except at the early stages, the rate of movement is not proportional to $\sqrt{\text{time}}$. The final size of the disc of precipitate, however, is linearly related directly to the antigen concentration, and inversely to antibody concentration (Mancini *et al.*, 1965). Hill (1968) should be consulted for theoretical considerations in radial immunodiffusion. Quantitative methods have been treated by Berne (1974) as well. Single diffusion tests have, for many applications, been replaced by double diffusion and immunoelectrophoretic techniques.

2.2.2. Double immunodiffusion

Double diffusion tests can be performed in tubes or on surfaces. On surfaces, the antigen and antibody can be placed in rectangular wells at 90° to one another, or in a variety of circular well arrangements where diffusion is radial. The number of bands formed in the test is interpreted as the minimum number of precipitating systems present, but does not necessarily represent the maximum number. As diffusion proceeds, steep gradients of each reactant form on either side of the precipitin zone. This factor tends to compensate for excesses in concentrations of one or the other reactants, although the position of the final band will vary depending upon the degree to which one reagent was initially in excess. The test, by its nature, tends to compensate for imbalances in the concentrations of reactants, within reasonable limits. The resolution and sensitivity of the test depend on there being sufficient quantities of reactants present at the position where the diffusing species meet, and sufficient time being allowed for this to occur, given a certain distance between the wells.

Double diffusion tests have been used to compare antigen identity. Interpretation of double diffusion patterns in terms of antigenic identity or nonidentity requires caution. The simplest cases are indicated in Fig. 2.1. A triangular arrangement is often employed for the wells. Antisera to one of the antigens is placed in one of the wells. The other two are occupied by homologous antigen and the antigen one wishes to compare with it. Fig. 2.1a shows the fusion or identity pattern. If antigen concentrations are not equal, a skewed identity pattern (Fig. 2.1b) may be the result. Nonidentical antigens, compared with an antiserum containing antibodies to each of them, give independent precipitin lines which intersect (Fig. 2.1c). The partial identity, or partial fusion, pattern (Fig. 2.1d) results when the non-homologous

antigen cross reacts to some extent with the antiserum to the homologous antigen. The size of the "spur" is related to the degree of cross reactivity. A double spur pattern (Fig. 2.1e) may form if two different antigens which are related to a third antigen are compared, and the antiserum is to the third antigen. The patterns indicating antigenic identity were originally called "identity patterns", but the term "fusion" is probably more preferable, since it describes the observation, rather than indicating its interpretation (Ouchterlony, 1968; Crowle, 1973). Many factors influence the formation and patterns of the precipitin lines in these systems, and there are known exceptions to the simple cases outlined above. Care must therefore be exercised in drawing conclusions about the identities of antigens based solely on double diffusion precipitin patterns.

Precipitin lines may be curved, and this behavior ordinarily reflects differences in the diffusion coefficients of the reactants, although parameters such as salt concentration can influence the outcome as well. There are many variations of the double diffusion test, and these are discussed in the specialized references (Ouchterlony, 1968; Crowle, 1973). Immunodiffusion techniques are less widely employed than before because of the improvements in immunoelectrophoretic methods.

2.3 Electrophoresis

2.3.1 Introduction

Elecrophoresis is the movement of charged particles in solution under the influence of an electric field. The technique for electrophoresis of proteins was devised and perfected by Tiselius (1930 and 1937) in liquid medium, an achievement for which he was awarded the Nobel Prize in chemistry in 1948. This technique is often called "moving boundary electrophoresis", "free electrophoresis" or the Tiselius technique (Longworth, 1959). The method has been almost entirely supplanted by techniques employing stabilizing media. These provide a matrix for the buffer in which the proteins travel, and serve as a "trap" for the separated proteins so that they can be detected, compared and examined. Paper, cellulose acetate, agar and agarose gels, hydrolyzed starch gels, polyacrylamide gels, ion exchange resins, sephadex and other molecular sieving media, and a number of other materials have been employed as electrophoretic media. Electrophoresis in stabilizing media is usually called "zone electrophoresis".

2.3.2 Factors influencing migration and separation

The fact that proteins are electrolytes allows them to be separated by electrophoresis. Net charge on proteins is a function of pH, and so, therefore, is their electrophoretic mobility. It was originally thought that buffer solutions used in electrophoresis served mainly to maintain the medium at constant pH, and insure that the polyelectrolytes retained constant charge throughout the experiment. While that is indeed true, the buffer ions can have other effects as well. Some types of buffer ions bind to proteins and alter their electrophoretic mobility. Concentration of buffer is important too. The more concentrated the buffering solutions, the more current will be carried by buffer ions, and the slower the separating compounds will be expected to migrate. Separation zones are often found to be sharper in more concentrated buffering solutions, however. Some workers quote the ionic strength of buffer solutions, rather than their concentration. Ionic strength is defined as

$$\mu = \tfrac{1}{2} \sum m_i c_i^2$$

where m_i is the molality of the i^{th} component and c_i is its charge. Sometimes, the term "ionic strength" is used incorrectly in describing buffers, and it is necessary to read methodological descriptions carefully so that concentration and ionic strength are not confused with one another. Support media offer a resistance to current flow which is a function of the medium, the type of buffer employed and its concentration. This resistance causes heat to be generated during electrophoresis in accordance with $H = i^2R/4.18$, where H is the heat generated in cal/sec, i is the current and R is the resistance. Heating of the support matrix causes resistance to decrease over the course of time. The rate of migration of the separating species therefore increases, and in constant voltage experiments, current increases as resistance decreases. Heating also causes evaporation of water from the support, generally decreasing resistance and increasing migration rates. The resistance of a strip of supporting medium is a function of its length, and voltages used for electrophoretic separations should be quoted as "per unit length", generally in V/cm. At lower voltages, with thin support media, such as paper or cellulose acetate membranes, heating may not present a serious problem. With gels, however, it is often necessary to provide for continuous cooling to prevent thermal gradients from developing within the medium. The resistance of the medium is equal to the sum of the resistances of all the individual components, including the wicks. The applied voltage, as read from the power supply unit, is not necessarily therefore the actual voltage being applied across the medium.

Another important effect in zone electrophoresis, resulting from the interaction of aqueous buffers with the support media, is called electroendosmosis, or electroosmosis. Water molecules become positively charged with respect to the support medium, and so stream toward the cathode carrying with them buffer ions and components of the mixture. In the case of many proteins, which have acidic pI, electroendosmotic flow is in the opposite direction to electrophoretic movement. Slow moving proteins, such as the γ-globulins, may be carried back toward the cathode, and thus appear to have moved in the direction opposite to that expected. Neutral molecules may be employed to determine the electroendosmotic properties of a medium. Glucose, urea or a variety of other molecules can be used to determine this effect for small molecules, and blue dextran can be used to test it for macromolecules. Different support media vary in

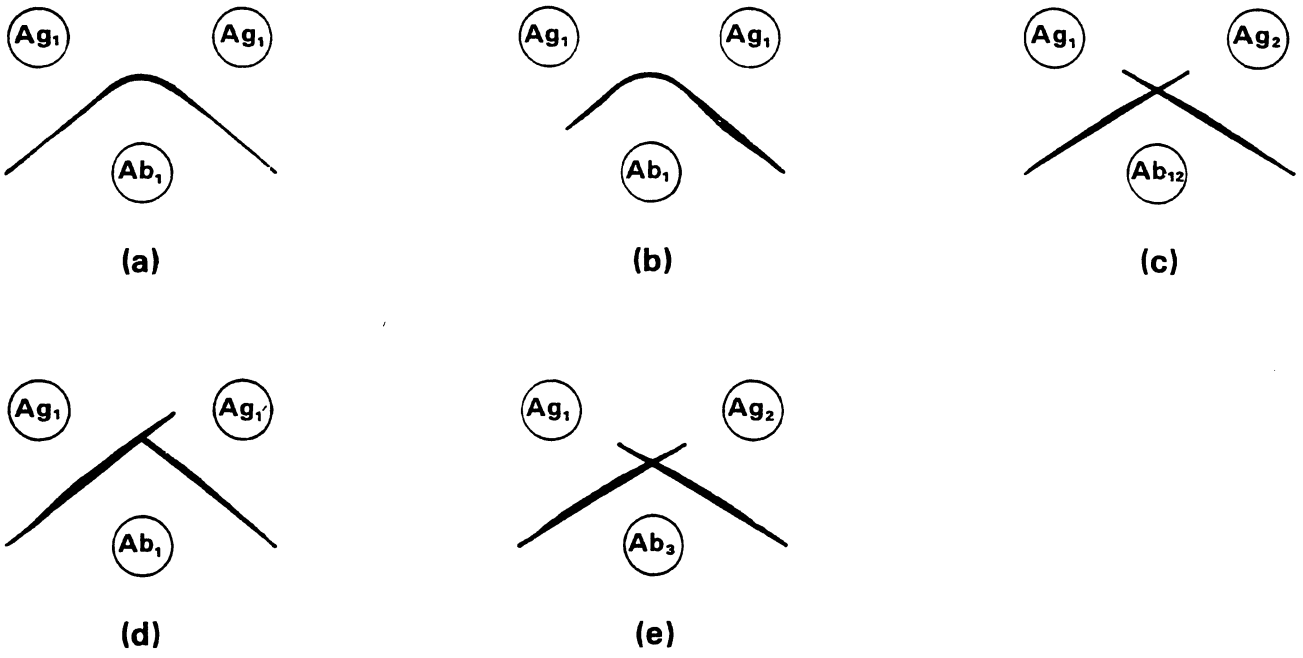

Figure 2.1 Precipitin Patterns in Double Diffusion Tests. Ag-antigen Ab-antibody Subscripts denote sameness or dissimilarity (see text).

their electroendosmotic properties, different types of agar probably being the most variable in this respect.

2.3.3 Paper electrophoresis

Paper was the first support medium employed for electrophoresis. The technique is still useful for smaller molecules. It is applicable to macromolecules as well, but the gel methods have largely supplanted its use for this purpose. According to Wunderly's (1959) review, König first suggested paper as a support medium for electrophoresis in 1937. Development of paper electrophoresis therefore preceded the development of paper chromatography, which grew out of the studies of Consden *et al.* in 1944. By 1950, the investigations of a number of workers had resulted in a clearer understanding of the technique, and its applicability to the study of inorganic ions (McDonald *et al.*, 1950; Kraus and Smith, 1950), as well as to amino acids, proteins and other macromolecules (Turba and Enenkel, 1950; Durrun, 1950; Cremer and Tiselius, 1950). A definitive paper on the paper electrophoresis of proteins by Kunkel and Tiselius appeared in 1951. Further details on the development of paper electrophoresis may be found in Wunderly's review (1959) and in Smith (1968).

2.3.4 Starch gel electrophoresis

Electrophoresis on starch gels has become an important technique in forensic biochemistry because of its wide applicability to a variety of polymorphic isoenzymes of the red cell (Cf. Unit VI). In 1952, Kunkel and Slater investigated a number of possible support media for zone electrophoresis, including glass beads and powders, special sands, resins and hydrolyzed starch. They found starch to be especially useful because of its low absorption properties for proteins. Their studies were carried out in starch gel blocks. Schoch (1961) has discussed the physicochemical basis for the gellation of starch suspensions. In 1955, Smithies reported that he had perfected a zone electrophoretic technique on starch gels which had good resolving power for serum proteins (Smithies, 1955a). The detection of proteins was also a relatively simple matter in this system. The gels were capable of resolving what we now know were the polymorphic variants of serum haptoglobin. This aspect of his studies was expanded (Smithies, 1955b), but a discussion of serum protein polymorphism will be deferred to Unit VII. Resolution of serum proteins could be greatly improved by a two dimensional technique, in which electrophoresis was first carried out on a paper strip, and the strip inserted into a starch gel in such a way that the direction of electrophoresis in starch was at right angles to that on the paper (Smithies and Poulik, 1956; Poulik and Smithies, 1958). In 1957, Dixon and Smithies showed that starch gel electrophoresis could be used to resolve enzymes in a mixture. The early techniques employed horizontal gels, and samples were usually applied on some type of support (e.g. filter paper) which was inserted into a precut slot in the gel. Smithies (1959a) reported that resolution could be improved using a vertical system in

which the samples were inserted directly into the gel. Early studies were generally carried out using boric acid-NaOH buffers. In 1957, Poulik introduced a discontinuous buffer system for starch gel electrophoresis which improved resolution and sharpened the separated zones. The bridge buffer was boric acid-NaOH, but a tris-citrate gel buffer was employed. In this system, the borate is fast moving, and a borate-tris interface appears and moves in the gel, eventually overrunning and passing the proteins. A voltage discontinuity exists at the interface which results in band sharpening. Wieme (1975) has discussed the theory of discontinuous buffer systems. The enhanced resolution of proteins on starch gels, as compared with paper and some other supports, is due in part to the pore structure of the gel. There is some molecular sieving effect with starch gels, depending upon the gel concentration and the molecular size of the proteins which are being separated. Smithies (1959b) reviewed the early work on starch gel electrophoresis of serum proteins.

Starch gel techniques have been widely applied to the separation of a wide variety of macromolecules. Details may be found in Smith (1968), Kunkel and Trautman (1959) and Michl (1975). Sometimes it is desirable to use denaturing gels, which are acidic, incorporate protein denaturing agents or both. Azen *et al.* (1966) carried out detailed studies to determine the most suitable buffers in such systems. Until 1968, starch gels used for electrophoresis were several mm thick. Various techniques were used to slice the gels along their long axis in order to stain the separated proteins. Gel slicing can present problems, and thicker gels are more difficult to keep cooled during electrophoresis. In 1968, Wraxall and Culliford showed that many of the separations could be carried out on thinner (about 1 mm) gels, and that these were both easier to cool during electrophoresis and did not require slicing prior to staining. Thin gels are applicable to a variety of polymorphic enzymes of the red cell, and are preferred where resolution is adequate.

2.3.5 Agar gel electrophoresis

According to Wieme (1965), the work of Field and Teague (1907a, 1907b) is often cited as the first in which electrophoretic separation was achieved on an agar gel. Because of the experimental set-up used, however, those workers were observing electroendosmosis rather than electrophoresis. The first unequivocal electrophoresis experiments on agar gels were those of Kendall (1928) with inorganic ions. Electrophoresis of proteins in agar gels was first reported by Gordon *et al.* in 1949.

Agar is a polysaccharide derived from the cell membranes of red algae. For a long time, its composition was not well defined. Studies on the structure of agar have been carried out, but its structure is sufficiently complex that information is still being developed. Commercially available examples of agar are, therefore, not normally well defined chemically, and may vary from supplier to supplier and even from lot to lot.

Araki's extensive studies on the structure of agar suggested that it was a mixture of two polysaccharides, which were called agarose and agaropectin, by analogy to amylose and amylopectin. Agarose is the simpler, being a linear molecule and said to consist of repeating units of β-D-galactopyranose and 3,6-anyhydro-L-galactose. Agaropectin was more complex, and contained sulfate and uronic acid residues (Araki, 1937; Araki, 1956; Araki and Arai, 1956).

Duckworth and Yaphe (1971a, 1971b) have indicated that the two component composition concept is probably oversimplified. Their studies showed that agar is a complex mixture of polysacharides with three extremes of structure: neutral polysaccharide; pyruvated polysaccharide with little sulfation; and sulfated galactan. Agarose is not regarded by them as a neutral molecule, but rather as a fraction with low charge and, therefore, good gelling abilities. Sulfate is not required for gelation of agar, but the electroendosmotic properties of agar gels are related to the sulfate content. Fractions which have little or no sulfate do not exhibit electroendosmosis. Because of the chemical variability of the material, commercial preparations should be evaluated for their applicability to particular procedures. Rees (1972) has discussed the molecular mechanisms involved in the gelation of complex polysaccharides, including agar.

Agarose gels do exhibit pronounced molecular sieving effects, depending upon the concentrations employed. Such gels can be used for molecular sieving chromatography (Hjerten, 1962a and 1962b). At the concentrations normally employed for protein electrophoresis, however, the sieving effect is minimal and less important than in starch gels. Agar gels often exhibit pronounced electroendosmosis, and this factor must be taken into consideration in selecting support media for particular applications. The electroendosmotic flow can be measured by subjecting a neutral molecule, such as blue dextran, to electrophoresis in the gel. In some cases, a certain level of electroendosmosis is required to achieve the desired result, such as in the case of crossed over electrophoresis on agar gels for determination of species of origin (see section 16.1.2). In this test, the immunoglobulins exhibit a net cathodic migration because of electroendosmosis, even though their intrinsic electrophoretic mobility is slightly anodic. In the absence of electroendosmosis, the antigen and antibody would migrate in the same direction. The best single reference work on agar gel electrophoresis is that of Wieme (1965) which should be consulted for further details.

2.3.6 Cellulose acetate electrophoresis

Cellulose acetate for electrophoresis is prepared by making "membranes" or "foils" from cellulose acetylated with acetic anhydride. Different cellulose acetates vary in the degree of acetylation. The membranes consist of a three-dimensional network of interlocking pores. Sometimes, the manufacturer supplies the "foil" itself with no inert support, or sometimes the foil is bonded to an inert supporting material such as a plastic. Cellulose acetate membranes are

rather brittle when dry, but acquire a gel like appearance and lose their brittleness when saturated with buffer solutions. The properties of cellulose acetate, such as pore size, density, thickness, and so forth, can be controlled quite well, making the medium chemically well defined and fairly uniform from batch to batch.

Cellulose acetate membrane electrophoresis was first reported by Kohn (1957 and 1958). He refined the method, and developed it on a microscale in 1959. It is applicable to a wide variety of proteins and other molecules, and has several advantages. Separations can usually be achieved much more rapidly on cellulose acetate membranes than on other common support media. Heating is usually not a problem, therefore, and the membranes can be rendered transparent and subjected to densitometric tracing for quantitation of the separated proteins. The manipulations involved are generally simple compared to other support media, so that for routine work cellulose acetate electrophoresis is very convenient. Many commercial cellulose acetate membranes are available, and the technique has found wide application in clinical biochemistry, and to a lesser extent in forensic biochemistry. Cellulose acetate may be used as a support for immunoelectrophoresis and immunodiffusion as well. Detailed descriptions of electrophoretic applications on cellulose acetate have been given by Chin (1970) and by Kohn (1968).

2.3.7 Polyacylamide gel electrophoresis

Polyacrylamide gels are synthetic gel polymers prepared prior to use in electrophoresis. They may be used for disc electrophoresis, which is carried out in cylindrical tubes, or in gel slabs, much like starch.

2.3.7.1 Polyacrylamide disc gel electrophoresis Disc gel electrophoresis was first devised by Ornstein (1964) and Davis (1964). Ornstein (1964) noted that preliminary results with the method had been reported at meetings as early as 1959. The name derives from the dependence of the technique on discontinuities in the electrophoretic matrix, and from the fact that the separated zones of ions have a discoid shape in the standard procedure. The technique is applicable to the separation of a wide variety of macromolecules of biochemical interest, including proteins. It has not as yet found wide applicability in medico-legal investigations.

Polyacrylamide combines to a great extent all the desirable properties of an electrophoretic medium. The gel is relatively easy to prepare over a range of concentrations. Concentration of the gel is inversely related to pore size so that molecular sieving effects can be controlled. The gels are stable, have good mechanical properties, and are inert and transparent. With disc gel electrophoresis, running times are generally quite short.

Polyacrylamide gels are prepared from acrylamide and the cross linking agent N,N′-methylene-bis-acrylamide (sometimes called "Bis"). The main factors determining pore structure are the w/v concentration of acrylamide plus Bis, and the ratio of Bis to acrylamide concentration. Usually, only the former is varied. Gels can be made from about 3% to about 30%, but the usual gel is in the range of 5 to 10 percent. Free radicals are required for polymerization to take place, and several different ways of providing for their presence have been used in practice. Chemical polymerization is brought about by the addition of ammonium persulfate and tetramethylenediamine (TEMED). Ammonium persulfate is a catalyst while the TEMED acts as an initiator. β-dimethylaminopropionitrile (DMAPN) may be substitued for TEMED. A small amount of oxygen is required for initiation, though the continued presence of oxygen inhibits the reaction. Polymerization rate is directly proportional to TEMED concentration. Polymerization may also be brought about photochemically by the addition of riboflavin, and exposure of the solution to UV light. A small amount of oxygen must be present for initiation of photochemical polymerization as well, but as in the case of chemical polymerization, its continued presence is inhibitory. Riboflavin is effective at low concentrations.

The original system consisted of a 7% gel prepared in a small cylindrical glass tube. This gel occupied much, but not all of the volume of the tube, and is called the "separation" or "analytical" gel. Above this gel is prepared a "spacer" or "stacking" gel of much larger pore size (about 3% gel), and about 3 mm in depth. The analytical gel was chemically polymerized, while the stacking gel was polymerized by photochemical means. The sample is then applied above the spacer gel. In the original experiments, the sample was made up in large pore gel medium, and polymerized into place ("sample gel"), but it may be layered on in solution with equally satisfactory results. If it is applied as a solution, sucrose or some other substance is used to make the solution dense, so that the material does not escape and mix with the upper buffer solution. The ions in the buffer systems are chosen according to their mobilities. Kohlrausch showed many years before that in two solutions, one layered above the other, the upper solution containing ions of lower mobility than those of the lower solution, a sharp boundary forms at the interface between the two ions upon passage of current. The boundary is maintained as the ions move at the same rate in the column. The theory indicates that at the pH values selected, proteins migrate with mobilities intermediate between glycinate in the upper buffer compartment and Cl^- in the gel buffer. In the spacer gel, the proteins "stack," i.e. each protein forms a discrete disc, and the separate discs are stacked with the glycinate ion running behind the slowest moving protein. The mobility of the proteins decreases as they enter the smaller pore analytical gel, and the glycinate ion overtakes them to form a sharp boundary with the Cl^- ion. The proteins continue to migrate as a series of thin discs. The use of a tris-chloride buffer for the gel and a glycine upper electrode vessel buffer causes a change in pH during the run as well. The analytical gel buffer is transformed into a tris-glycine buffer having a higher pH than that of the electrode vessel buffer. As a consequence of the

higher pH, the glycinate ion increases its mobility as it enters the analytical gel. More generally, the requirements for satisfactory separation as developed by Ornstein and Davis, were that the leading ion (in the analytical gel buffer) have the highest mobility in the system, that it be an ion of a strong acid, and thus unaffected by pH, that the trailing ion which overtakes all the proteins must be of a weak acid so that its mobility will increase as it passes into the higher pH analytical gel. Further, the buffer counter ions have to be capable of buffering both the trailing and leading ions within the range of pH of the running gel. It was said too that the "stacking" gel was required to allow stacking of the protein discs before migration into the analytical gel, and that a sample gel was needed to avoid diffusion. It is clear from much experimental work, however, that the spacer and sample gels are not required, and many workers dispense with them.

Polyacrylamide disc gel electrophoresis gives excellent separations of many proteins, and only a small amount of sample (μg quantities) is required. Further information on the details and applications of the procedure may be found in Ornstein (1964), Davis (1964), Wieme (1975), Tombs and Akroyd (1967) and Smith (1968).

2.3.7.2 Polyacrylamide flat gel electrophoresis. Polyacrylamide gels can be prepared in flat slab form, in a way very similar to starch gels. This technique was first described by Raymond and Weintraub (1959). Their paper was also the first in which polyacrylamide was proposed as a supporting medium for zone electrophoresis. Except for the discoid shapes of the zones in disc electrophoresis, many of the same considerations apply to slab polyacrylamide electrophoresis as do to disc electrophoresis. Very often, the buffer systems used for flat slab applications are continuous, however, and the ionic fluxes may be different in the gel depending upon this parameter. The gels may be prepared for either horizontal or vertical electrophoresis. One of the advantages of the flat slab technique is that it allows comparison of two different samples on the same gel side by side. The technique is discussed in some detail in Smith (1968), and by Tombs and Akroyd (1967). Parkin (1971) described the use of a horizontal slab polyacrylamide gel with a starch insert. Samples were applied to the starch gel, and then migrated electrophoretically into the acrylamide gel.

2.4 Immunoelectrophoresis

2.4.1 Simple immunoelectrophoresis

Immunoelectrophoresis combines the separation powers of electrophoresis with the specific and sensitive detecting powers of antibodies to the separated proteins by a precipitin reaction. The method was devised by Grabar and Williams in 1953. Its discriminatory capability depends both on the electrophoretic separation achieved and the specificity of the antiserum employed for detection.

In simple, qualitative immunoelectrophoresis, the sample is inserted into a gel and subjected to electrophoresis. Agar gels are often used, although the technique can be carried out on other supporting media as well. A trough is then cut in the gel a short distance away from the line of electrophoretic migration and parallel to it, and is filled with antisera. Because diffusion has occurred during the time required for electrophoresis, the separated antigenic proteins will occupy larger areas than was originally occupied by the applied sample. As a result, precipitin arcs form between the trough of antisera and the line of electrophoretic migration. The method has been used extensively for the study of normal and pathological serum and of other body fluids. Techniques and interpretation of the arc structure are discussed in detail by Ouchterlony (1968), Grabar and Burtin (1964), Cawley (1969) and Arquembourg (1975). A review of immuno-chemical methods used in clinical chemistry, including immunodiffusion and immunoelectrophoresis, is given by Grant and Butt (1970).

2.4.2 Some variations of immunoelectrophoresis

A number of variations of immunoelectrophoretic technique have been proposed, and various authors have applied their own terminology to the methods. Although it is generally quite clear from the descriptions what technique is being used, different authors may use similar terms to describe rather different techniques. In simple immunoelectrophoresis, the antigens are separated electrophoretically, and then detected in a second step by immunodiffusion.

Lang, in 1955, described a technique in which antigen and antibody are applied to the same support medium on parallel lines, the antigen being applied behind the antibody because of its greater mobility. During electrophoresis, the antigen overtakes the antibody and gives a precipitin line. This technique was called "überwanderungselektrophorese" (Lang, 1955; Haan and Lang, 1957). Quantitative results could be obtained by the use of radioactively labelled antigen.

In 1959, Bussard proposed a technique in which both antigen and antibody are placed in wells in an agar gel, the former being placed cathodically, the latter, anodically. Both antigen and antibody have a net anodic electrophoretic mobility at the pH used, but the antibodies show a net cathodic mobility because of electroendosmosis, resulting in the formation of precipitin bands between the antigen and antibody wells. The technique was called "electrosyneresis" by Bussard, but the term is not widely used in the literature. Culliford applied the technique to the immunological determination of species of origin (see Section 16.1.2). More often, the technique is called "cross over electrophoresis". Grunbaum (1972) applied the method to the determination of several serum proteins in bloodstains. The term "cross over electrophoresis" has a more general meaning, though. Any procedure in which two different materials are subjected to electrophoresis in such a way that their paths of migration intersect would fall under the general heading of "cross electrophoresis," according to Nakamura (1966) who has written a definitive reference work on the subject. Cross electrophoresis has been applied to a number of different types of materials on paper and on other types of supports.

Antigen-antibody cross electrophoresis is included in Nakamura's work, although Bussard's technique is not specifically mentioned.

In 1969, Alper and Johnson reported an immunoelectrophoretic technique in which the separated proteins were detected in agar or starch gels by overlaying the gels with antisera after electrophoresis, and allowing the precipitin reaction to proceed. The excess protein could then be washed out in salt solutions, and the antigen-antibody complexes stained with protein stain. They referred to this technique as "immunofixation electrophoresis". Wilson (1964) described a very similar technique, and called it "direct immunoelectrophoresis".

2.4.3. Quantitative immunoelectrophoresis

A number of techniques have been devised for quantitative immunoelectrophoresis. The best single reference to all of these is probably Axelsen *et al.* (1973). Some of the methods have been applied to medico-legal problems while others have not. Emphasis will be placed on the former in this section. All the techniques involve the electrophoresis of antigens in gels containing antibodies to them, under conditions where the immunoglobulins do not migrate, or do so only slightly, in comparison to the antigens. Precipitates form in the systems, and the area delimited by the precipitate is proportional to the antigen: antibody ratio. If either component is held at constant concentration, quantitation of the other is possible.

2.4.3.1 Rocket electrophoresis. The simplest technique involves the electrophoresis of antigens in antibody-containing gels. It was first described by Ressler in 1960. He applied the samples in linear slots. Laurell (1966) modified the procedure somewhat by applying the samples in very small wells in $\mu\ell$ quantities. Electrophoresis into antibody-containing gels then gives rocket-shaped precipitin lines, and for this reason the procedure is sometimes called "rocket electrophoresis". Independently, Merrill *et al.* (1967) described the same technique for quantitating immunoglobulins in body fluids. They called it "electroimmunodiffusion", or EID. In 1972, Laurell reviewed the procedure and its technical details. He pointed out that one of the points about the technique whereby it differed from conventional immunoelectrophoresis and immunodiffusion was that diffusion was minimized by the electrophoresis. He did not, therefore, regard the term "electroimmunodiffusion" as appropriate, and called it "electroimuno assay". A detailed discussion of the practical aspects of the procedure has been given by Weeke (1973a), and Driscoll (1973) discussed the principles underlying rocket electrophoresis.

The precipitin line (see Fig. 2.2a) represents the antigen antibody equivalence point (see section 1.3.4.2). Antibody is in excess in the agarose gel, ahead of the migrating antigen. As antigen migrates, equivalence is achieved and precipitation occurs. The precipitin line blocks further migration of antigen into the gel, so that antigen accumulates within the confines of the precipitin line. As the antigen concentration builds up behind the precipitin line, the precipitate is dissolved, but will be re-formed a little further along the path of antigen migration, where there is, again, equivalence. This process continues, and the precipitin peak, or rocket, is elongated until excess antigen is exhausted and the peak size stabilizes. Peak height thus bears a linear relationship to the amount of antigen originally present, at constant antibody concentration in the gel. This relationship is summarized graphically in Fig. 2.2b. Rocket electrophoresis has been applied to the immunochemical quantitation of acid phosphatase in attempting to differentiate seminal and vaginal acid phosphatases in samples from sexual assualt cases (see section 10.3.2).

2.4.3.2 Crossed immunoelectrophoresis. In 1965, Laurell described a modified immunoelectrophoretic method in which antigens were first separated electrophoretically, and then subjected to a second electrophoresis, at right angles to the direction of the first, and into an antibody-containing gel. This technique, he called "antigen-antibody crossed electrophoresis". A slightly modified technique was reported by Clarke and Freeman (1968). The type of pattern obtained when this technique is applied to serum proteins (or other complex protein mixtures) is shown in Fig. 2.3. Correspondingly less complex patterns are obtained with less complex mixtures. The principles underlying the relationship between peak area and antigen concentration are essentially the same as those underlying the relationship between peak height and antigen concentration in rocket electrophoresis (section 2.4.3.1). A number of different methods have been employed for the determination of peak area, and these are discussed by Weeke(1973b). Areas under the peaks are compared to those obtained with a reference antigen using the same reference antiserum under the same conditions. The procedure can be carried out on cellulose acetate membranes as well as on agarose gels (Miller and Mutzelberg, 1973).

Ganrot (1973) reviewed the procedure, and suggested that it be called "crossed immunoelectrophoresis", to indicate that it had developed from conventional immunoelectrophoresis, and that electrophoresis was employed in two dimensions. Sweet *et al.* (1973) used the method to examine serologically different proteins in a microorganism, and referred to it as "crossed electroimmunodiffusion", or CEID.

Whitehead *et al.* (1970) applied crossed immunoelectrophoresis to the study of serum proteins in bloodstains, and suggested that the technique might have potential in their discrimination. Saint-Paul *et al.* (1971) also suggested that medico-legal applications of the method might be devised, such as monitoring differential putrefactive degradation changes in bloodstains. Sweet and Elvins (1976a, 1976b) could detect pattern differences in bloodstains from 10 individuals as well as differences between the males and the females. They suggested that the technique could be useful in the individualization of bloodstains. These medico-legal applications will be discussed further in a subsequent section.

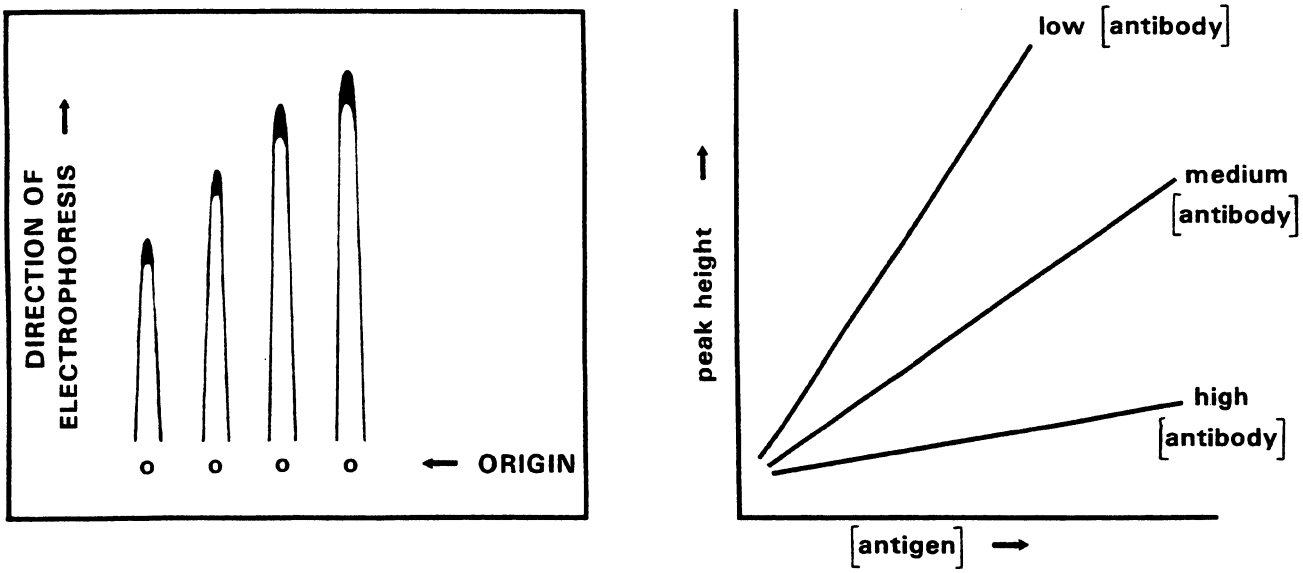

Figure 2.2 Rocket Electrophoresis

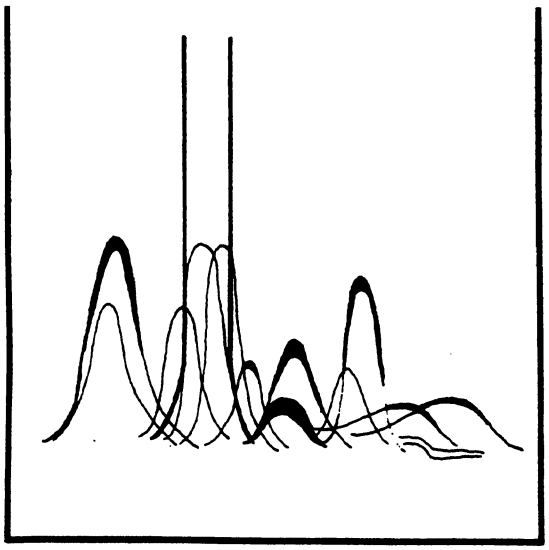

Figure 2.3 Schematic Representation of Pattern of Normal Serum Examined by Crossed Immunoelectrophoresis

2.4.3.3 Other methods of quantitative immunoelectrophoresis. A number of other quantitative immunoelectrophoretic techniques have been devised, as well as further variations of the methods discussed above. Details concerning these may be found in Axelsen *et al.* (1973). They have not thus far been applied directly to medico-legal investigations, but are used in immunologic studies. Axelsen and Bock (1972 and 1973) have reviewed and discussed quantitative immunoelectrophoretic techniques, particularly as they may be applied to the determination of antigen identity or partial identity.

2.5 Isoelectric Focusing and Isotachophoresis

2.5.1 Isoelectric focusing

Isoelectric focusing is not a new technique, but various technical problems prevented its becoming a widely employed analytical and preparative separation method until relatively recently. Molecules which carry both acidic and basic ionizable groups are called ampholytes. Amino acids and proteins are ampholytes, as are many other molecules. All ampholytes have an isoelectric point, or pI, which is the pH at which the molecule has no net charge in solution. In electrophoresis, a constant pH is ordinarily maintained with a buffer, and the protein molecules migrate in the electric field acording to their net charge.

If a variety of ampholytes with differing pI is dissolved and subjected to current flow, a pH gradient becomes established. The lowest pH will be near the anode and the highest near the cathode. The gradient forms in this way because the ampholyte of lowest pI will be anionic at all pH greater than its pI, and will migrate toward the anode under the influence of the current. It will collect in a narrow zone in its isoelectric condition and, due to a buffering capacity, will impart to the nearby solution a pH corresponding to its pI. The next most acidic ampholyte will behave similarly, forming another zone slightly cathodic to that of the most acidic component. It does not pass the zone occupied by the most acidic component for, if it did, its pI would be surpassed and it would acquire opposite charge. This process continues until a smooth pH gradient exists between the electrodes. The nature of the pH gradient is determined by the number of ampholytes in the mixture, their relative amounts, buffering capacities and isoelectric points. If large MW ampholytes, such as proteins, are applied to such a system, they will migrate under the influence of the current, and focus in narrow zones corresponding to their isoelectric points. The focusing is brought about by the electric field, and the technique is therefore usually called "isoelectric focusing".

There were a number of experiments carried out over the years on the isoelectric fractionation of ampholytes, including proteins. The earlier experiments are reviewed are reviewed by Svensson (1948), Haglund (1971) and Rilke (1973). Until the early 1960's, the major problem was in obtaining stable, reproducible pH gradients in the presence of the current flow. Svensson (1961, 1962a, 1962b, 1967) developed the theoretical and practical foundations for the development of electrofocusing methods. The properties of ampholytes required for stable, natural pH gradients were derived from theoretical considerations. Peptides were first used as carrier ampholytes, and allowed the theoretical arguments to be experimentally confirmed. Sucrose density gradients were also used in electrofocusing experiments to minimize convection currents. There are obvious disadvantages to using peptides as carrier ampholytes if one wishes to separate proteins. Vesterberg and Svensson (1966) and Vesterberg (1967) succeeded in synthesizing a series of aliphatic aminocarboxylic acids which had all the desired properties of carrier ampholytes. These are now commercially available as "ampholines". Properties of the ampholine chemicals and their applicability to isoelectric focusing are discussed by Vesterberg (1973) and by Haglund (1975).

Isoelectric focusing gives excellent resolution of proteins, and molecules with differences in pI of as little as 0.02 pH units can be separated. The method has become fairly widely applied in protein chemistry in recent years, and many variations have been developed. Electrofocusing can be carried out, for example, in polyacrylamide gels (Righetti and Drysdale, 1971; Wellner and Hayes, 1973; Vesterberg, 1975). Among other advantages, such procedures minimize convection and help stabilize the pH gradient. Detailed discussion of isoelectric focusing and its applications may be found in the specialized references (Arbuthnott and Beeley, 1975; Catsimpoolas, 1973; Haglund, 1971).

2.5.2 Isotachophoresis

Isotachophoresis is an electrophoretic technique for separating ions, and is related in its theoretical aspects to disc electrophoresis (section 2.3.7.1). In isotachophoresis, all ions of the same sign having a common counterion move at the same speed at equilibrium. They are separated into zones based on their mobilities. A leading ion, with the same sign as those being separated, and with the highest mobility in the system, must be present, as must a trailing ion which has the lowest mobility. In order to achieve separations of proteins, "spacer ions" are used, and the carrier ampholytes used for isoelectric focusing have been employed for this purpose.

The theory and some applications of isotachophoresis may be found in Martin and Everaerts (1970), Everaerts and Routs (1971), Beekers and Everaerts (1972), Everaerts *et al.* (1973) and Routs (1973). Protein separations by isotachophoresis using ampholyte spacers is discussed by Griffith *et al.* (1973) and reviewed by Catsimpoolas (1973). Grunbaum and Hjalmarsson (1976) employed isotachophoresis to examine the protein profiles in aqueous extracts of bloodstains from four different persons. 24 hour extraction times did not alter the pattern in the same individual as compared with brief extractions. The separated proteins were detected by their UV absorption. Different patterns were seen in different individuals, and it was suggested that the procedure might have medico-legal value if future studies confirmed the observed trends.

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† Known as H. Rilbe after 1968

UNIT II
IDENTIFICATION OF BLOOD

SECTION 3. HISTORY AND DEVELOPMENT OF MEDICO-LEGAL EXAMINATION OF BLOOD

There is a considerable literature concerning the history of legal, or forensic, medicine, but little mention is made of medico-legal examinations of blood and/or body fluids in most of the work. The National Library of Medicine's *Bibliographies of the History of Medicine* (U.S. DHEW, PHS, NIH, NLM, 1964–1969 and 1970–1974) contain some references to legal medicine; but Nemec's bibliography is perhaps the best and most complete single source (Nemec, 1974). One of the most well-known scholars of the history of legal medicine, the late Prof. R. P. Brittain, has done a useful bibliography of medico-legal works in English (Brittain, 1972), which contains some references to the medico-legal examination of blood and body fluids. Brittain was apparently engaged, at the time of his death, in the preparation of an extensive, closely-documented work on the origins of legal medicine. Some parts of this work have appeared as a series of papers in the *Medico-Legal Journal*; these papers deal with the medico-legal aspects of the *Constitutio criminalis Carolina* (Brittain, 1965a), the *Leges barbarorum* (Brittain, 1966a), the Assizes of Jerusalem (Brittain, 1966b), the *Capitularia* of Charlemagne (Brittain, 1966c), and the *Leges duodecim tabularum* of 449 B.C. (Brittain, 1967). In addition, there are papers on the origin of legal medicine in Italy (Britain, 1965b) and in France from the 12th to the 16th centuries (Brittain, 1966d). There are, however, no direct references in these discussions to the identification or individualization of blood or body fluids.

According to Kiel's interesting and well-documented review of the origins and development of legal medicine in China (Kiel, 1970), a Chinese doctor named Chich-ts'si wrote the first known treatise on forensic medicine. The work, entitled *Ming Yuen Shih Lu,* was written in the 6th century A.D. Around 1250 A.D., a more comprehensive and well known treatise on legal medicine, known as *Hsi Yuan Lu,* or "Instructions to Coroners," was compiled in China. Kiel notes that there are references in this work to the detection of bloodstains. It was said that an old bloodstain could be detected on a knife by heating the stain and treating it with vinegar, after which a brown stain, resulting from the formation of hematin crystals through the reaction of the hemoglobin with the acetic acid in the vinegar, would appear. A method for paternity testing was prescribed in the *Hsi Yuan Lu* as well. Apparently known as the "dropping test," this method called for the allegedly related persons, such as parent and child, to cut themselves and allow their bloods to drip into a single basin of water. Smooth mixing of the bloods was taken to mean relatedness, while clumping of the cells indicted incompatibility, and presumably, non-relatedness. Furuhata (1927) mentioned three other very early works in Chinese: *Sen-en-roku* (False charges cleared) in 4 volumes by Ji of the Sung dynasty (1247); *Kei-en-roku* by Cho of the Sun dynasty (year unknown); and *Mu-en-roku* (No false charges) by O Yo of the Gen dynasty (1308). The last was translated into Japanese in 1736 by Naohisa Kawai with the title *Mu-en-roku-jutsu* (Interpretation of no false charges). The "dropping test" was mentioned in these works, as was some method for determining affiliation between the living and the dead by dropping the living person's blood onto the bones of the deceased. While these methods were unreliable by any present standards, as noted by Wiener (1960), they are interesting precursors to present methods and ideas. The vinegar test for blood may have been a crude Teichmann crystal test.

Rosner (1973) has described an interesting passage in the *Talmud,* written in the 2nd century, which elaborates a method for the differentiation between bloodstain and a dye stain on a garment. According to the *Talmud,* seven substances were to be applied to the stain in a specified order; stains which then faded away or became fainter were bloodstains, while those which did not fade had originated from dyes. The seven substances, in order, were; tasteless spittle, the liquids of crusted beans, urine, natron (native sodium carbonate), *borith* ((lye, or perhaps sulfur, or a type of plant or grass), *kimonia* (Cimolean earth, or a type of clay, or salt, or pulverized dried grass), and *eshlag* (lion's leaf, said to be "found between the cracks of pearls, and . . . extracted with an iron nail," or possibly an alkali or mineral used in soap). In various sections of the *Talmud* and in various Talmudic commentaries, according to Rosner, the nature of these seven substances is discussed and specified. But, as will be clear from the parenthetical notes above, differences in translation and in various commentators' opinions have led to some confusion as to precisely what they were in all cases.

A few references are made to blood and body fluid examinations in Thomas' interesting and readable review of the highlights of the development of forensic science and medicine in Europe from the Renaissance to World War II (Thomas, 1974). This article contains some interesting material on M. J. B. Orfila, about whom we shall have more to say shortly. The contributions of Jules Bordet, Paul Uhlenhuth and Karl Landsteiner are discussed as well, and these, too, will be discussed in appropriate, subsequent sections of this Sourcebook.

It would be of interest to know when and where the medico-legal identification of bloodstains, using relatively modern chemical techniques, originated. It seems quite reasonable to draw a distinction between the ancient and medieval methods and those of more recent times on the basis

of the fact that the latter rely on at least some knowledge of the chemcial composition of blood. The fact that the knowledge of blood's chemical composition was very incomplete, and characterized by notions that have not stood the test of time, may make this distinction somewhat arbitrary. But it seems possible, nevertheless, to distinguish between the pre-19th century commentaries which are of purely historical interest, and those methods which appeared after 1800, and were based on what most people would regard as chemical priniciples, rather than on purely empirical observations having no theoretical framework whatsoever. One of the oldest books in English having to do with bloodstains is W. D. Sutherland's *Blood-Stains: Their Detection, and the Determination of Their Source* (Sutherland, 1907). The book is, in fact, one of the few in any language up to its time which treats what we now call forensic serology (in its broadest sense) exclusively. Sutherland, a Major in the British Indian Medical Service at the time of the book's appearance, was obviously something of a scholar, in addition to the fact that he had forensic casework responsibilities. The Preface of the book begins with the comment: "As there does not exist in any language of which I have knowledge a compendium of the modern tests by which the detection of bloodstains and the determination of their source may be carried out . . . ." From this remark, and from the nature and extent of his bibliographic reference list, it seems likely that Sutherland read a number of modern languages and probably Latin in addition of course to English. An interesting man, he later became a Lieutenant Colonel, and apparently stayed in India until he died in 1920. A brief obituary appeared in the *British Medical Journal* **2:** 189 (1920). He was very interested in the precise origins of medico-legal examinations of bloodstains, noting at the beginning of the second chapter:

> I have endeavoured to ascertain when the medico-legal importance of blood-stains first came to be recognized, but without success. The older medico-legists, whose works are cited in the bibliography, treat of most things from miracles to slight wounds, but none make special mention of bloodstains. And, as we see, even so late as 1834 the fourth edition of a popular German textbook contained no special reference to them, although in 1817 Orfila had dealt with the chemistry of the blood in his textbook of medical chemistry, and in 1808 Jacopi is said to have managed by the aid of the microscope to distinguish bovine from human blood. It is from the discussion which took place at the Academie Royale de Medecine in 1828 that I infer that for a considerable time the French experts had busied themsleves with the question of the detection of bloodstains.

The discussion to which Sutherland referred may be one which appeared in the *Journal generale de medecine de chirurgie et de pharmacie* in 1828 (Raspail, 1828a; Orfila, 1828a; Raspail, 1828b; Raspail, 1828c). It was based on a serious disagreement which had arisen between Raspail and Orfila over the value which was to be attached to the results of a series of chemical tests proposed by Orfila (1827a) for the identification of blood in medico-legal investigations. Mathieu-Joseph-Bonaventure Orfila was one of the most prominent medical scientists of his time. Born in Spain April 24, 1787, Orfila began his studies in that country, but completed them in France, receiving his doctorate at Paris in 1811. He remained in France until his death in 1853. He enjoyed a brilliant career, had considerable prestige, and his opinions and pronouncements carried a good deal of weight in the scientific community. Orfila was primarily a toxicologist; Prof. Dr. Muñoz, in a Spanish-language biographical tribute to Orfila, published in 1956 in Quito, Ecuador, called him the "founder of modern toxicology". A biographical sketch of Orfila's career appeared at the time of his death as well (Chevallier, 1853). There is no doubt that Orfila was one of the important figures in the development of legal medicine. At various times he focused his attention on blood and body fluids, and his contributions in this area are among the earliest systematic studies that can be found in the published literature.

The earliest methods for medico-legal identification of blood were chemical ones. Lassaigne is said to have published a paper on the discrimination of bloodstains and rust spots in 1825. This paper could not be located, though there is no doubt of its existence. In 1827, Orfila published a systematic series of tests for the recognition of bloodstains on various substrata, and their discrimination from rust spots, iron citrate, and a number of red dyes (Orfila, 1827a). He mentioned Lassaigne's work, but without giving a specific reference. Orfila said that, in order not to be accused of scientific plagiarism, he should point out that he had been engaged in this work since 1823.

Orfila's tests were based primarily on the solubility of bloodstain components in water, and the behavior of the aqueous extract toward a number of reagents, litmus paper, and so forth. Raspail disagreed that Orfila's criteria were sufficient to establish the identity of blood in a medico-legal investigation, and said that a stain could be constructed from ovalbumin and a red dye which would give a completely convincing set of false positive results using Orfila's tests. He noted further that there might exist other materials which would give results similar to the ones given by blood. Orfila responded to these objections in a point-by-point refutation which appeared in the discussion cited above (1828a), as well as in a separate paper (Orfila, 1828b). Orfila was convinced that he was correct, but Raspail did not accept the refutations as is clear from the discussions in the Royal Academy. It appears that Orfila's opinion on the matter prevailed to a large extent, at least in terms of acceptance by judicial tribunals. In 1835, Orfila and his colleagues J.-P. Barruel and J. B. A. Chevallier published a detailed report on a case in which they had been consulted. Three brothers named Boileau and a fourth man named Victor Darez were accused of having murdered a rural constable named Hochet. A number of suspected bloodstains were involved in the case, and the Court was interested in knowing whether certain of these stains were of human or animal origin, and

whether certain of them came from the same individual, assuming it could be established that they were stains of human blood. The experts established that a number of the stains were bloodstains using Orfila's chemical tests as criteria. They could not shed any light on the questions of species of origin, nor of common origin, nor on the question of the time interval which had elapsed since the deposition of the stains.

Around this time, some authorities were considering microscopical examination of bloodstains as a means of identification of blood in medico-legal inquiries. In some minds, microscopical results were more certain, and were preferable to the chemical methods. This subject is discussed in section 5.3. It was also recognized that carefully conducted microscopical observations of blood cells from bloodstains could, under some circumstances, help in diagnosing the species of origin. The blood of animals having nucleate red cells could, at least, be distinguished from mammalian blood. Some authorities, aware that the red cells of different species differed in size, thought that carefully conducted microscopical measurements of the red cells could, in some cases, serve as a basis for species determination, even among mammalian species. This subject is discussed in section 15.

Orfila (1827b) looked into microscopical methods for blood identification and species determination. He was unable to obtain satisfactory results, and concluded that the chemical methods were much more reliable. Around 1836, Prof. Persoz at Strasbourg introduced the use of hypochlorous acid as a reagent for discriminating bloodstains from other red stains, especially older ones which were not very soluble in water. Orfila evaluated this technique quite thoroughly in 1845, and found it unsatisfactory if used alone, but admitted that it might be useful as an auxiliary method in certain types of cases.

A number of the early papers on the subjects discussed in this section may be read in their entirety in the translations (Unit IX). There is not much doubt that the earliest scientifically systematic attempts to employ physical and chemical methods to the medico-legal examination of blood and body fluids are attributable to the French scientists in the early years of the 19th century. Some of the earlier papers on the identification of seminal fluid, other body fluids, menstrual blood, and so forth, are discussed in appropriate sections and some have been included in the translations as well (Unit IX).

SECTION 4. CRYSTAL TESTS

4.1 Structure and Nomenclature of Porphyrins and Hematin Compounds

The discussion of crystal tests and of spectrophotometric and spectrofluorimetric methods which follows will involve many terms which refer to porphyrin and hematin compounds. The history and development of nomenclature of these materials is somewhat complex and can lead to considerable confusion. Interested readers are referred to the specialized works by Lemberg and Legge (1949), Falk (1963) and Marks (1969). Most standard biochemistry texts also carry a discussion of the subject (e.g. White *et al.*, 1973; Pritham, 1968; Mahler and Cordes, 1971). The present discussion is designed primarily to provide an outline of the nomenclature, and give some indication of the different meanings that may be associated with various terminology.

Porphyrins are derived from the cyclic ring compound *porphin*, a structure with four pyrrole-like rings linked by methylene bridges. Represented in Figure 4.1 in two different ways, the structures (a) and (b) are identical, neither is "correct" or "incorrect", and neither is preferable for any particular reason. Both are encountered in the literature.

The porphyrins which occur in nature are all compounds in which side chains are substituted for some, or all, of the hydrogens at positions 1 through 8 in Figure 4.1. It is convenient in giving structural representations of these compounds to use a "shorthand" notation for the porphyrin nucleus, omitting structural detail, but allowing the different side chains to be shown in their correct positions. Figure 4.2 (a) and (b) shows the shorthand representations corresponding to the structures in Figure 4.1 (a) and (b), respectively.

Introducing side chains into the molecule gives rise to a large number of different structural isomers. If, for example, the eight numbered hydrogen atoms are substituted with four methyl- and four ethyl- groups, four isomers are possible. These are shown in Figure 4.3 according to both "shorthand" conventions, corresponding to Figure 4.2 (a) and (b), respectively. Only compounds deriving from structures I and III, Figure 4.3, are found in nature, those from III being more important. In representing the isomers according to the shorthand notation, it is usual to number the positions as has been done in Figure 4.2. Not all authors number the positions in the same way, however, and while any sequentially numbered system is interconvertible to any other by rotation in the plane of the paper, it eliminates confusion, in my opinion, if the numbering convention is clearly stated. Some of the different, naturally occurring porphyrins are indicated in Table 4.1. Only the type I and type III isomers (Figure 4.3) are given. If the number of types of substituent side chains is increased, then clearly the number of possible structural isomers increases as well. In the case of protoporphyrin, for example, with three substituent types, there are fifteen structural isomers. All the porphyrins derived from hemoglobin and naturally occurring hematin compounds are of protoporphyrin type III, Table 4.1. The molecule is more commonly referred to as protoporphyrin IX, since it was the ninth in a series of isomers listed by H. Fischer (White *et al.*, 1973). Protoporphyrin IX is shown in shorthand notation in Figure 4.4.

Porphyrins possess the ability to combine with many metals, the most important biologically active molecules being those in which the porphyrin is combined with Fe or Mg. These compounds are collectively referred to as metalloporphyrins. The hematin compounds, which are the only metalloporphyrins of major interest to this discussion, are all iron-protoporphyrin compounds. The iron atom in an iron-protoporphyrin complex is coordinated to the four pyrrole nitrogen atoms in a planar configuration (Figure 4.5), replacing the two dissociable hydrogens of the porphyrin nucleus. The detailed properties, including the nature of the coordinate binding and thermodynamic stability, or iron and other metal porphyrins were discussed by Phillips (1963). The iron complexes readily add two additional ligands, which coordinate to the metal forming an octahedral structure and in which the metal is then hexacoordinated.

The valence of the iron atom is specified by the prefixes *ferro-* (for Fe^{2+}) and *ferri-* (for Fe^{3+}) in naming the various compounds. *Heme* is *ferroprotoporphyrin. Heme* is spelled *haem* in some countries, the spelling variation carrying over to other terms derived from the word *heme*, e.g. hemoglobin/haemoglobin, hematin/haematin, hemochromogen/haemochromogen, etc. *Ferriprotoporphyrin*, obtained as the chloride, is called *hemin chloride*, or *hematin chloride. Ferriprotoporphyrin hydroxide* is simply call *hematin.* The use of the term *hemin* is restricted by some authors to ferriprotoporphyrin halides, especially the chloride, (Lemberg

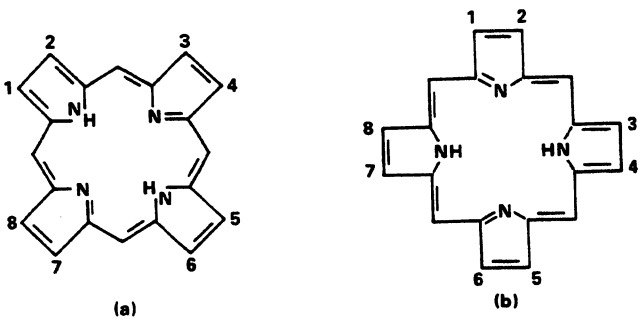


Figure 4.1 Porphin

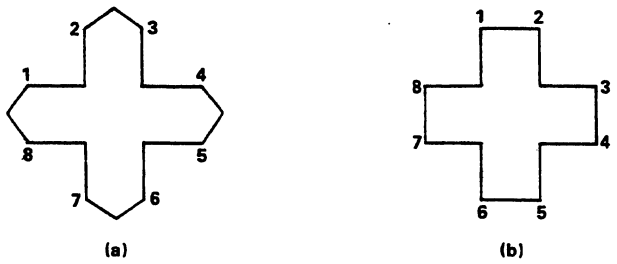
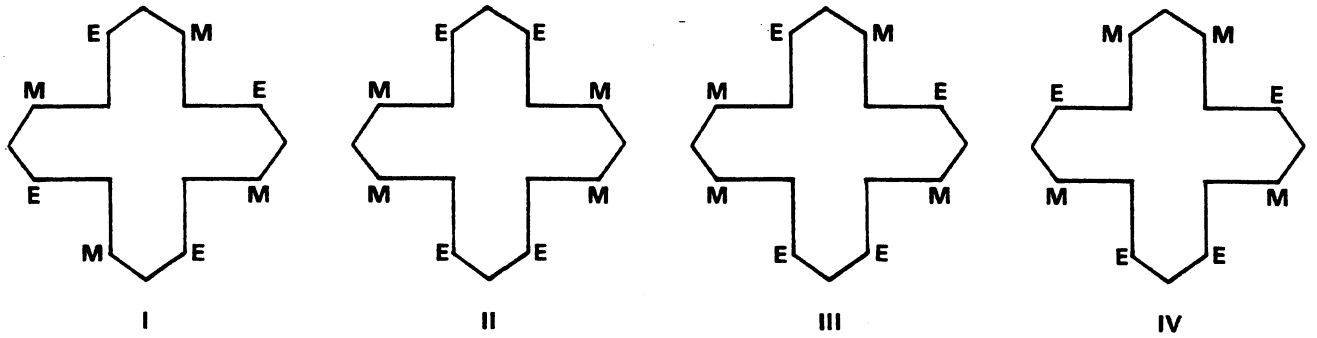


Figure 4.2 Porphin - Shorthand Notation



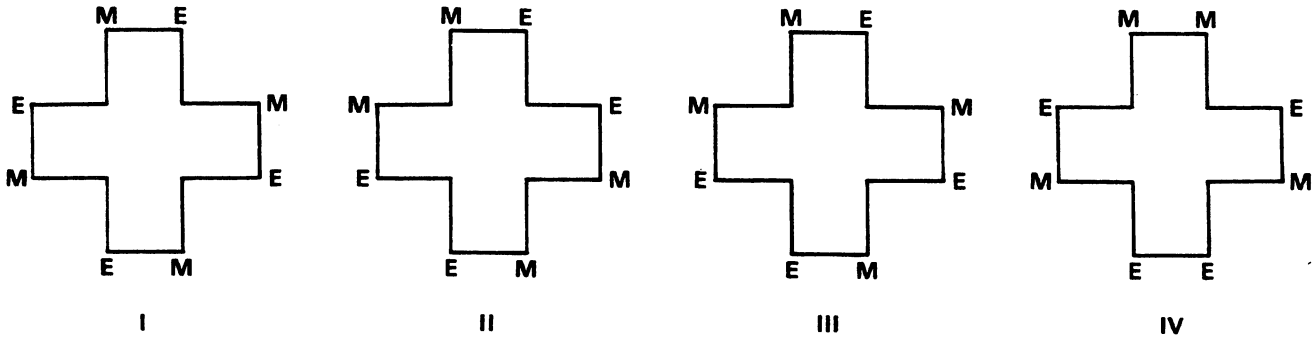


Figure 4.3 Structural Isomers of Etioporphyrin - Equivalent Representations

M - methyl E - ethyl

and Legge, 1949; Phillips, 1963; White *et al.*, 1973). Marks (1973) seems to be suggesting that the term *hemin* be reserved for ferriprotoporphyrin halide crystals (see Teichmann, 1853), and that it should not be used in place of the term *hematin*. *Ferroprotoporphyrin* may be called *ferroheme*, as *ferriprotoporphyrin* may be called *ferriheme* (or *ferrylheme*). In compounds in which the fifth and sixth liganding molecules are nitrogenous bases, the term *hemochromes* is often applied. The names *ferrohemochrome* and *ferrihemochrome* may be used to specify the valence of the iron atom. *Ferrohemochromes* have long been called *hemochromogens*, and the terms *hemichrome* and *parahematin* have been applied to *ferrihemochromes*. Table 4.2, a modification of Table I, Chapter V, of Lemberg and Legge (1949), gives a comparison of some of the different nomenclatures.

Another important consideration, which is not really a matter of nomenclature, but which may be worthy of brief discussion here, is that of the interconvertibility of the various hemoglobin derivatives. Both the crystal and spectral tests for the presence of blood in stains rely on these conversions. There are, in addition, a number of methods designed to determine the age of bloodstains which rely on the hemoglobin-methemoglobin interconversion. These are discussed in a later section.

The structure of hemoglobin will not be discussed here, but in a later section dealing with the determination of genetically-determined hemoglobin variants. Suffice it to say that native human hemoglobin is a tetrameric molecule, consisting of two α and two β polypeptide chains, having one heme per peptide chain, or four in the intact molecule, and a molecular weight of about 68,000. The iron atom is divalent in hemoglobin. Oxidation of the iron atom to the ferric state gives rise to methemoglobin (hemiglobin; fer-

Table 4.1. Side Chain Structures of Some Porphyrins

Porphyrin	Substituents	Type I	Type III
Etioporphyrin	4 M, 4 E	1,3,5,7-M 2,4,6,8-E	1,3,5,8-M 2,4,6,7-E
Mesoporphyrin	4 M, 2 E, 2 P	1,3,5,7-M 2,4-E 6,8-P	1,3,5,8-M 2,4-E 6,7-P
Protoporphyrin	4 M, 2 V, 2 P	1,3,5,7-M 2,4-V 6,8-P	1,3,5,8-M 2,4-V 6,7-P
Coproporphyrin	4 M, 4 P	1,3,5,7-M 2,4,6,8-P	1,3,5,8-M 2,4,6,7-P
Uroporphyrin	4 A, 4 P	1,3,5,7-A 2,4,6,8-P	1,3,5,8-A 2,4,6,7-P
Deuteroporphyrin	4 M, 2 H, 2 P	1,3,5,7-M 2,4-H 6,8-P	1,3,5,8-M 2,4-H 6,7-P
Hematoporphyrin	4 M, 2 HE, 2 P	1,3,5,7-M 2,4-HE 6,8-P	1,3,5,8-M 2,4-HE 6,7-P

Abbreviations used in the table: Numbering corresponds to Fig. 3.2. M- methyl E- ethyl P- propionic acid V- vinyl HE- hydroxyethyl A- acetic acid H- hydrogen

rihemoglobin). Methemoglobin does not bind oxygen, but will bind a number of other ligands, such as hydroxide, cyanide, azide and nitrite (Kiese, 1954). Figure 4.6 summarizes the interrelationships between the various derivatives (Lemberg and Legge, 1949; Pritham, 1968).

4.2 Crystal Tests

4.2.1 Introduction.

Parkes (1852) reported that, in examining microscopically the residual matter in a bottle which had contained partially putrefied blood, had been rinsed with water, and allowed to stand for a time, needle-like crystals could be observed in abundance. These crystals were insoluble in water and in strong acetic acid, but soluble in what I presume to be KOH. The crystals could be reprecipitated with strong acetic acid, but were less satisfactory and less abundant than the original crystals. Parkes noted that around this same time Funke had reported similar crystals from blood-water mixtures using horse spleen blood and fish blood. Drabkin (1946) has suggested that Funke may have been looking at hemoglobin crystals. Kölliker (1853-1854) reported that he had observed crystals in dog, fish and python blood in 1849. These were soluble in alkali and in acetic and nitric acids, and he said they were identical to Funke's crystals. Parkes subsequently attempted to prepare crystals similar to those which he had discovered by accident, but did not again obtain them in the same quantity. He did note that a number of different types of crystals are obtainable from putrefying blood, but could not identify them. He did not think they were identical to the hemoglobin crystals of Virchow nor to the albumin crystals of Reichert.

The preparation, microscopical and spectroscopic examination of crystalline forms of hemoglobin derivatives have occupied a great deal of attention in the development of methods for the medico-legal identification of blood. Many methods have been devised, and most are based on the preparation of either hematin or hemochromogen crystals. Many authorities have regarded crystal tests as methods of certainty in the identification of blood in stains (Beam and Freak, 1915; Brunig, 1957; Bertrand, 1931; Casper, 1861; Chiodi, 1940; Derobert and Hausser, 1938; Gonzales et al, 1954; Guarino, 1945; Lopez-Gomez, 1953; Lucas, 1945; Mueller, 1975; Olbrycht, 1950; Rentoul and Smith, 1973; Schleyer, 1949; Smith and Fiddes, 1955; Sutherland, 1907). Others who have considered the crystal tests in detail have been less explicit about the issue of whether the tests should be considered certain or not (Ziemke, 1938; Kirk, 1953). Dalla Volta (1932) took the position that the microscopical methods used to examine the crystals in routine forensic practice were inadequate to insure proof. Rather more sophisticated crystallographic analysis than would be routinely practical would be needed, in his view, to establish with certainty the presence of blood by these methods.

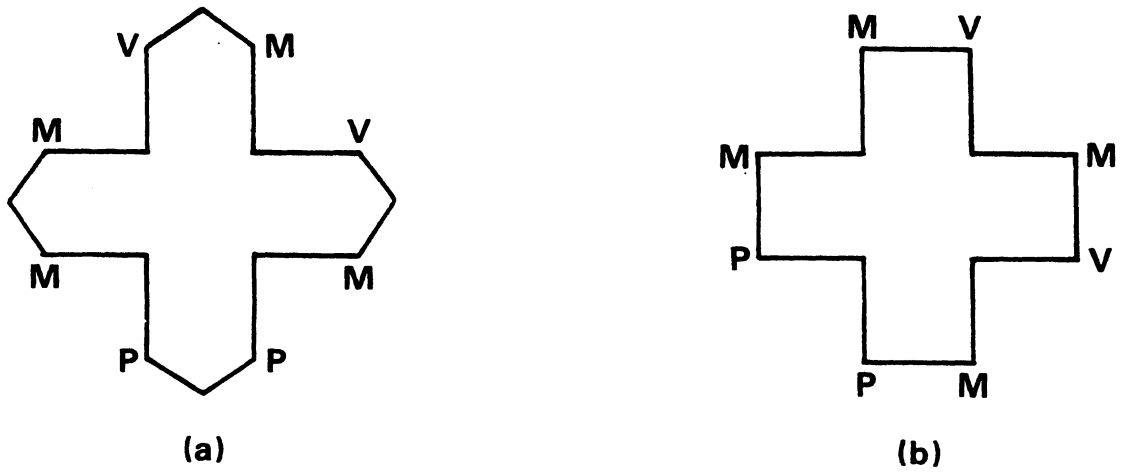


Figure 4.4 Equivalent Representations of Protoporphyrin IX

M - methyl V - vinyl P - propionic acid

4.2.2 Hematin crystal tests

In 1853, Teichmann published an extensive study of the formation of crystals in blood; this paper, still occasionally cited, formed the basis of the use of crystal tests for identification of blood in bloodstains. Teichmann's name has subsequently become synonymous with the hematin crystals which he obtained as well as with his own, and various modifications of the method for doing so. Teichmann's crystals are obtained by treatment of blood with glacial acetic acid in the presence of small quantity of salt and gentle heating. He called these crystals hemin, but they are now known to be hematin chloride. He suggested that the ability to form them reliably would provide the basis for medico-legal identification of blood in stains, as indeed it has done. Sutherland (1907) regarded a positive Teichmann crystal test as *"a sure proof of the presence of blood in the suspected stain"* (emphasis his own).

The extensive literature which developed on the subject has had mainly to do with the following matters: (a) development of different (hopefully improved) reagents for obtaining the crystals, including the use of halides other than chlorides, and of acids other than acetic; (b) variations in the way the test is carried out; and (c) experiments to determine the different conditions and substances that could interfere with the test, cause it to fail, or yield a result in the absence of blood. Casper (1861) in his Handbook mentions the test, noting that it is of value only when positive. The older literature on hematin crystals and crystals from blood in general was reviewed by Bojanowski in 1862. Table 4.3 is a modified version of one which was prepared by Lewin & Rosenstein (1895) in their review and reproduced by Sutherland (1907).

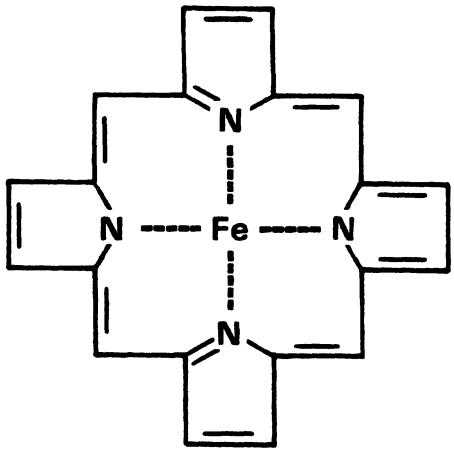


Figure 4.5 Iron Protoporphyrin

From it, one can get an idea of the different variations of the hematin crystal test in the older literature. It should be noted that some authorities felt that it was unnecessary to add salt because blood contains salts, which are present in sufficient quantity to yield crystals.

Henocque (1875) reported that an investigator named M. C. Husson had prepared hematin iodide crystals from the blood of a number of species. Sarda (1910) noted that Husson's work was reported in 1875, and that it involved both the iodide and the bromide of hematin. He reported very good results of his own with the potassium and ammonium salts of bromide and iodide.

Table 4.2. Comparison of Nomenclature of Hematin Compounds

Coordinating Ligands in iron protoporphyrin IX	Charge on over-all coordinate complex	Valence of Fe	Old Names	Nomenclature of Pauling & Coryell (1936) and Guzman Barron (1937)	Nomenclature of Clark (1939), Clark et al. (1940) and Drabkin (1938 and 1942a)	General Nomenclature
four pyrrole N	0	2	reduced hematin; heme	ferroheme	ferroporphyrin	heme
four pyrrole N, two additional N of nitrogenous base	0	2	hemochromogens; reduced hemochromogens; reduced hematin	ferrous or ferro-hemochromogens	base (e.g. dipyridine—, nicotine—) ferroporphyrin	hemochromes
four pyrrole N, water and OH^-	0	3	hematin; hydroxyhemin; oxyhemin	ferriheme hydroxide	ferriporphyrin hydroxide	hematin
four pyrrole N	yes★	3	hemins, e.g. chlorhemin, bromhemin	ferriheme chloride, bromide etc.	ferriporphyrin chloride, bromide etc.	hemins (Cl, Br, etc.)
four pyrrole N, two additional N of nitrogenous base	yes★	3	parahematins; oxidized hemochromogens	ferri- or ferric hemochromogens	base (e.g. dipyridine—, nicotine—, etc.) ferriprotoporphyrin	hemichromes

★ Charge depends on pH.

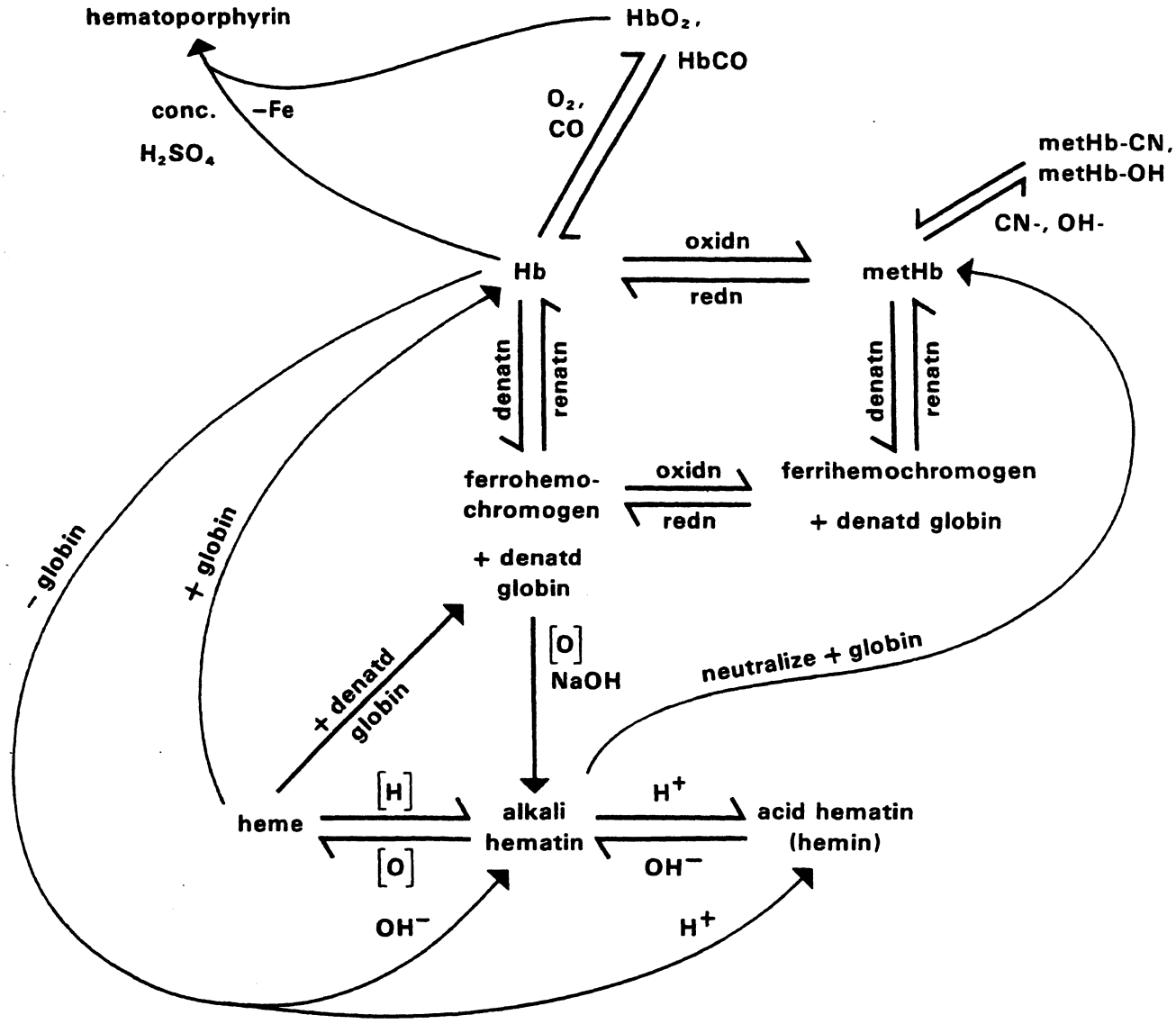


Figure 4.6 Interrelationships Among Hemoglobin Derivatives

Abbreviations used: Hb = hemoglobin HbO_2 = oxyhemoglobin Hb CO = carboxyhemoglobin metHb = methemoglobin metHb-CN = cyanomethemoglobin metHb-OH = hydroxymethemoglobin denatn = denaturation renatn = renaturation denatd = denatured [H] = reducing agent [O] = oxidizing agent oxidn = oxidation redn = reduction H^+ = acid OH^- = base conc = concentrated

Table 4.3 Some Modifications of the Hematin Crystal Test (modified from Lewin and Rosenstein, 1895)

Blood Preparation	Acetic Acid	Other Acid	NaCl	Other Salt	Heat or Temperature (°C)	Reference
dried,	much	oxalic, tartaric citric lactic	–	–	25 - 62.5	Teichmann (1853)
dried, fluid	slight excess	–	only if none in blood	–	cold or 40 - 60	Büchner and Simon (1858)
dried	to fill space under cover slip	–	yes	–	heat in flame	Virchow, 1857
–	yes	–	no	–	gentle heat or spontaneous evaporation	Morache, 1881
dried	few drops	–	no	–	heat until bubbles appear	Janert, 1875
dried, fluid	yes	–	few drops salt soln	–	heat in H_2O bath	Brücke, 1857
dried	yes	oxalic, tartaric in alcohol	yes if blood Cl free	NaBr, KBr NH_4Br, NaI KI	–	Bikfalvi, 1886
sediment prepared by copper sulfate pptn and extraction with alcoholic sulfuric acid	yes	–	yes	$BaCl_2$, SrCl, KCl LiCl, $CaCl_2$, NH_4Cl $MnCl_2$, $SnCl_2$, $FeCl_2$ $MgCl_2$	–	Teichmann, 1856
dilute solutions of blood pigment, add ammonia, tannic acid, then acidify with acetic acid, get hematin tannate precipitate	yes	–	no	NH_4Cl	–	Struve, 1880
alcoholic extract of dried precipitate by sodium carbonate pptn of defibrinated blood	yes	–	yes	$CaCl_2$	–	Gwosdew, 1886

According to Oustinoff (1929), who advocated the iodide crystals, Strzyzowski had prepared hematin iodide in 1902. Guarino (1945) employed iodoform in alcohol for the preparation of the crystals, and Lopez-Gomez (1953) reviewed, in some detail, the use of bromide and iodide salts in hematin crystal test reagents. Lopez-Gomez and Cantero (1942) are said to have carried out systematic studies on bromide and iodide crystals. Gouillart (1939) studied the formation of hematin iodide crystals in great detail. There is some confusion in the literature regarding the preparation of hematin fluoride crystals, Welsch and Lecha-Marzo (1912a) seeming to advocate their use, while Leers (1910 and 1912) believed that other halogens were greatly preferable. In any case, there does not seem to have been much subsequent use of fluoride-containing reagents. A number of authorities have recommended solutions containing 0.1g each of KCl, KBr and KI in 100 mℓ glacial acetic acid for the production of Teichmann crystals (Nippe, 1912; Kirk, 1953; Smith and Fiddes, 1955). Fiori (1962) said that there is no particular advantage to preparing hematin crystals from halogens other than chloride.

An extremely thorough study of the formation of both hematin and hemochromogen crystals was published by Mahler in 1923. He studied bloods from different species, including human, mostly as dried stains under a variety of different conditions and using a number of different methods. In these expriments, Mahler compared among other things the method of Wachholz (1901), utilizing alcoholic solutions of strong acids, with that of Nippe (1912) which called for a solution that is 0.1% (w/v) in KI, KBr and KCl in glacial acetic acid. Mahler got his best results from a combination of the two methods, wherein the blood-stained fragment was first warmed with alcoholic glacial acetic acid, and then warmed again following the addition of the Nippe reagent.

A similar set of comparative experiments was done by Kerr and Mason (1926). Some of the methods they studied were the same ones that Mahler had considered, but there were some differences. They preferred the method of Sutherland (1907), according to which a drop of saline is evaporated by heating on a clean slide, the stained fragment then being placed on the residue, and a drop of glacial acetic acid added. After applying a cover slip, the preparation is heated gently until bubbles just appear. It is then set aside and crytallization allowed to proceed.

Blood or bloodstains heated to temperatures in excess of 140° to 145° will not yield Teichmann crystals (Katayama, 1888; Hammerl, 1892; Wood, 1901). Bell (1892) discussed the hematin test in his review, and described the techniques then being used in this country by Formad (Formad, 1888) and by Prof. Tidy. Wood (1901) discussed his own experiences with the test in a paper read to the Massachusetts Medico-Legal Society. Any substance or condition which causes hemoglobin or hematin to form its decomposition product, hematoporphyrin (iron free hematin), he said, will interfere with crystal formation. Heat, long exposure to sunlight, and some organic solvents often cause difficulty. It is to be noted, nonetheless, that Muller *et al.* (1966) reported a positive Teichmann test from bloodstains on clothes that had been dry cleaned. Schech (1930), in his studies of the effects of ironing bloodstains on cloth on the subsequent ability to detect and analyze the bloodstain, noted that hematin crystals might be obtained if the iron had not been applied directly, but through a wet cloth for example. Rust and exposure, and especially the combination of these, interfere with the test (Sutherland, 1907).

Among the many modifications of the test that have been proposed, a few others will be mentioned. Oustinoff (1929) thought that the incorporation of gum arabic into the reagent improved the results. The heating step in the procedure, if done, is very critical. Bertrand (1931) discussed this point, noting that it is possible to err either in the direction of overheating or of underheating. He recommended a solution containing glycerol, apparently to lower the volatility of the reagent. Wachholz (1901) recommended a solution of a concentrated acid (lactic, sulfuric or acetic) in 95% alcohol because this boils easily, and reduces the chances of overheating. Sottolano and DeForest (1977) have described a technique utilizing Kirk's (1953) solution for hematin crystals which involves placing the test slides on a rack within a pressure cooker. Crystal formation is facilitated by the increased pressure, and the danger of total evaporation is overcome. The technique is applicable to the formation of hemochromogen crystals using Takayama's solutions (see Section 4.2.4) as well. Beam and Freak (1915) proposed a technique which, they stated, rendered crystal formation much more certain. The essential ingredient of this modification is very slow evaporation. The material to be examined is placed in the bottom of a flat, arsenic sublimation tube. A few drops of glacial acetic acid, containing 0.01 to 0.1% NaCl, are added, and a fine cotton thread is placed in the tube such that its lower end contacts the solution and its upper end is near the top of the tube. The thread is moistened if necessary to insure that it is everywhere in contact with the side of the tube, and evaporation is allowed to proceed at its own rate, without heating. The process which may require from 12 hours to more than a day, is accompanied by capillary movement of the solution within the thread, the crystals forming along the thread's length. This technique, recommended by Lucas (1945), is capable of giving large crystals suitable for crystallographic analysis (Fiori, 1962), but may not be very practical for routine work because of the investment of time required.

Although a number of materials and conditions interfere with the formation of hematin halide crystals, the age of the stain alone does not seem to be deleterious. Haseeb (1972) got a positive Teichmann test on a 12-year old stain kept on the laboratory bench in the Sudan. Beam and Freak (1915) obtained crystals from 10-year old human and 12-year old ovine bloodstains, as did Mahler (1923) from human stains over 20 years old. Dervieux (1911) mentioned that he had obtained hematin-iodide crystals from a 4000 year old bloodstained cloth from a mummy.

4.2.3 Acetone chlor-hemin crystal test

In 1935 Wagenaar recommended the preparation of acetone chlor-hemin crystals. A few drops of acetone are added to a fragment of bloodstain, followed by a drop of dilute mineral acid. Crystals form quickly at room temperature, even when the stains are old or the blood partially putrefied (Wagenaar, 1937). Dérobert and Hausser (1938) discussed this technique in their reference book. Chiodi (1940) carried out extensive studies on it with human, and different animal bloods, and stains exposed to adverse conditions. He recommended that it replace the Teichmann test. In 1949, Schleyer showed that the test could detect as little as 2 to 8 μg hemoglobin, and that methemoglobin and putrefied blood would give a positive test. Hematoporphyrin and blood heated above 200° do not give the test. Apparently, crystals are obtained from bile as well (due perhaps to the bilirubin content), but not from the urinary or fecal pigments (probably stercobilin, urobilin and urochrome) (Schleyer, 1949). Stassi (1945) reported that the test did sometimes fail in the presence of blood.

While the Teichmann and Wagenaar crystal tests are at least valuable, if not conclusive, tests for the presence of blood in stains, the crystals are not always easy to obtain. Even in experienced hands, these tests sometimes fail in the undoubted presence of blood (Sutherland, 1907; Corin, 1901; Dalla Volta, 1932; Olbrycht, 1950; Stassi, 1945; Mahler, 1923).

4.2.4 Hemochromogen crystal test

Hemochromogens are those compounds of ferroporphyrin (i.e., Fe^{2+}) in which the fifth and/or sixth positions of the hexacoordinate complex are occupied by the N atom of an organic base, such as pyridine, histidine, pyrrolidine, or various amines.

Hemochromogen was first prepared by Stokes in 1864. He obtained this material, which had a very characteristic spectrum by treating hematin with a reducing agent in alkaline solution. Stokes called the substance "reduced hematin." Hoppe-Seyler took up a number of further studies on the pigment finding, among many other things, that it bore as close a resemblance to hemoglobin as it did to hematin. He proposed that it be called "hemochromogen" (Hoppe-Seyler, 1877, 1879), this name having essentially supplanted that given the compound by the original discoverer. In 1889, Hoppe-Seyler prepared crystals by exposing hemoglobin to 100° temperatures in basic solution. These crystals have often been referred to in the literature as the first example of hemochromogen crystals. Leers (1910) noted that Hoppe-Seyler's student, Trasaburo-Araki reported obtaining similar crystals in 1890. Gamgee (1898) said that Hoppe-Seyler had not in fact obtained hemochromogen crystals, as had been reported in the textbooks for a number of years:

> It is quite erroneous to state, as is asserted in all textbooks, that Hoppe-Seyler succeeded in separating haemochromogen in a crystalline condition. He only succeeded (*at most*) in obtaining crystals of the CO-compound, and concluded that haemochromogen itself must be a crystalline body, but he never asserted that he had actually obtained the crystals, and a promise made in 1889 to describe the assumed crystalline haemochromogen, though implying that he had already obtained the body in this condition, was never fulfilled. Moreover, in the last systematic account of haemochromogen which he published in 1893 [Hoppe-Seyler and Thierfelder, 1893] Hoppe-Seyler does not refer to its being crystalline, but, on the contrary, speaks of it (as he had done in 1889) as separating in the form of a violet-grey powdery precipitate.

Copeman (1890) observed that hemochromogen crystals form from hemoglobin crystals upon long standing. Menzies (1895a) noted that allowing blood to stand in a water bath for some days in the presence of the chloride, bromide or iodide salts of potassium gave rise to a substance which could be converted to hemochromogen upon addition of a reductant, $(NH_4)_2S$. He further showed that ammonium sulfide would convert acid hematin to hemochromogen (Menzies, 1895b). Hüfner (1899) used hydrazine hydrate to convert alkaline hematin to hemochromogen.

Donogany (1893a) working in Budapest was the first investigator to note that pyridine reacted with hemoglobin to form hemochromogen crystals, and that these formed within a few hours if the material was placed on a microscope slide and sealed with a cover slip. He showed that this reaction occurred with dried blood, and suggested its application to the problem of medico-legal blood identification. In a subsequent paper (1897), Donogany suggested that the pyridine hemochromogen test would be useful for the determination of blood in urine, noting at the same time that he had first published his observations on pyridine hemochromogen in the Hungarian literature four years earlier (Donogany, 1893b).

Hemochromogen crystals may be obtained from either acid or alkaline solutions. Fiori (1962) stated that crystallization from acid solution was first carried out using acetic acid, pyridine and pyrogallol (1,2,3-benzenetriol), quite possibly a reference to the papers of Welsch and Lecha-Marzo (1912) or of Lecha-Marzo (1907), the latter of which was cited by Gisbert Calabuig (1948) who proposed the use of an improved solution: 0.5 mℓ glacial acetic acid, 1.5 mℓ pyridine and 1 mℓ 2% ascorbic acid.

It is far more common in the literature, however, to find descriptions of crystallization from alkaline solution. The older methods consisted of treating the stained material with pyridine and ammonium sulfide (Bürker, 1909; De Dominicis, 1902 and 1911), following upon the work of Menzies (1895a). Lochte (1910) suggested a modified version of the reagent containing NaOH and alcohol. Kürbitz (1909) and apparently Lecha-Marzo (1908) recommended extraction of the stain with alcoholic iodine solution prior to adding the pyridine-ammonium sulfide reagent. Leers (1910) used this method, but apparently employed an aqueous iodine solution. Alkaline solutions of pyridine containing hydrazine hydrate (Mita, 1910) or hydrazine sulfate (De Dominicis,

1909; Puppe, 1922) as reductants were described as well, being logical in view of Hüfner's earlier observations (1899). Nitrogenous bases other than pyridine will participate in hemochromogen crystals formation. Cevidalli (1905) successfully employed piperidine solutions, and Lochte (1910) noted that piperidine or picoline could be substituted for the pyridine.

The most comprehensive study of hemochromogen in the early literature is almost surely that of Dilling, published in 1910 in both German and English. This volume records the results of Dilling's extensive experiments carried out in Prof. R. Kobert's laboratory. Hemochromogen crystals were prepared from blood, hematin and other derivatives of hemoglobin utilizing pyridine, piperidine and a number of other nitrogenous compounds, with ammonium sulfide, hydrazine hydrate, and ammonium sulfide in NaOH as reductants. In each case the microscopic and spectral characteristics of the crystals obtained were described, as well as any problems encountered in the course of applying a particular technique. As nitrogenous bases, Dilling tested pyridine, piperidine, nicotine, methylpiperidine, ethylpiperidine, coniin (2-propylpiperidine), conhydrine (2-(α-hydroxypropyl) piperidine), and its isomer pseudoconhydrine, 2-methyl- and 3-methyl-pyridine (α- and β-picoline, respectively), α-dimethylpyridine (α-lutidine), as well as trimethylpyridine (collidine)† and tetramethylpyridine (parvoline), the two last mentioned probably consisting of mixtures of the isomers. Pyridine and piperidine were found to be the most satisfactory of all these compounds for crystal formation from blood. In addition to presenting his results, Dilling gave a good, comprehensive review of the pre-1910 literature. Somewhat less comprehensive reviews have been given by Kalmus (1910) and by Kurbitz (1910).

A great many authorities since 1912 have preferred to prepare hemochromogen crystals using the reagents described by Takayama in that year (Akaishi, 1956; Brünig, 1957; Gonzales *et al.*, 1954; Greaves, 1932; Hunt *et al.*, 1960; Kerr and Mason, 1926; Kirk, 1953; Lopez-Gomez, 1953; Mahler, 1923; Olbrycht, 1950; Rentoul and Smith, 1973; Thomas, 1937; Ziemke, 1924). Takayama's name has come to be used to describe not only the reagent he devised, but also the procedure and the hemochromogen crystals thus obtained, in much the same way as did Teichmann's name in the case of hematin halide crystals. The original paper in 1912 is cited in several different ways in the literature‡ and some difficulty was encountered in locating it. The paper, written in Japanese, appeared in *Kokka Igakkai Zasshi.*

Curiously, Takayama's paper was published in 1912 in Japan, but it does not appear to be mentioned in the European literature until Strassmann's paper appeared in 1922. It would be of interest to know how the information got from Japan to Germany. Takayama was in Germany around the turn of the century, but before 1912 (see in Unit IX, Translations). We had some correspondence on this point with Prof. Dr. Hiroshi Hirose of Kyushu University in Fukuoka, Japan. After some extensive searching in the early literature, Prof. Hirose discovered the paper by Strassmann (1922), and kindly shared the fruits of his search with us. His letter to me, in which the historical details are given and fully documented, has now been published (Hirose, 1979). Strassmann learned of the Takayama test from Prof. Fujiwara who was a student of Takayama's, and who was in Europe from 1920–1923. Takayama had noted that Reagent I required that the preparation be warmed for best results, while Reagent II did not require warming. Perhaps for this reason, many authorities preferred the second reagent, even in Japan where the test was widely used before its introduction in Europe. As mentioned in the footnote, the citation of the original paper was not correct in the German and English literature. Neither we nor Prof. Hirose could find any journals with the titles given in the incorrect citations. Takayama proposed two solutions, II being a sort of improved version of I.

Solution I:		Solution II:	
10% dextrose	5 mℓ	Saturated dextrose solution	3 mℓ
10% NaOH	10 mℓ	10% NaOH	3 mℓ
pyridine	10–20 mℓ	pyridine	3 mℓ
water	65–75 mℓ	water	7 mℓ

The crystals, obtained by treating a small amount of blood or stain fragment with these solutions are shallow rhomboids, salmon-pink in color. Gentle heating was needed with the first reagent, but not with the second.

As mentioned previously, Mahler's (1923) extensive study of blood crystals included hemochromogen crystals as well as hematin crystals. He compared a number of different methods and solutions for obtaining the crystals from a large number of different types of dried blood specimens. Kerr and Mason (1926) discussed the test in some detail as well, concluding, as had Mahler, that Takayama solution II was the most reliable reagent for obtaining hemochromogen crystals. The speed with which the crystals appear depends on temperature, being almost immediate if the material is heated, somewhat slower at room temperature (1 to 6 minutes), and slower yet if in the cold (e.g. if the reagent has been kept in the refrigerator). The reagent is stable for 1–2 months, crystals taking somewhat longer to form with older reagent. Greaves (1932) recommended the use of Takayama solution II for hemochromogen crystals as the method of choice for blood identification. He preferred not to heat the material, and suggested waiting up to several hours if necessary before deciding that the result is negative. Puppe (1922) also noted that good results were obtained without heating. Oustinoff (1930) suggested that gum arabic be included in the reagent, 1 part to 3 parts pyridine.

There are a number of advantages to the hemochromogen

† 2,4,6-trimethylpyridine is now called γ-collidine, while α-collidine is 4-ethyl-2-methylcollidine and β-collidine is 3-ethyl-2-methyl-collidine.

‡ Kerr and Mason (1926) and Greaves (1926) cited the paper as having appeared in the Japanese Journal of Toxicology. Ziemke (1924) and Wagenaar (1929) cited it as having appeared in Japan. Zeitschrift für Staatsarzneikunde, this last having probably been taken from Strassmann (1922).

test, as compared with the hematin test. A technical advantage is that heating is not required to obtain results within a reasonable amount of time; and even if one does prefer to apply heat, the test is not subject to being ruined by overheating. The test also yields positive results under some of the circumstances where the Teichmann test fails. Thus, Mahler (1923) obtained a positive Takayama test in cases of various 22 year old bloodstains on linen and of stains on rusty knives up to 23 years old, which failed to give Teichmann crystals. Similarly, Kerr and Mason (1926) showed that stains on linen and glass which had been heated to 150° for 30 minutes, stains (relatively fresh) washed in hot water, and stains on rusty metal surfaces up to 45 years old, all yielded hemochromogen crystals but did not give hematin crystals by the Sutherland technique.

According to Kirk (1953), both the specificity and sensitivity of the hemochromogen test are about the same as those of the hematin crystal test. Kerr and Mason (1926) and Greaves (1932) say that the hemochromogen test never failed in their hands in the known presence of blood. Mahler (1923) got some failures, but with solutions other than the Takayama II. The proof value of a positive hemochromogen test, with spectroscopic confirmation of the identity of the product, is not widely disputed. A negative crystal test, however, should not necessarily be interpreted as meaning that blood is absent (Kirk, 1953; Olbrycht, 1950). That bloodstains which have been exposed to heat, or are old or weathered, become increasingly insoluble has been known for a long time (Katayama, 1888; Hammerl, 1892). In cases such as these, solubility can be a problem in itself; if it is not possible to solubilize any hemoglobin or hemoglobin derivatives, it will obviously not be possible to obtain a positive crystal test.

Comparisons of sensitivity present some difficulty because various different authors express sensitivities in different ways. It is not always possible to convert one set of units or reference frame to another. This problem is encountered in many of the identification and serological and biochemical tests used in this field.

As to hemochromogen crystal tests, Greaves (1932) mentions only that the fragment of stain or stained material should be very small, only just large enough to be seen and manipulated onto a slide. Antoniotti and Murino (1956) noted that the test is still positive with 1 $\mu\ell$ of blood or about 0.1 mg hemoglobin, while Hunt *et al.* (1960) could obtain crystals from a stain fragment containing only 0.2 $\mu\ell$ of blood. Akaishi (1965) stated that a positive test could be obtained from a stain made from a 1:30 dilution of whole blood, but did not state how much stain was taken for the test. Miller (1969) thought that the Takayama test was considerably more sensitive than the Teichmann test. He could obtain Takayama crystals from 5 $\mu\ell$ of a 1:1000 dilution of whole blood, provided that the material was dispensed onto a slide in 10 separate 0.5 $\mu\ell$ aliquots in order to keep the area occupied by the test material as small as possible.

Not long after Kerr and Mason (1926) published their article on the Takayama test, Dilling (1926), in a letter to the Editor of the *British Medical Journal,* suggested that the hemochromogen test should not supplant the hematin test, but rather be used as to supplement it. He brought up two other points: (1) Kerr and Mason had said that the only discussion of hemochromogen tests in English prior to their paper was Sutherland's discussion, and Dilling correctly pointed out that he had published an extensive study of the subject in 1910; and (2) he believed that the purpose of the sugar in the Takayama reagent was to decrease the solubility of hemochromogen, and not, as Kerr and Mason had suggested, to serve as a reductant. Kerr (1926a) replied to the letter, saying that he agreed with Dilling's interpretation of the mechanism of action of the sugar. He further said that there was no prior account of the Takayama method in English, and that he still believed it to be much superior to the hematin test for medico-legal work.

Recently, Blake and Dillon (1973) have investigated the question of false positive hemochromogen crystal tests. They correctly note that the question has received virtually no attention in the medico-legal literature. Of particular interest was the issue of whether other iron-protoporphyrin containing substances, such as the enzymes catalase and peroxidase, would give misleading false positive crystal test reactions. Three presumptive tests were also carried out on the material, including the benzidine and phenolphthalin catalytic tests (sections 6.3 and 6.4) and the luminol test (section 6.7). A number of microorganisms were tested, since these are known to be particularly rich in catalase and peroxidase activities. Pure samples of both the enzymes gave Takayama crystals, those formed with catalase being virtually indistinguishable from the crystals obtained with blood. These materials also gave positive results with all three presumptive tests. The effectiveness with which different bacteria reacted with benzidine, in a two stage test, was directly related to the catalase content of the cells. One drop (about 0.05 mℓ) of a suspension of Citrobacter having a cell concentration of 400 $\times$ 10^6/mℓ gave a positive benzidine reaction, and several microbial suspensions which had been dried as spots on filter paper gave positive benzidine reactions after 2 months. Heating a number of different bacteria at 100° for 30 min or at 150° for 15 min did not abolish the benzidine reaction. Although pure catalase and peroxidase could give a positive crystal test, none of the bacteria tested contained sufficient concentrations of these enzymes under the test conditions to give a false positive Takayama test. Blake and Dillon cautioned that great care should be used in the interpretation of catalytic and crystal tests, and in combinations of them, since it is not only possible, but even likely in some situations, that case material will be contaminated with bacteria.

SECTION 5. SPECTRAL AND MICROSCOPICAL METHODS

5.1 Spectroscopic and Spectrophotometric Methods

Spectroscopic and/or spectrometric analysis of hemoglobin and its various derivatives is considered to be among the best methods for the certain identification of blood in stains (Brünig, 1957; Dérobert and Hausser, 1938; Ewell, 1887; Gonzales *et al.*, 1954; Gordon *et al.*, 1953; Lopez-Gomez, 1953; Mueller, 1975; Olbrycht, 1950; Rentoul and Smith, 1973; Simonin, 1935; Sutherland, 1907; Walcher, 1939; Ziemke, 1924). The methods are not technically difficult in practice, but as with so many of the methods and tests, a good deal of care should be exercised in the interpretation of results. Most of the older workers employed hand spectroscopes or microspectroscopes or both, since spectrophotometers were not widely available until relatively recently. Some authorities have said that the identity of hemochromogen crystals (section 4.2.4) should be confirmed microspectroscopically.

While the spectral methods might be thought to have the singular advantage of being nondestructive, they really do not if the tests are carried out properly. It does not suffice in the opinion of most experts to determine the spectrum of an extract of a stain, and infer from that alone the existence of blood in the stain. Even in cases where one can be reasonably certain that the material being examined is pure, the identity of a substance should never be inferred solely on the basis of the observation of its absorption spectrum. Lemberg and Legge (1949) state this concept especially cogently:

> While it is certainly true that under identical conditions the same substance cannot have different absorption spectra, spectra which are apparently identical are insufficient evidence for chemical identity.
> . . . . . It is always necessary to demonstrate identical *alterations* in spectra when chemical reactions are performed, before identity of two substances can be considered in any degree certain.

This *caveat* must be considered especially relevant to medico-legal identifications, not only since there are a large number of porphyrin compounds in nature, many of which have common spectral features, but also since it can almost never be assumed that stain evidence is uncontaminated. Most of the spectral methods, therefore, involve the preparation of various hemoglobin derivatives, followed by verification that these have actually been obtained by measuring the spectra. This subject, along with the crystal tests and other chemical tests, has been excellently reviewed by Fiori (1962). I am indebted to this work for source material as well as for lucid explanations of many aspects of the various tests.

Since there is quite a large number of different hemoglobin derivatives that may be used in identification procedures, and a fairly extensive literature on their absorption spectra, it seemed most profitable to present a cross section of this information in tabular form (Lemberg and Legge, 1949; Fiori, 1962). When there are multiple absorption maxima, the bands are sometimes called I, II, III, etc., in going from longer to shorter wavelengths; another convention is to refer to the major visible bands as α and β, the α-band being at longer wavelength. A very intense band in the region of 400 nm, characteristic of all conjugated tetrapyrrole structures, is known as the "Soret band". Table 5.1 gives the absorption maxima reported by different workers for a number of hemoglobin derivatives. A composite representation of the spectra of some of the derivatives, as presented by Lemberg and Legge (1949), is given in Figure 5.1. The absorption bands for a number of derivatives, as seen in the spectroscope, are given in Fig. 5.2, as originally shown in the review of Hektoen and McNally (1923). Representations such as that in Figure 5.2 are still seen in textbooks of legal medicine.

Among the earliest papers on the spectral properties of the "coloring matter of blood" was that of Hoppe (1862). He observed and reported the absorption bands of hemoglobin and several of its derivatives in the visible range of the spectrum, and suggested that the spectral method be employed for the forensic identification of blood. More elaborate studies were done by Stokes (1864) who recognized the difference between Hb and HbO_2, described the spectral properties of hematin and, for the first time, of hemochromogen. Sorby (1865) independently studied the spectra of a number of hemoglobin derivatives and advocated the spectral method for the identification of bloodstains. In 1868, Herepath discussed the techniques in some detail. He was able, using the microspectroscope, to identify blood on the wooden handle of a hatchet which had lain exposed in the country for several weeks. In this case (Reg. v. Robert Coe, Swansea Assizes, 1866), the amount of blood remaining on the handle was very small. The technique was stated to be sufficiently sensitive to detect ". . . . less than one thousandth of a grain of dried blood, the colouring matter of which had been dissolved out by a drop and a half of distilled water." Bell (1892) noted that Dr. Richardson of Philadelphia had said he was able to detect the blood on an ax handle equivalent to $\frac{1}{3000}$ grain of blood in a case that he had had. Sorby (1870) described his own technique, and stated that it was equally applicable to medico-legal blood identification and to the clinical identification of blood in urine. These applications were based on his earlier studies of the spectra of the various derivatives (Sorby, 1865), in which he had first

Table 5.1 Summary of Absorption Maxima of Hemoglobin and Some Derivatives

Compound	Abbreviation	Method of Formation	Conditions/Remarks		Absorption Maxima (nm)	References ★
Hemoglobin	Hb	from HbO_2 by reduction with $(NH_4)_2S$, 10% dithionite, 0.2% hydrazine sulfate, etc.	–		270-280 430 555 755	6,7,17,20,21,24 26
Oxyhemoglobin	HbO_2	–	–		275 350 412-415 540-542 577 920-930	6,7,11,13,17,18,19,21,24,25
Carboxyhemoglobin	HbCO	CO readily replaces O_2 in HbO_2	spectrum similar to that of HbO_2		270-280 330-340 418 538-540 568-572	6,11,13,17,18,21,25,26
Methemoglobin	MetHb	oxidation of hemoglobin by $KMnO_4$, $K_3Fe(CN)_6$, etc. – forms in bloodstains upon aging	spectrum is pH dependent; the 540 and 577 bands increase in intensity as pH increases and the red band shifts to 600 and decreases – acidic compound is brown – basic one is red	acidic	266-275 404 500 570 630 (pH 7)	1,11,13,15,17,19,20,25
				basic	411 540 577 600 (pH 10)	
Cyanmethemoglobin	MetHb-CN, HICN	addition of neutral solutions of cyanide to MetHb solutions	compound is red		412-421 540	7,18,25
Hematin	–	acid: addition of glacial acetic acid containing ethanol to solution of hemoglobin prolonged exposure of blood-stains yields this product	spectrum is pH dependent	acid	400 510 540 630-635 (in acetic acid)	11,21
					278 376 526 550-558 626 662 (in HCl)	11,22,25
		alkaline	pH dependent spectrum	alkaline	220 365 385 492 606-615 (NaOH) 585 (borate, pH 10)	2,8,10,11,12,13,14
					402 590 (alcoholic $NaHCO^3$)	
Hemochromogen (hemochrome)	–	from blood or hematin compounds by addition of reducing agent in presence of nitrogenous base	(Fe^{2+}) 20% pyridine and pH 11.6 solutions		419-424 525-530 550-560	3,5,8,9,16,21
			(Fe^{3+})		545 575	8
Cyanide hemo-chromogen (Dicyano ferroporphyrin)	–	from bloodstains heated with 10% KCN, then treated with reducing agent	–		430 537-540 565-570	4,5,10,27
Sulfhemoglobin	–	treatment of Hb with H_2S in presence of O_2	–		400 617-623	19,20
Hematoporphyrin	–	treatment of Hb, blood with conc. H_2SO_4	–	acid	404 553 577 598	23
		treatment of acid hematoporphyrin with KOH	–	alkaline	504 539-541 566 618	11

★ Numbers in reference list in Table refer to the following references:

1. Austin and Drabkin (1935)
2. Clegg and King (1942)
3. DeDuve (1948)
4. Drabkin (1942a)
5. Drabkin (1942b)
6. Drabkin (1946)
7. Drabkin and Austin (1935a)
8. Drabkin and Austin (1935b)
9. Falk (1963)
10. Guzman Barron (1937)
11. Heilmeyer (1933)
12. Herzfeld and Klinger (1919)
13. Hicks and Holden (1929)
14. Hogness et al. (1937)
15. Holden (1943)
16. Holden and Hicks (1932)
17. Horecker (1943)
18. Kennedy (1927)
19. Klese (1954)
20. Lemberg and Legge (1949)
21. Mahler and Cordes (1971)
22. Newcomer (1919)
23. Schmidt (1940)
24. Sidwell et al. (1938)
25. Van Assendelft (1970)
26. Winegarden and Boorsook (1937)
27. Ziemke (1910)

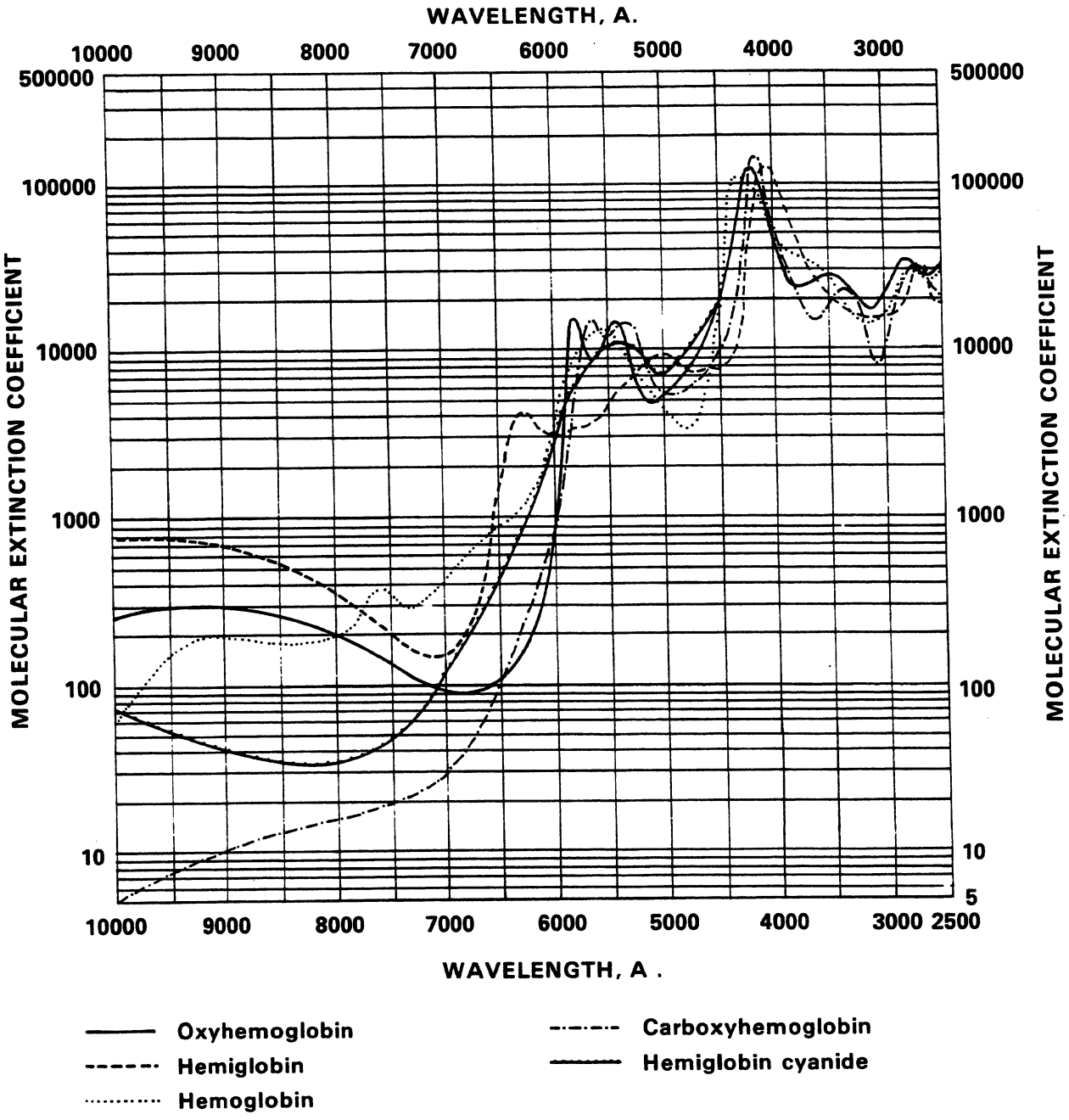


Figure 5.1 Absorption Spectra of Some Hemoglobin Derivatives (after Lemberg and Legge, 1949) (Reprinted by permission of Interscience Publishers, Inc.)

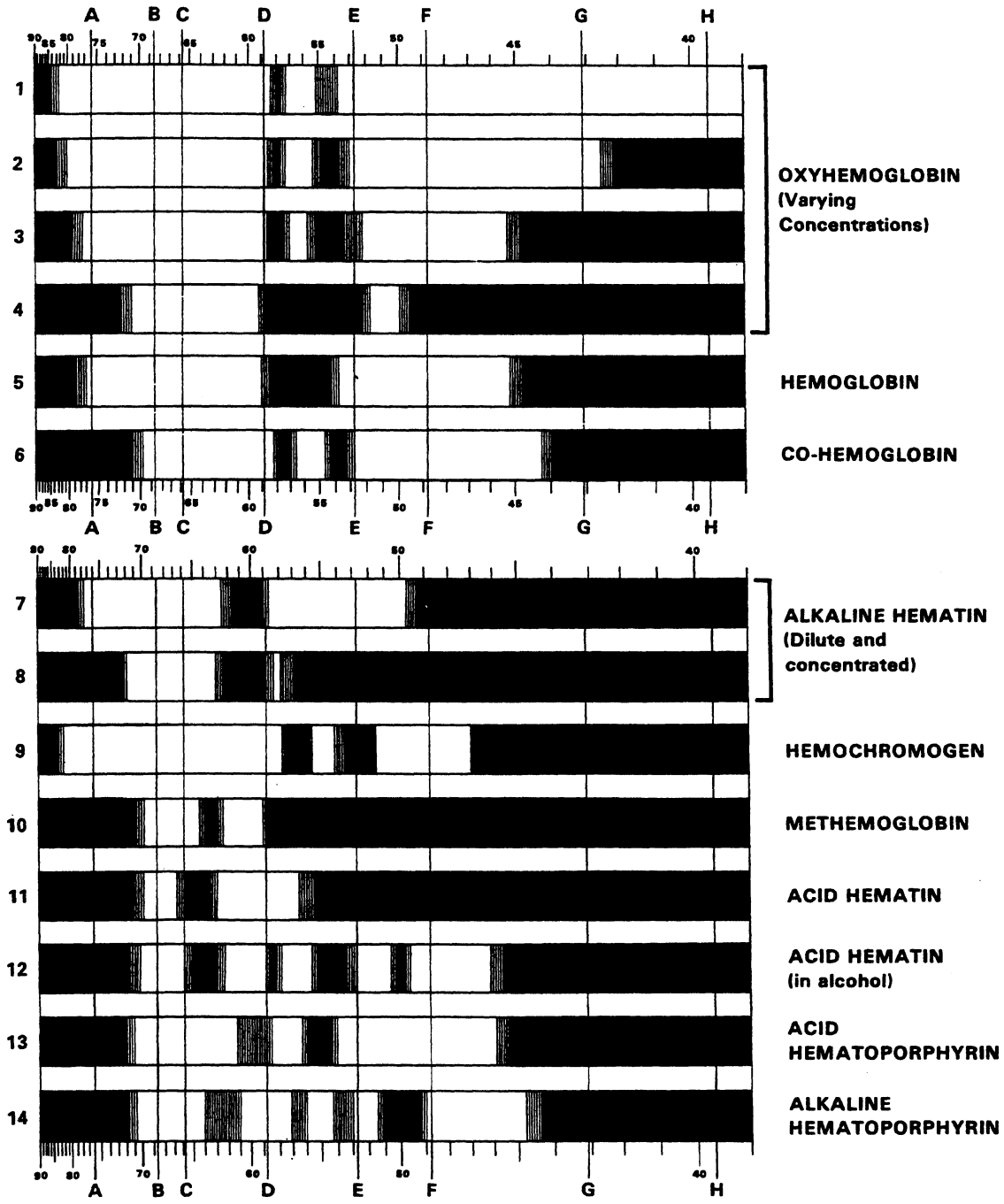


Figure 5.2 Absorption bands for hemoglobin and some derivatives. Wavelengths in nm for Fraunhofer lines: B 686.7, C 656.3, D 589.3, E 527.0, F 486.1, G 430.8. From: L. Hektoen and W. D. McNally *Medicolegal Examination of Blood and Bloodstains* in: F. Peterson, W. S. Haines & R. W. Webster (eds.) *Legal Medicine and Toxicology*, 2nd ed., V. II, 1923. Reprinted by permission of W. B. Saunders Co.

recommended this technique for forensic examinations. Dr. Taylor recommended Sorby's technique for medicolegal cases, and wrote a sort of introduction to the 1870 paper (Taylor, 1870).

The spectral methods were incorporated into the repertoire of techniques for examining bloodstains fairly quickly. The earlier texts of Chapman (1892), Ewell (1887) and Reese (1891) carry discussions of them. Relatively fresh, reasonably well-preserved stains may still show hemoglobin spectra. As aging proceeds, the hemoglobin is converted into methemoglobin and later into hematin. The last-mentioned is exceedingly less water-soluble than hemoglobin, it being necessary with old stains to extract with acid, base or solvents. The extracts may then be examined for the appropriate hemoglobin derivative. Reese (1891) was apparently recommending the preparation of alkaline methemoglobin in cases of stains, though present-day terminology for the derivatives was not then used. In the early English literature, as noted by Gamgee (1868), hemoglobin was called *cruorine,* after Stokes' suggestion (1864). HbO_2 was called "scarlet" cruorine, while Hb was "purple" cruorine. The early workers regarded the $Hb + O_2 \rightleftharpoons HbO_2$ reaction as one of oxidation-reduction, and they seem to have had difficulty squaring this concept with their results using oxidizing or reducing agents which were affecting the valence of iron. Sorby (1865) apparently suggested that the term "brown cruorine" be applied to methemoglobin. It was Hoppe-Seyler (1864) who introduced the present-day name "hemoglobin". "Um Verwechselungen zu vermeiden, nenne ich den Blutfarbstoff Hämoglobulin oder Hämoglobin," he wrote. [In order to avoid confusion, I call the blood pigment hemoglobulin or hemoglobin].

All hematin compounds can be transformed into hemochromogens by additon of base, reducing agent and a nitrogenous compound (Lemberg and Legge, 1949). The derivative, characterized spectrally by a sharp α-band in the 550–560 rm region, is therefore one of the most useful in diagnosing bloodstains. As discussed previously in section 4.2.4, heme will combine with a variety of nitrogenous bases to form hemochromogens. Bloodstain extract may be treated with strong alkali and ammonium sulfide to form hemochromogen (Sutherland, 1907). Simonin (1935) suggested extracting the bloodstain with 10 mN HCl, and treatment of the extract with KOH and hydrosulfite. The characteristic band at 560 nm is detectable by this method at 1:500 to 1:1000 dilutions of whole blood. Smith and Simpson (1956) and Glaister and Rentoul (1957) recommended a reagent made up by shaking 2 g $Na_2S_2O_4$ in 5 mℓ 10% NaOH or KOH and adding 1 mℓ alcohol. This solution must be freshly prepared before each use. Pyridine hemochromogen may be obtained using Takayama's solution (Smith and Simpson, 1956; Fiori, 1962), as discussed in Section 4.2.4. Riegler (1904) used a solution of hydrazine sulfate in alcoholic NaOH. A drop of hydrazine sulfate in 20% KOH, introduced under the cover slip on a slide containing the sample, was recommended by Hesselink (1931). Olbrycht (1950) dissolved the stain directly in pyridine, then added hydrazine sulfate as reducing agent. Meixner (1927) recommended KOH in glycerol as a good medium for extracting older stains. Hankin (1906), who was concerned with stains that underwent rapid putrefaction when damp, because of the tropical climate, recommended treatment with boiling water, followed by addition of ammonium sulfide on a slide. Cyanide hemochromogen may be prepared as well (Hankin, 1906; Fiori, 1962) if KCN solution is used to dissolve the bloodstain.

The use of the hematoporphyrin spectrum for bloodstain identification was first suggested by Struve in 1880, but not until Kratter published the results of his experiments in 1892 did the method begin to enjoy more general use. Hematoporphyrin is formed from bloodstains upon treatment with concentrated H_2SO_4. The iron atom is removed by this treatment and the vinyl residues of the porphyrin nucleus are oxidized to hydroxyethyl groups (see Table 4.1). This material was originally called "iron-free hematin". The treatment with sulfuric acid can cause charring of the fabric, and carbonized fabric fragments interfere with spectral examination. Ipsen (1899, 1900) thought that the treated material could be washed in order to get rid of such particles, and more acid added. But Ziemke (1901) found that this procedure did not work well. He advocated 24 hr sulfuric acid treatment of the stain, glass wool filtration and neutralization to form a precipitate. The precipitate is then washed, dried and an ammonia-alcohol extract of it examined spectrally. Takayama (1905) allowed the acid to act on the stain for 5–7 days, after which the material was heated, diluted to about three times its volume with water, filtered through glass wool, and examined.

The hematoporphyrin procedure may well be the least desirable of all spectral tests, if proof of the presence of blood is wanted. There are probably more naturally occurring substances with spectra similar to hematoporphyrin than to the other derivatives. When bloodstains are very old, however, or when they have been exposed to high temperatures for extended periods, they become very difficult to dissolve, and often fail to give hematin or hemochromogen. In these cases, hematoporphyrin preparation may be the only recourse. Fiori (1962) notes that strong acids should be avoided for spectral determination, and that hematoporphyrin is, therefore, best prepared by the milder method of Dotzauer and Keding (1955). The stain is dissolved in 0.1N HCl, with heating if necessary. After 15 minutes, concentrated thioglycollic acid is added and the material heated to boiling for 1 minute. Hematoporphyrin formation is complete in about a half hour. Olbrycht (1950) has pointed out that bloodstain extracts can easily be contaminated with substances from the substratum which may then interfere with the identification tests. The use of concentrated sulfuric acid would only tend to aggravate such contamination problems because the acid is such a strong solvent. This consideration strengthens Fiori's contention that strong acids should be avoided.

Many conditions to which bloodstained material can be exposed affect the spectral tests (as, of course, they affect any of the identification or grouping tests). Weathering,

submersion in water, exposure to sunlight, heat, washing, or rust can all cause great difficulty. Rust formation is accelerated on bloodstained ferrous metal surfaces (Buhtz, 1933), and rust contamination often precludes the identification of blood. Scheller (1937) studied the spectral identification of bloodstains on various substrates after exposure to air and weathering in different humidities. Damp environments cause more rapid deterioration of the stain than moderate ones, and accelerate the formation of rust on bloodstained ferrous metal surfaces and of $ZnCO_3$ on bloodstained galvanized sheet metal surfaces. Green plants are particularly difficult contaiminants of samples to be subjected to spectral tests because of the chlorophyll, which, being itself a magnesium porphyrin derivative, shares spectral characteristics with hematin compounds and which may be convertible under some circumstances to similar derivatives (Mayer, 1933). A bloodstain on a green leaf may therefore present special problems in this regard. Hirose (1966a, 1966b) studied the effects of soil on bloodstained fabrics buried in earth. Hemoglobin leeches out of such fabrics, the rate being directly proportional to soil moisture content, but independent of soil temperature. Hemoglobin solubility decreases with time in such samples as well. In the case of a bloodstained cotton cloth buried in soil of pH 5.3, Hirose found the order of efficiency of extraction of hemoglobin (from most efficient to least efficient) to be as follows: 20% pyridine 1% NaOH > Veronal buffer, pH 9.2 > Veronal buffer, pH 7 > Veronal buffer, pH 5.3 > water. The hemochromogen spectrum could be obtained in some samples after 40 days in the soil. Olbrycht (1950) carried out extensive studies on spectrophotometric and spectrographic identification of stains at various dilutions of blood. Microspectrometric determination can fail if stains on linen were made from blood diluted more than 1:150; spectrographic tests in the UV region of the spectrum, however, can detect stains made from up to 1:400 dilute non-hemolyzed blood and 1:750 dilute hemolyzed blood, these being too dilute to give any evidence to the naked eye of there being a bloodstain on the cloth. Sunlight, metal oxides (especially rust) and admixture with fine, aluminum and iron containing soils were found to be the most deleterious conditions in Olbrycht's studies. Haseeb (1972) reported spectral identification of Hb and metHb in a 12 year old bloodstain kept on the laboratory bench in the Sudan. Hirose (1976) mixed blood with iron powder and allowed the mixture to age in order to mimic the aging of a bloodstain on an iron surface.

The pyridine-hemochromogen spectrum test was carried out at 1, 11 and 51 days, with the results shown in Table 5.2. The 20% pyridine-1% NaOH extraction medium was found to be best one under these circumstances, and the higher pH veronal buffer more efficient than the lower pH one, just as he had found in the case of the bloodstained cloth buried in soil (Hirose, 1966b).

5.2 Spectrofluorimetric Methods

In 1916, Heller proposed the use of hematoporphyrin fluorescence, resulting from excitation by ultraviolet light, for the identification of bloodstains. Methods of preparing hematoporphyrin have been discussed in the previous section. Hematoporphyrin gives a red fluorescence when illuminated with 366 nm light (Fiori, 1962). Dotzauer and Keding (1955) studied this method in some detail, and their method for preparing hematoporphyrin is given above, in section 5.1. The fluorescence is dependent on the solution used, the substrate concentration, the pH and the temperature. Scheller (1973) suggested this method for identifying bloodstains on oxidized metals. Dotzauer and Kedding (1955) showed that bloodstains up to 10 years old, exposed to heat, sunlight or humidity can give positive results. Further, chlorophyll does not interfere under their conditions because the acid concentration is too low to bring about its conversion to hematoporphyrin. Ju-Hwa and Chu (1953) obtained hematoporphyrin and its characteristic fluorescence by adding a solution of 50 ml glacial acetic acid and 50 ml 0.1N HCl, containing 1 g hydrazine di-HCl, to very dilute blood solutions (1:5000 - 1:20,000).

Direct application of the reagent to stains on fabrics or paper was unsuccessful, but treatment of saline extracts gave good results. Chlorophyll, however, gave positive results in this system as well.

Schwerd (1977) recommended hematoporphyrin fluorescence as a certain test for the presence of blood in stains. He preferred the sulfuric acid method of preparation to the HCl/thioglycollic method of Dotzauer and Keding (1955). Amounts of blood in stains corresponding to 0.6 μg Hb could be detected, and Schwerd indicated that he thought the possibility of false positive results with this test, when properly carried out, had been overstressed.

The same constraints concerning interpretation must, of course, apply to the spectrofluorimetric test for hematoporphyrin as do to the spectrophotometric test for it. The ubiquitous biological occurrence of porphyrin compounds must be kept in mind. Bile, feces and meconium may give positive results in these tests because of porphyrin compounds contained in them (Fiori, 1962).

5.3 Microscopical Methods

5.3.1 Blood identification by microscopical techniques

Microscopical tests have been applied to bloodstains for a number of different reasons, including:

- Identification of blood in stains
- Determination of species by noting whether red cells have nuclei or not and/or structure of white cells
- Examination of leucocytes for chromatin bodies for cytological determination of sex of origin of the stain
- Examination for epithelial and other cells which might indicate the origin of the bloodstain from a particular organ or tissue
- Examination for the presence of pathological conditions, such as hematological diseases, presence of parasites, etc.

The discussion presented in this section has primarily to do with microscopical methods as means of identifying blood.

Table 5.2 Pyridine Hemochromogen Spectrum After Aging of Blood-Iron Powder Mixtures As Percent of Zero Time Control (after Hirose, 1976) Reprinted by permission of *Forensic Science* and Prof. H. Hirose.

Extraction Medium	Age in Days		
	1	11	51
20% pyridine - 1% NaOH	67	36	33
1.25% NaOH	41	14	11
25% pyridine	30	5	4
Veronal buffer, pH 9.4	22	4.2	3.1
Veronal buffer, pH 7.8	14	0.6	0.4
Saline	18	0	0

Microscopical measurement of red cells as a means of identifying the species of origin of blood will be discussed in a later section (Section 15) as will cytological methods for determination of sex of origin (Section 48).

In one of the earliest systematic investigations of the use of microscopy in medico-legal inquiries, Mandl (1842) discussed the identification of blood in stains through the recognition of blood cells. Although he appeared to think that microscopical examination should supplement chemical tests where possible, he clearly thought that the finding of blood cells provided an unequivocal identification criterion if the chemical tests failed to give results. Robin and Salmon (1857) strongly advocated microscopy for the identification of blood in stains. They said that identification of white and red blood cells in the stain along with fibrin threads yielded unequivocal proof of the presence of blood. In the paper, they were reporting on a case in which a suspect named Doiteau had been accused of killing an old woman. The defendant claimed that the stains on his clothing had come from a duck, but the experts said in their report that, based upon the microscopical examination, the stains were of human origin. Roussin (1865) said that microscopical examination of suspected stains was the only sure method of arriving at an identification of blood, and that chemical tests were unreliable. A positive Teichmann crystal test (Section 4.2.2) gave at least strong presumptive evidence of the presence of blood in his opinion, but he regarded the crystal test as unpredictable, and said that in many cases it did not work.

Over the years, a large number of different solutions have been proposed for treatment of suspected stains. Various claims were made for their properties, these mostly having to do with the ability of the solutions to bring about the extraction of "intact" red cells, and protect or restore their shape and size to the *in vivo* condition. A number of these solutions have been reviewed by Formad (1888), Sutherland (1907) and Ziemke (1938). They are very common in the old literature, mostly being named for their proposers, e.g. Hayem's fluid, Roussin's liquid, etc. A few examples of these are given below.

1. 4 g NaCl, 26 g glycerine, 2 g $HgCl_2$ and 226 mℓ water; diluted 2-3X with water before use. (Pacini, 1972)
2. 1 vol conc. H_2SO_4, 2 vol glycerine, and water to yield S.G. = 1.028 at 15° (Roussin, 1865)
3. 32% aqueous KOH (w/v) (Donders, 1847-48). This solution was recommended by Virchow (1857) and by Malinin (1875) and is sometimes called Virchow's fluid
4. 5 g Na_2SO_4, 1 g NaCl, 0.5 g $HgCl_2$ in 100 mℓ water (Hayem, 1889)
5. 2 g NaCl, 0.5 g $HgCl_2$ in 100 mℓ water (Vibert, 1911)
6. Artificial serum of Malassez and Potain: equal parts of aqueous solutions of gum arabic, NaCl and Na_2SO_4, all of S.G. = 1.020 (Sutherland, 1907, Formad, 1888)

These solutions are now very seldom used or encountered, though as recently as 1954, Kerr was still recommending Roussin's solution in the 5th edition of his text, *Forensic Medicine*. He stated that the microscopical finding of red cells in stain extracts constituted unequivocal proof of the

presence of blood. There can be no doubt that the statement is true. The difficulty lies in being sure that one is, in fact, looking at red blood cells. Even under the most ideal circumstances, the red cells of most mammals do not exhibit many characteristic structural features, being enucleate and quite small. Their shape is exceedingly sensitive to changes in the osmotic strength of their medium; within a certain range of osmolarity they behave as almost perfect osmometers. Red cells are readily crenated in hypertonic media, and swollen and lysed by hypotonic ones. Membranes can "re-heal" after lysis or disruption, forming membrane-bound vesicles which may or may not bear any resemblance to intact erythrocytes.

The changes that occur in the structure of red cells upon dessication, as in the formation of dried bloodstains, and subsequent "reconstitution" with one or another solution, are probably extreme, and little documented. Most of the available information is now quite old, and consists of "before-and-after" studies and observations. Lucas (1945) mentioned Vibert's solution for the reconstitution of red cells from bloodstains, but noted that the results were often highly unsatisfactory. These difficulties seem to have been recognized long ago, at least by some workers; Sutherland (1907) quotes Tourdes as having written in 1878 that it is of little use to look for red cells in bloodstain extracts that resemble those found in blood, because what will more likely be seen is ". . . . rounded bodies, more or less spherical or flattened, and of a more or less deep tint, mixed with amorphous debris, the result of the destruction of other erythrocytes." The difficulties accompanying the method were long ago underscored by Liman as well. In 1863, he wrote:

> "It is exceedingly difficult and risky to try to find blood cells with the microscope in old, dried and contaminated stains. The method is of no use if the blood cells have been destroyed by being washed or pulverized."

In spite of all the problems, the experienced observer can sometimes locate red cells or white cells in stain extracts leaving no doubt as to the origin of the stain.

In more recent times, investigators who have advocated microscopical examinations of stains have not restricted their attention to red cells, but have suggested techniques which would enable the visualization of leucocytes as well. A number of suggested methods have thus involved the use of various histological stains. Rojas and Daniel (1927) and Nicoletti (1933) suggested the staining of the cells with hematoxylin and eosin *in situ*. Fiori (1962) suggested that this sort of histological staining be avoided if material is precious. Such as is available in these cases should be preserved for more reliable methods of identification, he said, noting also that interpretation of results requires great care since various structures might be mistaken for red cells. Rojas and Daniel (1927) called for fixation of the stained material with equal parts by volume of alcohol and ether, followed by hematoxylin-eosin staining. Cevidalli and Dalla Volta (1923) followed a similar procedure except that fixation was effected by treatment with a series of aqueous alcohol solutions of decreasing alcohol concentration. They also employed Giemsa and May-Grünwald stains. These methods were especially recommended for bloodstains on relatively transparent and non-colored material. Otherwise the fibers had to be teased apart carefully. In cases of bloodstains on smooth surfaces (e.g. knife, floor tile, etc.) or on nontransparent textiles, transfer methods must be used. Fiori (1962) classifies the transfer methods as "fast" and "slow". The former consist essentially of scraping or chipping a bit of bloodstain onto a slide, crushing it up to give fine fragments or powder, reconstituting with a solution such as albumin-glycerol (Cevidalli and Dalla Volta, 1923) or human serum (Undritz and Hegg, 1959), and fixation followed by staining. The "slow transfer" method (De Dominicis, 1917) consists of covering the suspected stain with a thin layer of celloidin in alcohol-ether solution and allowing it to dry. The resulting film can be removed and will have blood cells attached to it. Celloidin, incidentally, is a form of cellulose nitrate contained in a solvent and used extensively in histology for embedding microscopic sections. Cevidalli and Dalla Volta (1923) discussed the celloidin method in detail, and it was recommended by Romanese and Pinolini (1922).

As suggested earlier, there are different reasons for making microscopic preparations and the selection of histological stains is determined by what is wanted. Staining of the red cells and white cells can serve to identify the stain as blood. The structure of certain of the leucocytes can help determine species of origin, while the polymorphonuclear leucocyte nuclei must be stained to look for Barr bodies to determine sex of origin. Leucocyte granules also possess a peroxidase activity that may be of limited value in assessing bloodstain age.

Stain preparations like many other things in biology, have tended to acquire the names of their proposers or early users. Hematoxylin is a very common histological stain. As discussed above, it is not itself a dye, and must be converted to hematein. Many preparations have been proposed over the years. Hematein itself, while colored, has little affinity for tissues, except in the presence of various metals, with which it forms variously colored so-called "lakes". Thus, for example, Hansen's hematoxylin is prepared by mixing 45 mℓ 10% iron alum, 7.5 mℓ 10% hematoxylin in absolute alcohol and 47.5 mℓ water, boiling, filtering, and making the filtrate up to 100 mℓ with water (Gurr, 1960). Eosin is a very common counterstain for hematoxylin. Hematoxylin-eosin preparations will stain leucocyte nuclei. Leucocytes may be structurally distinguished using May-Grünwald or Giemsa stains as well. Biebrich scarlet (Fig. 5.3) and the Feulgen method have been used to stain the red cell nuclei of invertebrate, avian, reptile, etc. bloods, and leucocyte nuclei. Giemsa stain is recommended in thicker blood smears for visualization of parasites (Clark, 1973). Neutrophils, basophils and eosinophils have granules possessing peroxidase activity which may be stained with preparations such as that of Graham (1916): blood smears are treated successively with solutions of α-naphthol in alcohol and peroxide, pyronin Y in alcohol

and aniline, and methylene blue. Neutrophil granules are stained purple-red, eosinophil granules, lighter red, and basophil granules, a deep purple. The nuclei are stained dark blue and the cytoplasm light blue. It is said that the monocyte granules become peroxidase negative in bloodstains in about a month, about 8–12 months being required for neutrophil granules to be negative, while eosinophil granules may be positive in up to 5-year old stains (Undritz and Hegg, 1959). Environmental influences can greatly influence these values. Undritz and Hegg also noted, as did Ziemke (1938), that tissue and organ cells could be histologically differentiated in blood stains, such as in cases on blood crusts on a knife which had been used in a stabbing, or where stains may contain nasal or epithelial cells. Le Breton did not share the same level of confidence in or enthusiasm for the microscopical methods (Fiori, 1962).

De Bernardi (1959) suggested paraffin embedding, thin sectioning and staining with hematoxylin preparations containing alum for bloodstains in deeply absorbed in wood. Däubler (1899) had employed a similar procedure much earlier. If blood crusts are detached from rusty surfaces by the celloidin technique, the film may be fixed and soaked in a saturated solution of oxalic acid containing a bit of uranium nitrate, then exposed to light to dissolved the rust, prior to histological staining (Romanese, 1930).

Some of the more recent texts in forensic medicine do not mention microscopical methods for identification of blood at all, e.g. *Glaister's Medical Jurisprudence and Toxicology*, 13th ed. (Rentoul and Smith, 1973). It should be kept in mind that these techniques require a comparatively large amount of material, and that a certain degree of skill with histological technique is doubtless required if good results are to be expected. In some instances, however, some workers prefer these methods. If whole (liquid) blood is encountered, microscopical identification might well be the method of choice, since a smear could be quickly and easily prepared. Fiori (1962) said emphatically, however, that microscopical methods should never be employed *in place of* other, more reliable procedures, such as spectral, chromatographic and immunological ones. Discussions of microscopical methods are, in any case, of more than purely historical interest, because of the newer methods for the cytological determination of sex of bloodstains (Section 48).

5.3.2. Biological stains and dyes

The histological stains employed in this work have known, and often differential, staining affinity for blood cells. Most of the stains commonly employed in histological work have been in use since the last century. Considerable information about the structure, properties and mode of action of biological stains is available (Clark, 1973; Conn, 1933; Gurr, 1960; Gurr, 1962; Lillie, 1977). In 1922 a Commission on Standardization of Biological Stains was set up in this country to devise standards of uniformity for commercially available products and techniques, and to disseminate information. The handbook of biological stains, published under the Commission's auspices, has gone through nine editions (Lillie, 1977). Conn's *History of Staining* (1933) was authorized by the Commission, and a guide to recommended staining procedures, now in its 3rd edition, is published as well (Clark, 1973). A brief discussion of the more commonly used stains is given here. Readers interested in histological stains should consult the above-cited specialized works on the subject.

Hematoxylin was first employed as a stain in 1863, apparently without success. It was successfully employed two years later by Böhmer (Clark, 1933). Hematoxylin itself is a naturally occurring glycoside from logwood, and is not a dye. It is easily oxidized to the dye form, however, by oxidizing agents or exposure to air. The oxidation product, which is the dye, has the unfortunate name *hematein*, and should *not* be confused with the hemoglobin derivative *hematin*. Fig. 5.4 (a) and (b) shows the structures of hematoxylin and hematein. Eosin, also called eosin Y, is a tetrabromofluorescein (Fig. 5.5), and was discovered in 1871. Giemsa stain, which came into use in 1902, is a mixture of methylene blue and its oxidation products, the azurs, in combination with eosin Y. May-Grünwald stain (1902) is a mixture of eosin Y and unoxidized methylene blue (Fig. 5.6), and is equivalent to Jenner stain (1899), after the latter of whom it should no doubt have been named. Wright's stain is the result of heating methylene blue in the presence of $NaHCO_3$, adding eosin, collecting the precipitate which forms and dissolving it in methanol. The so-called Feulgen reaction for staining of nuclei relies on the acid hydrolysis of the purine residues from DNA, and reaction of the liberated aldehyde groups with Schiff's fuchsin-sulfurous acid reagent to form red-purple complexes. Basic fuchsin is a mixture of pararosanilin and related compounds.

Biological stains and dyes will come up again in this book in other contexts, for example in the visualization of spermatozoa under the microscope (Section 10.2.1) and as coupling reagents for the visualization of the naphthols liberated when naphthyl phosphates are employed as substrates for acid phosphatase (Section 10.3.2). These dyes and stains have frequently had a number of names over the years. It is, thus, not always apparent to the uninitiated that two very different sounding names may refer to the same material. This problem has existed for a long time. There have been standardized lists of dyes and stains prepared over the years, but different lists have not been consistent with one another, nor has there always been consistency in the same list as it was revised and re-revised over time. Dye manufacturers and trade associations have usually designated products by number. There is a lengthy history to the various dye indexes (see Lillie, 1977), but for purposes of this book, suffice it to say that there is now a fairly widely accepted numbering system which is derived from the *Colour Index* in its most recent edition. Most stains and dyes are assigned a five digit "CI number".

In Table 5.3 are listed a number of stains and dyes which come up in medico-legal biology. The preferred name is given, along with synonyms and CI number.

Table 5.3 Biological Stains and Dyes

Preferred Name	Synonyms	C.I. Number	Chemical Name or Nature
Amido Black 10B	Naphthol Blue Black; Naphthalene Black 10B	20470	Acid Diazo Dye
Aniline Blue WS	Water Blue I; Cotton Blue; China Blue	42755	Acid Triphenylmethane Dye
Biebrich Scarlet	Scarlet 3B of B of EC; Croceine Scarlet	26905	Diazo Dye
Brilliant Indocyanin G	Coomassie Brilliant Blue G-250; Supranolcyanin G;	42655	Arylmethane Dye
Carmine; Carminic Acid	Cochineal	75470	Derivative of Anthraquinone Glycoside
Crystal Violet	Methyl Violet 10B; Gentian Violet	42555	Hexamethylpararosanilin
Eosin B, BMX	Eosin BN, BA, BS, BW or DHV; Saffrosin; Eosin Scarlet	45400	Dinitro derivative of dibromofluorescein
Eosin	Eosin Y or G;	45380	Tetrabromofluorescein
Erythrosin	Erythrosin R or G; Pyrosin J	45425	Diiodofluorescein
	Erythrosin B; N or JN; Pyrosin B	45430	Tetraiodofluorescein
Fast Blue B	Diazo Blue B; Dianisidine Blue; Fast Blue Salt BN; Naphthanil Blue B; Brentamine Fast Blue B	37235	See Fig. 10.3
Fast Blue RR	Blue RR; NRR; Diazo Blue RR	37155	Diazonium Dye
Fast Red AL	Naphthanil Diazo Red AL; Red AL; ALS	37275	See Fig. 10.2
Fast Red RC	Red RC; RCS; Red Salt I; Diazo Red RC; RS: Fast Red 4CA	37120	See Fig. 10.4
Hematoxylin; Hematein	Logwood	75290	See Fig. 5.4

Table 5.3 (cont'd)

Preferred Name	Synonyms	C.I. Number	Chemical Name or Nature
Kernechtrot	Nuclear Fast Red; Calcium Red	60760	Anthraquinone Derivative
Malachite Green	Solid Green, O; Victoria Green, B; Malachite Green BXN	42000	See Fig. 6.6
Methyl Blue	Helvetia Blue; Cotton Blue; Soluble Blue; Ink Blue; Sky Blue	42780	Aminotriarylmethane Dye
Methylene Blue	Methylene Blue Chloride	52015	A Thiazin Dye
Methylthiazolyldiphenyl Tetrazolium (BS8)	MTT; Chelating Tetrazole MTT	———	3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide
α-Naphthylamine	Fast Garnet B	37265	See Fig. 10.1
Nitro Blue Tetrazolium (BS8)	NBT; Nitro BT; Ditetrazolium Chloride	———	3,3'(4,4'-di-o-anisylene)-2,2'-di(p-nitrophenyl)-bis(5-phenyl)
Pararosanilin	Magenta O; Basic Fuchsin	42500	Triaminotriphenylmethane chloride
Ponceau S	Fast Ponceau 2B	27195	Polyazo Dye
Procion Blue HB	Cibachron Blue F.3GA	61211	Aminoanthraquinone Derivative
Procion Brilliant Red M-2B	Mikacion Brilliant Red 2BS	18158	A Monoazo Dye
Pyronin Y	Pyronin G	45005	Xanthene Derivative
Remazol Brilliant Blue R	Remalan Brilliant Blue R; Ostazin Brilliant Blue VB; Primazin Brilliant Blue RL	61200	Aminoanthraquinone Derivative
Rhodamine B	Brilliant Pink B; Rhodamine O	45170	Xanthene Derivative

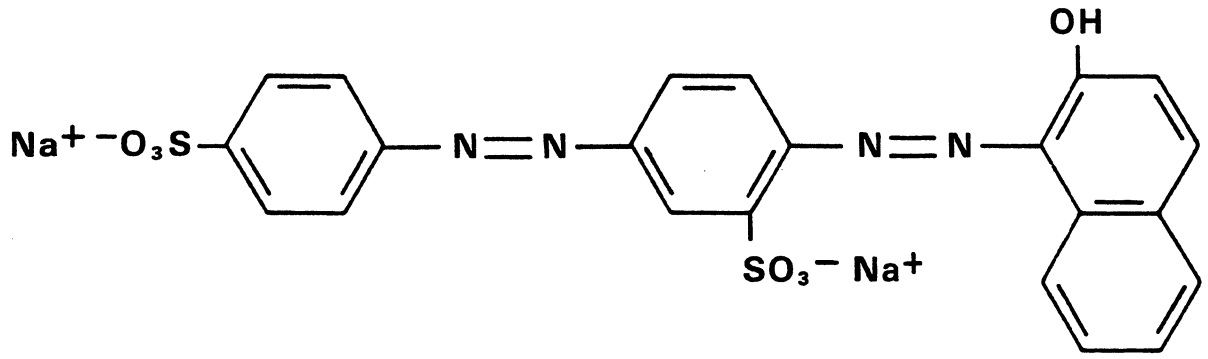
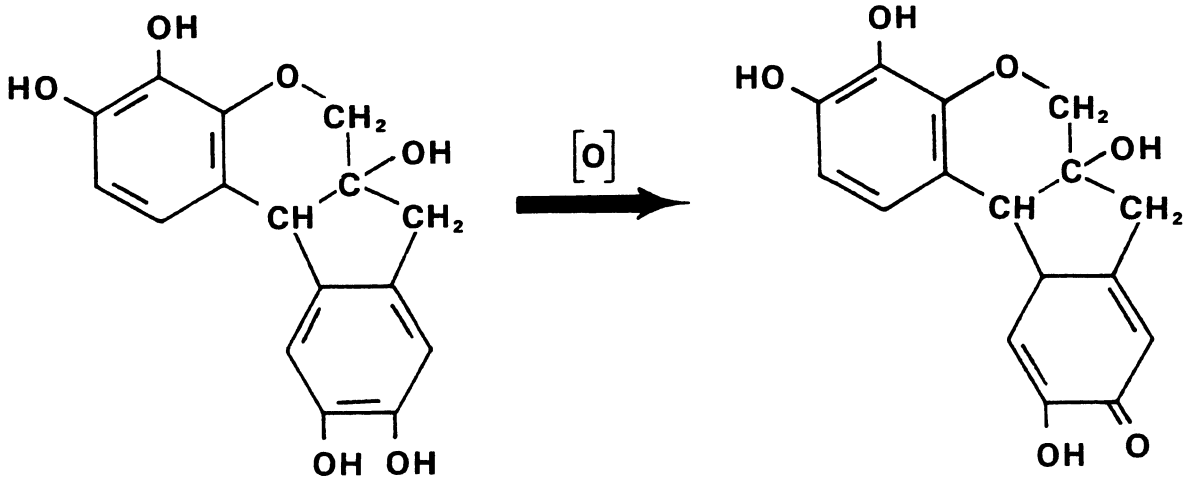


Figure 5.3 Biebrich Scarlet



(a) hematoxylin

(b) hematein (colored dye)

Figure 5.4 Hematoxylin and Hematein

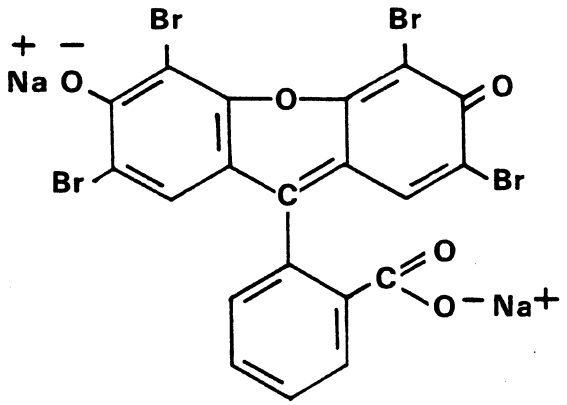


Figure 5.5 Eosin or Eosin Y

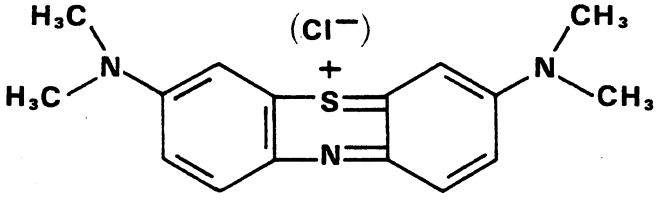


Figure 5.6 Methylene Blue

SECTION 6. CATALYTIC TESTS

Catalytic tests for blood are all based on the fact that hemoglobin and a number of its derivatives exhibit a peroxidase activity. If enzymes are defined as proteins which have *in vivo* catalytic functions, then hemoglobin is not an enzyme. It behaves as an enzyme in these tests, however, as do some of its derivatives, in that they catalyze the oxidation by peroxide of a number of organic compounds to yield colored products. Enzymes which catalyze the peroxide-mediated oxidation of organic compounds *in vivo* are called peroxidases; hemoglobin and the other derivatives which show this catalytic property are thus said to have "peroxidase activity". The "tests" based on the property are generally named after the compound undergoing oxidation (e.g. the guaiacum test, the benzidine test, the phenolphthalin test), or after the discoverer(s) (e.g. van Deen's test, Adler's test, the Kastle-Meyer test). The majority of tests which have been devised for the medico-legal identification of blood are based on the peroxide mediated oxidation of guaiacum, aloin, phenolphthalin, benzidine, the leuco base of malachite green, p-phenylenediamine, eosin hydrate, rhodamine, o-tolidine, o-toluidine, o-dianisidine or tetramethylbenzidine. Some of the tests have enjoyed wide use and great popularity, while others have been used by only one or a few workers. Likewise, some are very much in use today, while others have become a part of the historical archives of this field.

The generalized peroxidase reaction may be written

$$AH_2 + ROOH \rightarrow A + ROH + H_2O$$

where AH_2 is the donor and ROOH is the peroxide. Hydrogen peroxide is frequently used, in which case R = H, and the products would be $A + 2H_2O$. The AH_2 donor can be any oxidizable substrate yielding a detectable (usually colored) product A. In the special case of the catalase reaction, $AH_2 = H_2O_2$ and $2H_2O_2 \rightarrow O_2 + 2H_2O$. Additional information on the peroxidase and catalase reactions may be found in Mahler and Cordes (1971).

6.1 The Guaiacum Test

The subject of the peroxidase activity of blood was opened by Schönbein in 1857. He had shown that ozone and certain inorganic peroxides would cause guaiacum to turn blue, but that H_2O_2, "ozonized turpentine", and "ozonized ether" would not do so by themselves (Schönbein, 1848a, 1848b). The last three mentioned substances were called "antozones", this terminology being based on a theory of oxidation he held which said that "ozone" represented oxygen in a "positive polar state" while "antozone" represented it in a "negative polar state". Substances which behaved like ozone itself were called "ozonids" or "ozonides" while those behaving like "antozone" were called "antozonid(e)s". It was thought that the two forms of "polarized" oxygen reacted in some way to give oxygen. Ozone and "ozonides" would cause guaiacum to turn blue by themselves, while the so-called "antozonides" would not. The addition of a catalytic quantity of platinum black, however, would bring about the bluing reaction with H_2O_2, and the "antozones", and it was soon found that this same catalytic activity was possessed by blood and wheat gluten, neither of which worked by themselves (Schönbein, 1857). The catalytic principle in blood was demonstrable in red cell lysates, in the absence of fibrin and serum, and was not destroyed by boiling or drying.

Guaiacum is a resin isolated from *Guaiacum officinale* and *Guaiacum santum*, trees indigenous to Mexico, South America and the West Indies. It consists of a mixture of substances, one source saying that "guaiacum" is 70% guaiaconic acid, 10% guaiaretic acid and 15% resin (Snell and Snell, 1962). Guaiaconic acid has the formula $C_{20}H_{24}O_5$ according to Grant (1969) and Kastle and Loevenhart (1901), and can be substituted for guaiacum in the reaction (Mitchell, 1933; Buckmaster, 1907). Guaiacum is also sometimes called "guaiac".

In 1858, Schönbein showed that $FeSO_4$ catalyzed the guaiacum reaction. His colleague, Prof. Hiss, had shown that the catalytic activity of the red cells was proportional to their iron content, and he believed, therefore, that the catalytic principle in blood depended more on its iron content for activity than on any specific structural or organizational feature. In 1863, Schönbein suggested that the power of blood to blue guaiacum might serve as the basis of a medico-legal identification test. Van Deen (1862) extended the studies on the guaiacum reaction with blood, and was the first to suggest it as the basis of a medico-legal test. He showed that it occurred with minimal amounts of old, putrefied samples, as well as with blood which had been dried or boiled. Dilutions of whole blood of up to 1:40,000 in water still gave positive reactions. A number of inorganic salts gave positive reactions too, but could be eliminated using other methods. Van Deen used oil of turpentine as oxidizing agent. This so-called "ozonized turpentine", as well as "ozonized ether" could serve as oxidants because of the presence of organic peroxides which form upon exposure to air. According to Taylor (1868), not much notice was taken of Van Deen's work until Liman's extensive experiments on the subject were published in 1863. Liman thought that a negative reaction could be taken as an indication of the absence of blood without further testing. A positive result, however, did not constitute proof that blood was present, since various vegetable gums, milk casein, tanned sheep leather and some inorganic salts gave "false positive" reactions. Dr. Day in

Geelong, Australia, confirmed most of the results of earlier workers in 1867. He employed "ozonized ether" for the test, and correctly surmised that it worked because of the peroxides which formed when it was exposed to the air. In what may well have been the first reported use of the test in a medico-legal case, Dr. Day reported that he had detected blood on the trousers of a Chinese man suspected of a murder in a place called Scarsdale, Australia, on October 19, 1866. The trousers had been washed by the time they were taken as evidence, and the Government's forensic chemist could find no traces of blood on them by microscopical examination. Day wrote of this case to Dr. Taylor in London, and even enclosed a portion of the trousers on which he said he had detected blood. Dr. Taylor re-examined the cloth, some months old by then, by means of the guaiacum test, and confirmed Day's findings (Taylor, 1868). He reported this result in a rather lengthy study of the guaiacum test, prompted apparently by Day's communication. Taylor also noted that he gave the bloodstained cloth from the trousers in Day's case to Mr. Sorby, who was unable to confirm the presence of blood using his microspectroscopic method (see section 5.1). Taylor regarded the test as a useful one, to be used in conjunction with microscopical and microspectroscopic methods. Negative results were conclusive in his view, while a positive result ". . . enables a chemist to speak with reasonable certainty to the presence of blood . . .". The "false positive" results caused by oxidants which blued guaiacum in the absence of peroxide were easily eliminated by applying the guaiacum tincture first, and the peroxide after a short time if no reaction had taken place.

The guaiacum test was the first catalytic test devised for forensic blood identification, and, except for the aloin test which enjoyed very little popularity, was the only one in use for about 40 years. It is often referred to in the literature as Van Deen's test. Some English authors have called it Day's test. The old literature on this test was extensively and excellently reviewed by Kastle (1909). Kastle and Loevenhart (1901) stated that the product of the reaction, the so-called "guaiacum blue", had the formula $C_{20}H_{20}O_6$ and that guaiaconic acid, $C_{20}H_{24}O_5$, was the component of guaiacum resin which underwent oxidation. Guaiaconic acid can be separated, as it turns out, into α and β compounds (Richter, 1906), having the formulas $C_{22}H_{24}O_6$, and $C_{21}H_{45}O_5$, respectively. Guaiacum blue was said to be the oxidation product of the α compound, and to have the formula $C_{22}H_{24}O_9$. The guaiacum test has been applied to the detection of blood in urine (Schumm, 1909) and feces (Messerschmidt, 1909) for clinical purposes.

Medico-legal investigators were divided as to the relative value of the test as proof of the presence of blood. Buckmaster (1907) believed that, if the test were carried out on boiled samples (to eliminate vegetable peroxidases), a positive result was meaningful. Negative results indicated the absence of blood with certainty. Jenne (1896) thought that a positive result in a carefully performed test, using proper controls, warranted the conclusion that the stain was "surely blood". Siefert (1898) stated that a positive guaiacum test indicated a "high probability" of the stain being blood, while a negative result insured that it was not. Hemphill (1875) thought the test was a good and useful one, but did not clearly state that a positive result constituted conclusive evidence of the presence of blood. Schumm (1907) thought the test was trustworthy with certain precautions.

A larger number of authorities did not believe that a positive result was to be taken as proof of blood, but they did think the test had value as a preliminary or sorting technique (Chapman, 1892; Delearde and Benoit, 1908; Dérobert and Hausser, 1938; Ewell, 1887; Macnamara, 1873; Marx, 1905; Sutherland, 1914; Wood, 1901). Other workers believed that the primary value of the test was in eliminating stains that were not blood. They stressed the importance of negative results, which warranted the conclusion that blood was absent (Liman, 1863; Mialhe *et al.*, 1873; Mecke and Wimmer, 1895; Palleske, 1905a; Siefert, 1898; Whitney, 1909). A few investigators believed that the test was virtually worthless (Alsberg, 1908; Dervieux, 1910). Breteau (1898) said that very great caution should be used in interpreting results because of the large number of substances that would give a positive test.

The objections to the value of the test were based on the relatively large number of substances other than blood which had been reported to give positive results (Kastle, 1909). A number of inorganic elements and compounds, vegetable extracts, milk, gelatin, bile, gastric secretions, nasal mucus, saliva, pus, leather, soap, and certain types of papers have all been reported to give false positive reactions. It must be said that the way the test is carried out, the nature of the guaiacum, and of the oxidant used all make a difference in this regard. Carrying out the test on boiled samples (to eliminate vegetable peroxidases), addition of the guaiacum and peroxide in two steps (to eliminate inorganic oxidants), and the use of guaiaconic acid and H_2O_2 (to eliminate variability in the resin, ether and/or turpentine preparations) excludes the possibility of most, but not all, of the interfering substances. Workers who believed in the value of positive results recommended all these precautions, in addition, of course, to substratum controls.

Various claims have been made for the sensitivity of the test, it being difficult to compare the values in some cases because of the different ways in which they were expressed. Van Deen (1862) reported a positive test with a 1:40,000 dilution of whole blood in water. Mitchell (1933) and Kastle (1909) both say that Liman (1863) confirmed this result, but I have quite the opposite impression from Liman's paper. I understand him to say that he got a positive result with a 1:6,000 dilution of fresh blood in water, but that the reaction failed at dilutions of 1:40,000. However that may be, Schumm (1909) reported 1:40,000 to 1:100,000 dilutions of blood in water and 1:20,000 to 1:40,000 dilutions of blood in urine as the limits of sensitivity. Nicolesco (1934) gave 1:20,000 as the value. The most extravagant claim is that of Vitali (1903) who said that a $1:10^{11}$ dilution of dessicated blood in water still gave a positive result; this value is greater by six orders of magnitude than any other published figure.

Expressed as a dilution of whole blood in water, the range most often quoted for sensitivity is 1:20,000 to 1:100,000. The measurements are obviously affected by the reagents used.

The guaiacum test is now no longer employed as a catalytic test for the presence of blood in the forensic practice, having been supplanted by benzidine, phenolphthalin, etc. Schwarz (1936) used guaiacum as a substrate for testing the peroxidase activity of bloodstains in a series of experiments designed to correlate the color intensity with the age of the stain. In more recent times, guaiacol, which is o-methoxyphenol, and component of guaicum resing, has been employed as a staining substrate for haptoglobin (as the hemoglobin complex) in gels following electrophoresis (Reich, 1956; Queen and Peacock, 1966).

6.2 The Aloin Test

The aloin test for identification of blood in stains (and in urine and feces) is, like the guaiacum test, of primarily historical interest. In many ways, it is quite similar to van Deen's test. Aloin is a mixture of pentosides in the extracts of aloes, a genus of plants of the Family Lilaceae. Barbaloin, the major ingredient, is a hydroxyanthroquinone derivative of glucose. The structure is shown in Fig. 6.1 (Merck Index, 1968). Hemoglobin and some of its derivatives catalyze the oxidation of this material by H_2O_2 to yield a bright red product. Klunge (1882 and 1883) first noted that this test could be employed as a test for blood. A number of studies concentrated on chemical reactions of aloin, and the analogy between these and comparable guaiacum reactions (Neuberger, 1899; Schaer, 1900). Rossel (1901) noted that the test could be used for the detection of blood in urine. Buckmaster (1907) found that the test was less sensitive than the guaiacum test, but that the oxidation product (i.e. the color) was quite a bit more stable. The test was never as widely used as van Deen's, and not as much was written about it. Sutherland (1907) regarded it as a good confirmatory negative test.

6.3 The Phenolphthalin Test

In 1901, Kastle and Shedd showed that preparations of cellular "oxidases" would catalyze the oxidation of phenolphthalin to phenolphthalein in slightly alkaline solutions. At the time, the cellular enzymes responsible for the catalysis had not been purified to any great extent, nor had there been any attempt to systematize the enzyme nomenclature. The crude enzymatic preparations were often referred to as "oxidizing ferments". Phenolphthalein is, of course, pink to red in alkaline solution, while phenolphthalin is colorless. Thus, the latter was an excellent artificial substrate for assaying the "ferments" because the colored oxidation product was soluble and readily quantitatable colorimetrically. Meyer (1903) utilized phenolphthalin to detect the "oxidases" in leucocytes. In particular, he found differences in this activity between normal and leukemic samples. He noted further that this test could be used for the qualitative and quantitative determination of blood in urine. The first unequivocal suggestion that the test be applied to medico-legal blood identification was made later in 1903 by Utz. He reported that the test served well on bloodstains up to 1½ years old, gave a negative reaction with rust, but, not surprisingly, gave "false positive" reactions with pus and other leucocyte-containing secretions. The test became known as the "Kastle test", the "Meyer test" or the "Kastle-Meyer test."

It was soon quite clear that the test relied on the peroxidase activity of hemoglobin. Kastle and Amoss (1906) showed that the catalytic activity of blood toward the peroxide oxidation of phenolphthalin in alkaline solution was directly proportional to the hemoglobin content. The reagent, phenolphthalin, was prepared from phenolphthalein by reduction in the presence of Zn and strong NaOH or KOH. Kastle (1909) recommended the precipitation of the phenolphthalin by acidifying the reaction mixture, and collection of the precipitate. This material was recrystallized several times from minimal alcohol by cold water, and stored as a solid. Liquid solutions of the compound were stable for a matter of weeks if kept dark, and stability was greatly increased by the presence of a small quantity of zinc dust. One of the advantages of this test, in comparison to guaiac and aloin, was that the reagent was a pure compound. Kastle, who was very partial to this test for blood, discussed its many aspects in great detail in his review in 1909.

Deléarde and Benoit (1908a) studied the phenolphthalin test and showed that it was positive with hemoglobin, methemoglobin, hematin chlorhydrate, reduced hemoglobin, and old, putrefied blood. They got a positive test on a control bloodstain 26 years old, and believed that the test, properly controlled, was both sensitive and specific (1908b), in addition to its usefulness in detecting blood in urine, feces and gastric juice. Boas (1911) indicated that the test was useful for occult blood. Pozzi-Escot (1908), however, thought that no value should be attached to the test for blood because saliva, pus, malt extract, vegetable extracts and the salts of heavy metals such as Co, Mn, Pb and Fe could give false positive reactions. Dervieux (1910) agreed with this view, suggesting that the test had no value at all, positive or negative.

The phenolphthalin to phenolphthalein oxidation reaction, and the structures of the latter compound in both acidic and basic solution, are shown in Fig. 6.2. Because phenolphthalein is colorless in acidic solution but pink to red in basic solution, it has been widely employed as a pH indicator. Many have noted that the phenolphthalin test is more sensitive than either the guaiac or aloin tests. Deléarde and Benoit (1908a) and Nicolesco (1934) have indicated positive reactions with blood diluted $1:10^6$, as has Girdwood (1926). Gettler and Kaye (1943) reported a sensitivity of $1:10^7$ dilution of whole blood, but of $1:10^6$ dilution for old, decomposed blood. Glaister (1926a) noted that saline extracts of 1 year old stains reacted at 1:212,000 dilutions but that a 1:800,000 dilution of a water extract of the stain gave a positive result. Kastle (1909) did sensitivity experiments by dissolving

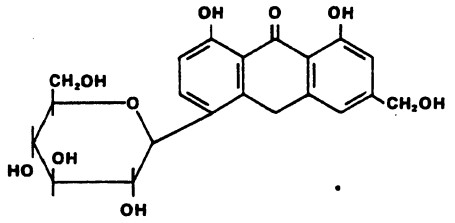


Figure 6.1 Barbaloin (1,8 dihydroxy - 3 - hydroxymethyl - 10 - (6 - hydroxymethyl - 3,4,5 - trihydroxy - 2 - pyranyl) - anthrone)

3.8 mg of blood in 100 mℓ water as a first dilution and making three serial ½ dilutions in addition. These, he denoted solutions (1), (2), (3) and (4), and they contained 38μg, 3.8 μg, 0.38 μg and 0.038 μg of blood per mℓ, respectively. One mℓ of each solution was tested by the addition of 2 mℓ reagent. Solution (3) which contained 0.38 μg/mℓ could be readily distinguished from the control colorimetrically. Solution (4), the weakest, could not be, but Kastle noted that two independent observers (he, presumably, being one of them) were able to distinguish the difference between solution (4) and the control by eye. In terms of dilutions of whole blood, therefore, since the assays were carried out in a total volume of 3 mℓ, solutions (3) and (4) correspond to about $1:8 \times 10^6$ and $1:80 \times 10^6$ parts blood to parts water. Kirk (1953) noted that $1:10^5$ dilutions of blood gave a positive reaction within 3 sec while $1:5 \times 10^6$ dilutions required 20 sec to do so.

Glaister (1926a) tested a variety of substances and body fluids, including rust, urine, saliva, semen, perspiration and milk, and got negative results with the phenolphthalin test. Kerr (1926b) took exception to Glaister's confidence in the method, noting that feces from patients taking aspirin gave a false positive test. Glaister (1926b) replied that, while he did not question the need for corroboration of the presence of blood, his own experience with the test had convinced him of its value in medico-legal cases. Girdwood (1926) noted that he did not think the test should be relied upon by itself as an indication of blood in stains, nor of occult blood in stool samples. Gettler and Kaye (1943) thought the test was more specific than guaiacum, benzidine or o-tolidine, and Gradwohl (1956) said that he preferred this test to benzidine. More recently, Higaki and Philp (1976) re-evaluated the test for blood in terms of sensitivity, reagent stability and specificity. The reagent was prepared essentially as recommended by Camps (1968), which follows almost exactly the original method of preparation used by Kastle (1909) except that the product is not isolated and recrystallized. Phenolphthalein (2 g) is dissolved in 100 mℓ water containing 20 g KOH and boiled with a reflux condenser in the presence of 20 g zinc powder until colorless. The resulting solution is kept in a brown bottle with some Zn dust present. The test was carried out in a number of different ways, with and without ethanol or methanol, and with peroxide or perborate. A so called one-stage test amounted to the addition of the combined reagents to the sample; a two-stage test consisted of the successive addition of reagent and either peroxide or perborate; a three-stage test involved the addition of the alcohol, the reagent, and the peroxide or perborate successively. Benzidine was employed as a control, because the experiments were designed to evaluate phenolphthalin as a substitute for benzidine in routine practice. It may be noted here that Camps (1976) recommended that the phenolphthalin test be substituted for the benzidine test. Dilutions of whole blood as well as stains prepared from them were tested. Stains were tested by applying reagents to a stained thread on filter paper. All results were recorded after 5 sec of observation. With liquid dilutions, ethanol or methanol, reagent and perborate, used in a one-stage test, proved to be most sensitive (in excess of $1:10^6$). A one-stage phenolphthalin-perborate test was sensitive to about $1:10^5$–$1:10^6$ dilutions, there being no advantage to a two-stage test with these reagents, nor to a three-stage test involving either alcohol. Reagent-peroxide combination, with or without alcohol, regardless of the number of stages, gave

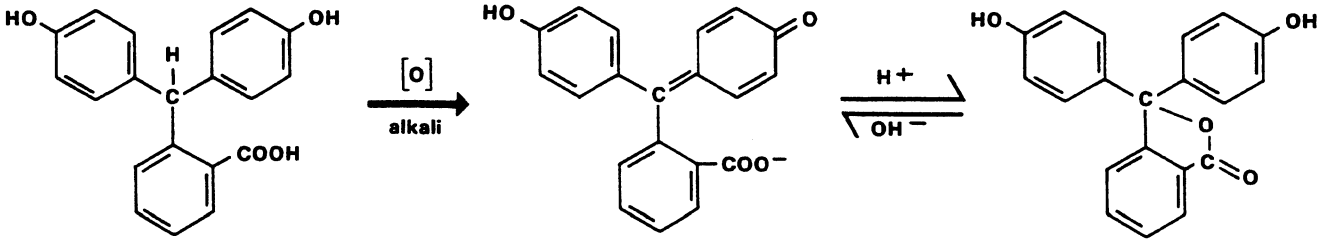


Figure 6.2 Phenolphthalin Oxidation and Phenolphthalein

sensitivities of about $1:10^4$–$1:10^5$ dilutions, fairly comparable to the benzidine control. With bloodstains, the one-stage methanol-reagent-perborate test was most sensitive, to about $1:10^3$ with the benzidine control being about $1:10^4$. Reagent, with peroxide or perborate, was considerably less sensitive in a one-stage test (neat-$1:10^2$ dilutions), and was unsatisfactory in a two-stage test, giving weak reactions at all dilutions to $1:10^2$. From the standpoint of stability, all perborate and one-stage reagents were relatively unstable. A three-stage test with peroxide, reagent and either alcohol, stored separately, was most desirable from the point of view of reagent stability. This test was recommended for screening, in spite of the fact that its sensitivity was of the order of $1:10^3$, because if greater sensitivity were required, the stain could be extracted, and the extract tested. Under these conditions, the sensitivity is increased by one to two orders of magnitude. The tests were performed on a number of vegetable extracts as well, the phenolphthalin test being found to be more specific than benzidine, particularly if the three-stage test with ethanol and peroxide was employed. Hunt *et al.* (1960) found that phenolphthalin gave positive reactions at blood dilutions of $1:10^7$, but that the reagent was useful for the examination of bloodstains only if extracts were made. The results obtained directly on stains, or on filter paper rubbings, were found to be less than satisfactory. These investigators noted further that the test appeared to be more specific for blood than some of the other catalytic tests, false positives having been noted only with copper salts, as had been noted much earlier by Glaister (1926a).

6.4 Benzidine Test

This test may well be the most familiar and widely used of the catalytic tests for blood. Its employment for that purpose began with a quite extensive series of experiments by Rudolf and Oscar Adler, published in 1904. A large number of oxidizable organic compounds were tested for their ability to form colored products in the presence of H_2O_2 and a $1:10^5$ dilution of rabbit blood in water. Among the substances tested were: aniline, its monomethyl-, dimethyl- and diphenyl- derivatives, p-toluidine, xylidine, the o-, m- and p-isomers of phenylenediamine, dimethyl-p-phenylenediamine, tetramethyl-p-phenylenediamine, phenol, p-amidophenol, the cresols, thymol, catechol, resorcinol, guaiacol, hydroquinone, pyrogallol, benzoic and salicylic acids, benzidine, tolidine, α- and β-naphthols, α-naphthylamine, the leuco bases of malachite green, brilliant green and acid green, methyl violet, crystal violet, a number of triphenylmethane dyes, eosins and rhodamines. The leuco base of malachite green (section 6.5) was used to test bloodstains, and this reagent, as well as benzidine and the leuco base of crystal violet, were recommended for the identificaiton of dilute blood in aqueous solutions. Benzidine and leucomalachite green were also recommended for testing for the presence of blood in urine and feces.

Benzidine is p-diaminodiphenyl (Fig. 6.3). The blue oxidation product obtained with peroxide in the presence of blood is often called "benzidine blue". The course of benzidine oxidation by peroxide is generally carried out at acid pH; a possible mechanism for the reaction is indicated in Fig. 6.4. The benzidine blue is an intermediate in the reaction. Eventually, the diimine compound forms, and it is brown. This latter may, in turn, polymerize. For histochemical work, the blue intermediate is more desirable than the brown end product and there have been efforts to find conditions which render it more stable. It is known that the stability is maximized at pH 4.5, and the blood test is usually carried out in acetic acid to achieve approximately this condition. The structure of benzidine blue is given by Feigl (1966) as consisting of one mole of amine, one mole of imine and one mole of whatever acid is present. Van Duijn (1955) looked into this question. He found that if milk peroxidase is used to form benzidine blue from benzidine and H_2O_2 in the presence of 5% NH_4Cl, the product had the composition $C_{24}H_{24}N_4Cl{\cdot}2H_2O$. The ammonium chloride had been found to stabilize the benzidine blue intermediate, a desirable goal in histochemical staining work. The amounts of time involved in the transition from benzidine blue to the brown diimine, which are of the order of minutes at acid pH, are too long to be of any practical concern for the medico-legal blood test, which occurs instantaneously or within a few sec.

This test quickly gained favor, and has become widely used. Messerschmidt (1909) preferred the test for detecting occult blood in feces, and recommended the method of Schlesinger and Holst (1906). Lyle *et al.* (1914) conducted experiments to determine the optimal concentrations of reagent, acetic acid and H_2O_2, primarily for the clinical test. They could achieve positive results with blood diluted $1:5 \times 10^6$ under optimal conditions, but were willing to allow up to 5 min for color development. In testing for the presence of blood in medico-legal exhibits, where little is known of the history of the article, the benzidine test has suffered the same criticisms levelled against other catalytic tests concerning specificity. Michel (1911) addressed the specificity problem in regard to metals. He reported that iron, copper and certain of their oxides, will oxidize guaiac, benzidine and leucomalachite green in the presence of H_2O_2. He noted, however, that treatment of the colored product obtained in the test with 2,4-diaminophenol would cause formation of a red-colored product if the original oxidation had been catalyzed by metals, but no such change would result if the original catalyst had been blood or pus. He added, however, that chloride or permanganate can oxidize the testing reagents, but are not subsequently affected by the 2,4-diaminophenol. Glemser (1939) noted that a variety of iron oxides cause the oxidation of benzidine, and concluded that the test could not be regarded as specific in the presence of rust. In 1973, Eisele tested approximately 50 substances for false positive benzidine reactions, and confirmed many of the observations of earlier workers. In addition, he confirmed the fact that fruit and vegetable extracts which contain peroxidases can give the reaction. Hunt *et al.* (1960) in their extensive studies noted that feces often gave a false positive,

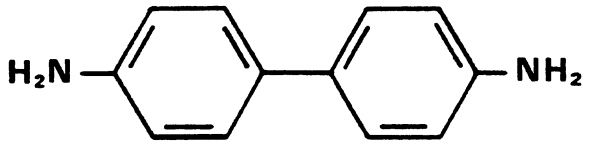


Figure 6.3 Benzidine

as did green leaf material smeared on filter paper. Smears of shoe polish (or leather) from used shoes gave a false positive reaction in one case of 30 tested. The fingernail scrapings from a grocer who handled fruits and vegetables gave the test, as did axillary wipings from normal men. These workers decidedly regarded the test as presumptive, and unequivocally stated so:

> . . . occasions do occur when a garment which is expected to be contaminated with blood gives a positive presumptive test for blood, although the deposit is insufficient or unsuitable for more specific tests. However tempting it may be to use this as evidence, it is scientifically and morally incorrect to do so, for it is clearly recognized that such tests are not specific and their introduction into evidence may well mislead. It is no argument that the evidence can be challenged, for to do so implies scientific advice which is not always available.

This strong remark was prompted in part by a case, which the authors review in the paper, in which a suspect willingly submitted to scientific investigation. His clothes and a leather container, alleged to have been used to transport the murder weapon (which was not recovered), were examined using the benzidine test. Material was insufficient for further tests. The Crown's expert testified that the benzidine test was presumptive, but upon being questioned, said that in skilled hands, it could be accepted as proof of blood's presence. That judgment, he said, would be based in large part on the time taken for the test to come up, but would not commit himself to a specific time period. In the estimation of Hunt *et al.*, the impression was left with the jury that blood had indeed been found, and there was an unstated implication that it was human. An opposite point of view on this question is discussed below.

In 1951, Grodsky *et al.* did a study of the catalytic tests and the luminol test (see Section 6.7). In addition to a number of important points discussed below, they noted that the use of sodium perborate instead of H_2O_2 for the benzidine, phenolphthalin and leucomalachite green tests represented a great improvement for the simple reason that the concentration of the perborate could be accurately determined, once reagent concentrations had been optimized. It should be noted that sodium perborate had been recommended previously as a substitute for H_2O_2 in the benzidine test (Lucas, 1935), but that Grodsky *et al.* (1951) were the first to suggest its use with phenolphthalin (Cf Higaki and Philp, 1976) and leucomalachite green. Grodsky *et al.* did not deny that a positive benzidine test alone should not be interpreted as positive evidence of blood without corroboration, but they did suggest that the use of several catalytic tests along with a luminol test provided far more convincing evidence than any one test alone (see Section 6.8).

There is no doubt that the majority of authorities have regarded the benzidine test as a sorting, or presumptive, test (Camps, 1968; Dérobert, 1974; Fiori, 1962; Gonzales *et al.*,

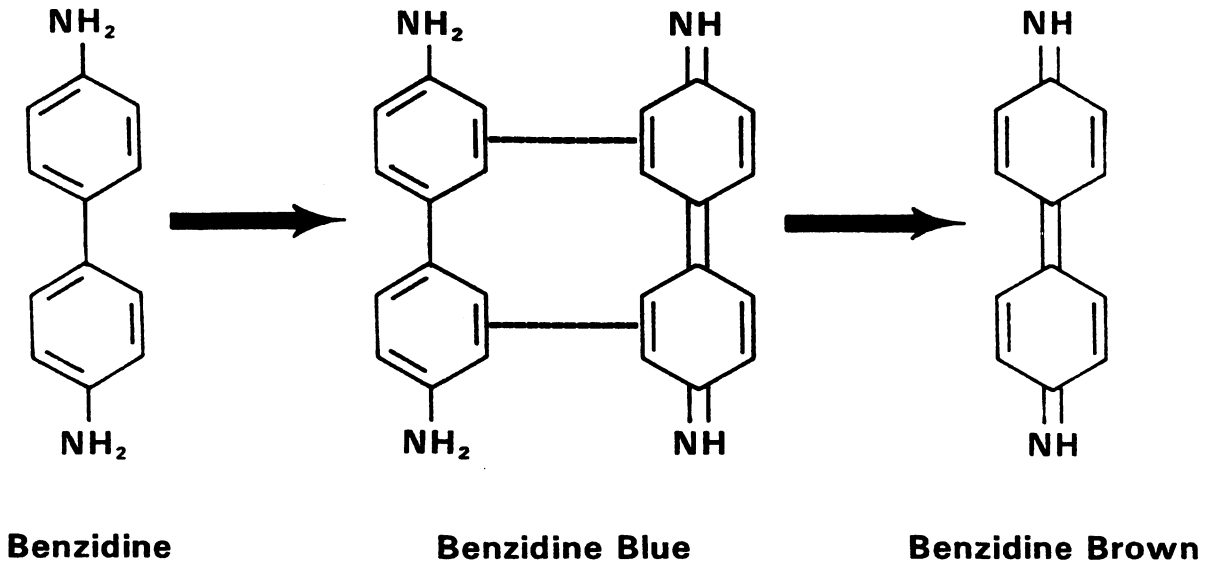


Figure 6.4 Course of Benzidine Oxidation by Peroxide

1954; Hunt *et al.*, 1960; Kerr, 1954; Lucas, 1935 & 1945; Mikami *et al.*, 1966; Prokop, 1966; Simpson, 1965; Thomas, 1937). In 1964, the benzidine test was critically examined by Culliford and Nickolls. They noted that by 1931, the test had fallen into disfavor, at least in some quarters, as a certain test for blood, Glaister having written in that year in the 5th edition of *Medical Jurisprudence and Toxicology:*

> While some employ this test, it has the disadvantage that, like the Guaiacum test, it can only be of value as a negative test, in that if no colour reaction occurs—blue or green—on applying it to a stain, it indicates the absence of blood. Should the colour reaction take place, it only suggests the presence of blood, since gluten, many plant juices as horseradish, and hypochlorites will give the blue colour reaction, although these may give the reaction either before or without the addition of ozonised ether. We do not put our trust in this test.
>
> We have abandoned completely the Guaiacum and Benzidine tests for the reason chiefly that the reaction obtained in the presence of minute amounts of known blood is uncertain and doubtful, and also because a reaction may be produced by it by substances other than blood. These objections do not apply to the Kastle-Meyer test.

On the other hand, Gradwohl (1954) wrote in his book, *Legal Medicine,* that positive reactions with benzidine (or phenolphthalin), assuming properly negative controls, do indicate "the presence of blood." This statement suggests that many laboratories regarded the test as considerably more valuable than did Dr. Glaister. Culliford and Nickolls point out, as did Grodsky *et al.*, (1951), that the judgment made about the test must be evaluated in the context of a number of variable parameters, such as the precise way in which the test was done (i.e., one-stage, two-stage, what controls were used, testing stains directly, testing extracts, etc.), the concentration and purity of the reagents used, whether peroxide or perborate was used, and the experience and judgment of the person carrying out the procedure. The major sources of "false positives" were categorized by these workers as: (1) blood contamination; (2) chemical oxidants or catalysts; and (3) vegetable or fruit peroxidases. Contamination should not be a problem in practice if the reagents are pure, the glassware clean, and the examination area kept scrupulously uncontaminated. The test is exceedingly sensitive, and contamination does not have to be very great to be a serious problem. Chemical oxidant interference is readily dispensed with by adding the reagent and the peroxide in successive steps; if color develops upon the addition of reagent alone, the presence of a chemical oxidant is indicated. Chemical catalysts, which work only in the presence of the peroxide, are not eliminated by the two-stage procedure, but Culliford and Nickolls argued that reactions caused by these materials appear quite different to the experienced eye than do those caused by blood. The plant peroxidases are heat-labile, and testing samples that have been heated to 100° for a few minutes serves to differentiate them from blood, which still reacts readily after the heating step. It was further shown that the majority of plant peroxidases which would give the reaction were quite labile in the dried state. These gave weak to negative benzidine reactions after three days in the dried state, whether the test was done directly on cloth, on a rubbing, or on an extract. Finally, these investigators described a simple electrophoretic procedure, carried out on 1% agar gels, for the differentiation of a great number of substances which could give misleading results in the benzidine test. The procedure is also described by Culliford (1971). It should be noted that these authors took strong exception to the statements of Hunt *et al.* (1960) above, to the effect that the reporting of a positive presumptive test in a case where there was no additional material available for testing would be scientifically and morally incorrect, because it could be misleading. Failure to report such a result, Culliford and Nickolls argued, would be to usurp the prerogatives of the Court, and in such a case as the one discussed by Hunt *et al.*, the result should be reported with a suitable explanation of its meaning.

Reports of the sensitivity of the benzidine test vary in the literature. Adler and Adler (1904) originally reported a sensitivity limit of $1:10^5$ dilution of whole blood in water. Nicolesco (1934), Dérobert and Hausser (1938) and Thomas (1937) all cite 1:200,000 dilutions as the limit. Grodsky *et al.* (1951), Hunt *et al.* (1960) and others have noted that the sensitivity quotations can be misleading, because the results depend on so many different parameters. Unless the technique and the reagents used are fully described, it is not at all certain that the results will be able to be duplicated exactly. Indeed, while it is easy enough to compare the sensitivities of reagents in terms of the maximal dilutions of whole blood which still give positive tests, it is not always clear how such values translate in their applicability to bloodstains. Hunt *et al.* (1960) noted great variability in different lots of commercially obtained benzidine. Expressed as the highest dilution of blood, dried onto filter paper, which gave the test, the sensitivity ranged from 1:20,000 to over 1:150,000, and was even more variable if the amount of time required for the color to develop were taken into account. Grodsky *et al.* (1951) noted that stains made from 1:300,000 dilutions of blood came up within 10 sec while those made from 1:100,000 dilutions came up within 1 sec. Akaishi (1965) reported a sensivity of only 1:12,800 for benzidine in stains made at that dilution, and noted that 20 sec was allowed for color development.

It may be noted here that Alavi and Tripathi (1969) recommended that blood testing in the field (e.g. at scenes etc.) could be done with benzidine-impregnated filter papers. The suspected material was moistened with water, the benzidine paper then pressed against it, and peroxide added to the paper from a sealed vial. The papers were said to be stable for up to 1 year and could be regenerated by soaking again in benzidine solution and drying.

The foregoing discussion of the benzidine test, without which the sourcebook would obviously be incomplete, may nevertheless be almost purely academic from a practical point of view. That benzidine was a chemical carcinogen has

apparently been known for some time. Hunt *et al.* (1960) mention that the manufacture of Analar Benzidine was discontinued in 1951 for that reason. Camps (1976) notes that phenolphthalin has replaced benzidine in routine practice. Rentoul and Smith (1973), in the 13th edition of *Glaister's Medical Jurisprudence and Toxicology,* suggest a saturated solution of amidopyrine in 95% ethanol as a benzidine replacement. (See Section 6.6.8). Apparently, therefore, the use of benzidine has been largely discontinued in England. Higaki and Philp (1976) carried out their study of the phenolphthalin test (see Section 6.3) primarily to check its applicability as a substitute for the benzidine test, suggesting that benzidine has been abandoned in Canada as well.

In this country, the use and manufacture of benzidine has become subject to extremely stringent restrictions and controls, according to regulations issued by the Occupational Safety and Health Administration of the Department of Labor (Code of Federal Regulations, 1976). While manufacture and use have not been ordered to cease, the regulations and restrictions are prohibitively involved for a laboratory doing routine work. Regardless of the merits of the test, or the qualifications that should or should not be place on interpretation of the results obtained with it in medico-legal cases, it is probable that the substance will shortly be unavailable. Supervisors will probably be increasingly unwilling to place their examiners at risk by continued use of the reagent, and the OSHA Regulations may also be sufficiently constraining that manufacturers will consider them prohibitive as well. It is likely, therefore, that the benzidine literature will shortly become a part of the archives of this field.

6.5 Leucomalachite Green and Leucocrystal Violet Tests

The use of the leuco base of malachite green as a blood testing reagent was first reported by Adler and Adler (1904), as noted above. The term "leuco compound", or in this case, "leuco base", comes from the literature of biological stains and dyes (Lillie, 1969). Compounds to be employed as stains or dyes obviously have to be colored. Although they differ greatly from one another chemically, all contain a chromophore group, a structure which renders them colored. They all share in common additionally the property of being reducible and reduction alters the chromophore group rendering the compound colorless. These colorless reduction products are referred to as "leuco compounds". Clearly, the leuco compounds are oxidizable to the dye forms. The "leuco bases" are particular types of leuco compounds, usually carbinols, and characteristic of the triphenylmethyl derivatives.

In the original work, the Adlers used leucomalachite green and leucocrystal violet, in addition to benzidine, for blood detection in aqueous solution. The structures of crystal violet (hexamethylpararosanilin) and malachite green are shown in Figs. 6.5 and 6.6, respectively. Only the leuco base of the latter has been widely used in forensic practice. The leucomalachite green test had a sensitivity limit, like

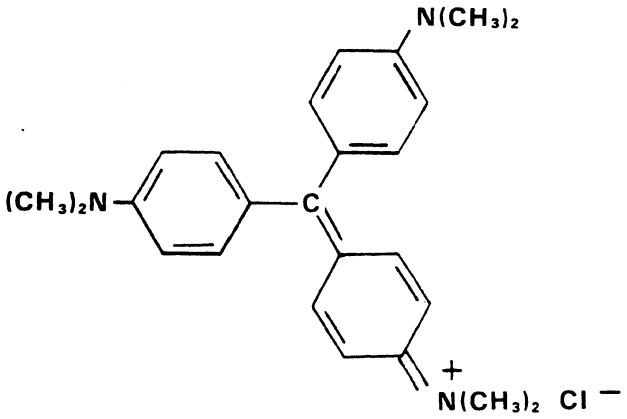


Figure 6.5 Crystal Violet

benzidine, of 1:10^5 dilution of blood. Michel (1911a) recommended this test and said that it was more sensitive then phenolphthalin. Von Fürth (1911) utilized the test on bloodstain extracts prepared by digesting the bloodstained material with 50% KOH in ethanol, and extracting that solution with pyridine. The test was then performed on a piece of filter paper, moistened with the pyridine extract. Medinger (1933) strongly recommended the reagent, and tested various physiological fluids, plant extracts and inorganic compounds, all with negative results, provided the peroxide was added in a second step after no color had developed in the presence of reagent alone.

White (1977) showed that leucocrystal violet was as sensitive in detecting iron (III) mesoporphyrin IX as a spot on filter paper as tetramethylbenzidine, guaiacum and aminodiphenylamine.

Alvarez de Toledo y Valero (1935) review the test rather extensively, and tested a large number of organic and inorganic compounds for false positive reactions. He found that there are many chemical oxidants that will give the reaction in the absence of peroxide, as well as some that catalyze the

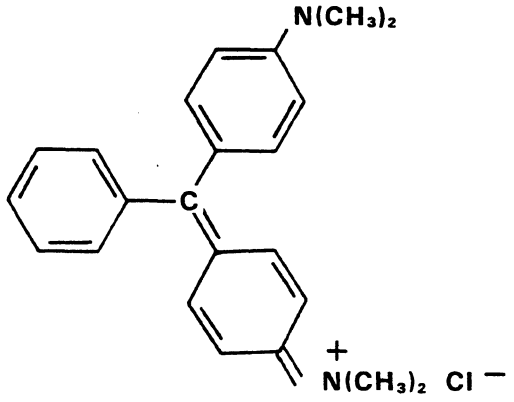


Figure 6.6 Malachite Green

reaction in its presence in much the same manner as does blood. The test is, therefore, not more specific for blood than most of the other catalytic tests.

The sensitivity of the test was originally reported to be $1:10^5$ dilution of blood by Adler and Adler (1904). Alvarez de Toledo y Valero (1935) reported the same value, but Nicolesco (1934) quoted the sensitivity as a 1:20,000 dilution of blood. Alvarez de Toledo y Valero noted that more dilute solutions of blood required longer times for color development, 1:1000 dilutions being instantaneous, 1:2,000 dilutions requiring 15–20 sec, and so on until 55 sec was necessary at $1:10^5$ dilutions and 25 min was needed for $1:2 \times 10^5$ dilutions. Grodsky *et al.* (1951) reported that stains made from $1:10^5$ dilutions of blood came up in 15 sec, using a reagent prepared by dissolving 0.1 g leucomalachite green and 0.32 g sodium perborate in 10 mℓ of 2:1 (v/v) glacial acetic acid in water. The benzidine test on the same sample, by contrast, came up in 1 sec. Hunt *et al.* (1960) similarly reported leucomalachite green to be less sensitive than benzidine, and apparently more specific. But the apparent increase in specificity was attributed to the lower sensitivity, and was thus not considered an advantage.

6.6 Other Catalytic Tests

Over the years a number of catalytic tests have been proposed for the detection of blood in stains, or of blood in feces or urine, which have enjoyed only limited use, or about which there is not a great deal of literature. All these tests are briefly discussed together in this section.

6.6.1 Peroxide

It should be mentioned that peroxide was sometimes used as a reagent for blood detection, especially in the older literature. That blood possessed a peroxidase activity, and acting as a catalase was thus capable of evolving oxygen from peroxide, has been known since the experiments of Schönbein (1863). All the catalytic tests are based on this principle, as discussed in the preceding sections; but all have relied upon the coupling of the peroxidase activity to the oxidation of a compound which formed a colored product.

Zahn (1871) specifically noted that peroxide could be used to detect blood in stains, though it is not clear that he was aware of Schönbein's work. If peroxide is brought into contact with a bloodstain the peroxidase reaction takes place after a minute or so, and is evidenced by the formation of large numbers of tiny bubbles. Gantter (1895) suggested that the test had substantial value if negative, i.e., was a good indication of the absence of blood. Sutherland (1907) referred to the test as the Zahn-Gantter Test, and noted that many of the substances now known to give false positive catalytic tests, such as vegetable extracts, gave this test as well. Cotton (1904) studied the evolution of oxygen in the presence of H_2O_2 from the blood of a number of different species. Palleske (1905b) also conducted studies on the test with different bloods. A positive test could be obtained with a drop of blood in 1500 mℓ of water, which, if we assumed that 20 of his drops would equal 1 mℓ, would represent a sensitivity of a 1:30,000 dilution. Sutherland (1907) thought the test was useful when negative, except where the stain had been heated above 120°. Leers (1910) presented the test as a presumptive one, noting that other substances than blood gave positive results. He called the test "Die Vorprobe mit Wasserstoffsuperoxyd" or, simply, "the preliminary test with hydrogen peroxide."

6.6.2 Eosin

In 1910, Ganassini proposed the use of an eosin reagent, prepared from crystalline eosin by heating in strong base, and collection and washing of the acid precipitate. This reagent in alcoholic solution in the presence of strong alkali and H_2O_2 gave a momentary yellow to red colored product. He believed the reagent to be specific for blood. Belussi (1911) disagreed, noting that other substances gave positive tests, and that the sensitivity of Ganassini's test was far lower than that of benzidine.

6.6.3 2,7-Diaminofluorene

There is a brief report in the literature by Schmidt and Eitel (1932) on blood identification using 2,7-diaminofluorene (also called 2,7-fluorenediamine). This report has mainly to do with a problem concerning the stability of the reagent, but the implication that the reagent was in use for the detection of blood is quite clear. The structure of 2,7-diamonofluorene is indicated in Fig. 6.7. The 7th edition of the *Merck Index* indicates that the reagent is used to determine halides, nitrate, persulfate and several metals. It seems likely, therefore, that these might be expected to give false positive reactions in the test for blood using this reagent.

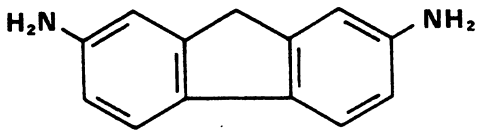


Figure 6.7 2,7 - Diaminofluorene

6.6.4 Rhodamine B

In 1917, Fuld recommended the use of a Rhodamine B reagent for blood detection. The reagent was prepared from Rhodamine B (Fig. 6.8) by reduction in base in the presence of zinc. This reagent detected blood at a dilution of $1:10^7$, according to Fuld. Alke (1922) studied the reaction in some detail, and reported that it is not given by semen, saliva, urine or a number of other biological substances. The usual inorganic oxidants will oxidize the reagents in the absence of H_2O_2. The reaction was negative with rust, and Alke reported a sensitivity limit of $1:10^5$ to $1:10^6$ dilution of blood. Ziemke (1938) notes that an investigator named Diels showed that chlorophyll gives the test. The reaction is apparently still positive with blood which is putrefied, or which has been heated above 200°.

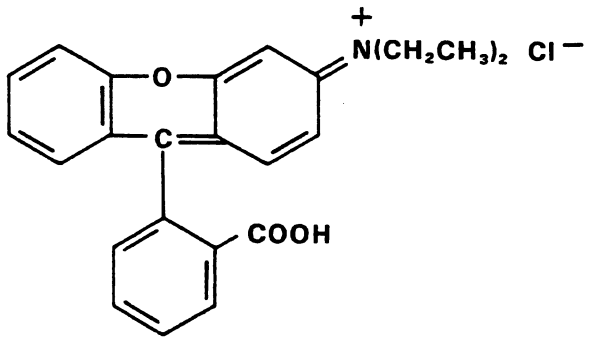


Figure 6.8 Rhodamine B

6.6.5 Para-Phenylenediamine

Boas, in 1906, recommended p-phenylenediamine (p-diaminobenzene) as a reagent for detecting occult blood in feces. Schumm and Remstedt (1906) evaluated the reaction, noting that under their conditions, the sensitivity could be as high as $1:10^5$ dilution of blood. The colors obtained varied, however, with different blood dilutions. Adler and Adler (1904) had tested this material, as well as the o- and m-isomers, in their experiments and obtained color reactions at sensitivities of 7:100,000 dilutions of blood, or about 1:14,285.

6.6.6 Ortho-Tolidine and ortho-toluidine

Ruttan and Hardisty (1912) recommended o-tolidine as a reagent for detecting occult blood. This reagent had a sensitivity limit of $1:7 \times 10^6$, an order of magnitude greater than that for benzidine, but somewhat less than that for phenolphthalin, which they reported as a $1:10^7$ dilution. The test was also recommended by Kohn and O'Kelly (1955) as a substitute for the benzidine test for occult blood, and Jacobs (1958) found the reagent to be a good substitute for benzidine for the staining of leucocyte peroxidases. In 1939, Gershenfeld wrote a paper noting that there had come to be some confusion in the literature as to the difference between o-tolidine and o-toluidine. The former is 3,3′-dimethylbenzidine (Fig. 6.9), while the latter is o-methylaniline (or 2-aminotoluene) (Fig. 6.10). Gershenfeld mentioned that some writers had erroneously credited the employment of o-toluidine for blood testing to Ruttan and Hardisty (1912), while these workers were responsible only for the employment of o-tolidine. Adler and Adler (1904) had tested p-toluidine but not o-toluidine in their studies. Either o-tolidine or o-toluidine may be used as reagents for blood testing, the former being more sensitive and giving a blue-colored oxidation product, while the latter gives a violet-colored one. The distinction is important, and should be kept in mind. Holland *et al.* (1974) confirmed Ruttan and Hardisty's earlier finding that o-tolidine is more sensitive than benzidine for blood testing, although the comparison in the more recent study was made on the basis of molar extinctions at maximum wavelength of visible absorption,

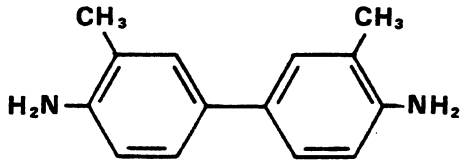


Figure 6.9 o-Tolidine

rather than in terms of maximal dilutions of blood which still gave positive reactions. Although o-tolidine has apparently not been placed on the OSHA regulations list (Cf:29 Code of Federal Regulations 1910.1000 *et seq.*), Holland *et al.* (1974) reported that it is carcinogenic in rats. Ferretti *et al.* (1977) reported that o-tolidine was mutagenic in the Salmonella/mammalian microsome test, indicating a high probability of carcinogenicity. Ortho-toluidine was not mutagenic in the test. Hunt *et al.* (1960) tested o-tolidine and found it to be quite similar to benzidine in most respects, and weak but slow positive reactions were obtained at $1:10^6$ dilutions of blood. Culliford (1971) mentioned that o-tolidine could be substituted for benzidine for routine identification testing.

6.6.7 Ortho-Dianisidine

Ortho-dianisidine is 3,3′-dimethoxybenzidine (Fig. 6.11). Owen *et al.* (1958) tested a number of compounds for the detection of the hemoglobin-haptoglobin complex in gels following zone electrophoresis. They found o-dianisidine to be the most sensitive, and to give the most stable color. The latter property led to its being adopted in clinical situations for the quantitative, colorimetric determination of heme compounds (Lupovitch and Zak, 1964; Ahlquist and Schwartz, 1975). Compton *et al.* (1976) used it to detect Hb-Hp complexes on cellulose acetate membranes following electrophoresis. Rye *et al.* (1970) said that there was no evidence that o-dianisidine was carcinogenic in humans, and that it was in fact metabolized by a different pathway than is benzidine, but Ferretti *et al.* (1977) reported that it behaved like benzidine and o-tolidine in the Salmonella/mammalian microsome test indicating that it might well be found to be carcinogenic. Culliford (1971) noted that this compound could be substituted for benzidine in identification tests for blood stains.

6.6.8 Amidopyrine

Amidopyrine, also known as aminopyrine, pyramidon, aminopyrazine, 4-dimethylaminoantipyrine, and a variety of other names, is 4-dimethylamino-2, 3-dimethyl-1-phenyl-3-pyrazolin-5-one (Fig. 6.12). Caplan and Discombe (1951) tested amidopyrine for the detection of blood in urine, and found it to be more sensitive than guaiacum, but less so than o-tolidine, benzidine or phenolphthalin. White (1977) found it to be among the least sensitive of a number of compounds he tested for the detection of Fe(III) mesoporphyrin spotted on filter paper. Owen *et al.* (1958) tested this material for

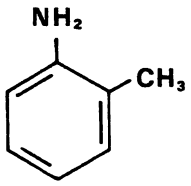


Figure 6.10 o-Toluidine

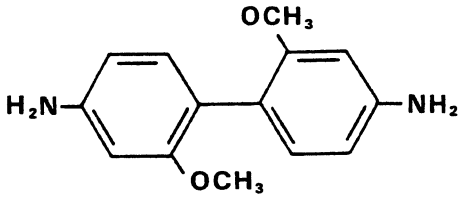


Figure 6.11 o-Dianisidine

the detection of Hb-Hp complexes following electrophoresis, but got poor results with it. As noted briefly above, however, Rentoul and Smith (1973) recommended amidopyrine as a benzidine substitute in blood identification tests. The suspect material is touched with a piece of filter paper, which is then first treated with a saturated solution of amidopyrine in 95% ethanol, and then with H_2O_2. A purple color appears in a few seconds if the test is positive, and the test was regarded as presumptive only. The test was said to work better with higher concentrations of peroxide, but these were not used because they were caustic to the skin.

6.6.9 Benzylidine dimethylaniline

In 1956, MacPhail reported that p,p′-benzylidine bis (N,N′-dimethylaniline) was an excellent reagent for the catalytic blood identification test. The compound is also known as 4,4′-tetramethyldiaminotriphenylmethane. Saline-moistened filter paper was placed in contact with the stain and then tested in a two-stage reaction. Reagent was made up 2 grains/oz 40% acetic acid. Sodium perborate (23 grains/oz 40% acetic acid) was used in the second stage. The oxidized product is momentarily green, passing quickly to a dark blue-green. Sensitivity was said to be in excess of $1{:}10^6$ dilution of blood, and the reaction only occurred with mammalian blood. False positives were not observed if the two-stage technique was employed. Williams (1974) recommended the reagent and said that it was superior to benzidine, there being less chance for false positive reactions.

6.6.10 3,3′,5,5′-Tetramethylbenzidine (TMB)

In an effort to find a safe substitute for benzidine, Holland *et al.* (1974) synthesized and tested 3,3′,5,5′-tetramethylbenzidine (TMB). The compound was prepared from 2,6-dimethylaniline, and its structure is shown in Fig. 6.13. There was some evidence, according to these authors, that carcinogenicity in these aromatic amines is related to the ability of the compound to undergo metabolic, or *in vivo,* o-hydroxylation. Methylation at all four o-positions renders this reaction impossible, and was expected, therefore, to decrease carcinogenicity. TMB was found to be much less carcinogenic in rats than either benzidine or o-tolidine, and more sensitive than either of them for blood testing. Garner *et al.* (1976) evaluated TMB as a reagent for the identification of blood in stains in comparison to benzidine. The sensitivity of the reagents, expressed as the weakest dilution of blood in saline that gave a positive test, was found to be identical at reagent concentrations of 0.05M, 0.1M and 0.2M, the last-mentioned being the saturating concentration of TMB in glacial acetic acid. At 0.2M strength, both reagents detected $1{:}10^6$ dilutions of blood. Studies on specificity were carried out by testing a number of plant extracts deposited on cotton cloth for false positive reactions. Testing was carried out on rubbings, extracts, and directly on the cloth. Positive reactions were obtained with both reagents on rubbings of horseradish stain, and with TMB but not with benzidine on rubbings of garlic stain. All other "false positive" reactions on rubbings or cloth were distinguishable from those of blood on the basis of the time of reaction or nature of the color developed. All saline extracts tested were negative with both reagents. Some types of papers gave positive reactions as well, but these too were qualitatively distinguishable from the blood reactions. Still, blood identification in stains on paper should be carried out with great care if TMB is employed. The reagents showed parallel decreases in sensitivity as a function of storage as 0.2M solutions in glacial acetic acid. The sensitivity had decreased to $1{:}10^5$ dilution of blood by the second day of storage, and to $1{:}10^4$ dilution by the eighth day. It made no difference to this decrease if the reagents were stored dark, or dark and refrigerated.

Nardelli (1976) has noted that parts of tangerine peels give positive but slow TMB reactions, a result which is not altogether surprising in view of the observation by Grodsky *et al.* (1951) that the white pulp, but not the juice, of the orange gave false positive reactions with benzidine. Nardelli also mentioned that saliva and asbestos glove material gave false positives. Garner *et al.* (1976) pointed out that as demand for and supply of TMB increases, and price decreases, the reagent may come to be used as commonly in forensic laboratories as was benzidine.

6.6.11 Chlorpromazine

Chlorpromazine is 2-chloro-10-(3-dimethylamino propyl)-phenothiazine. It can be oxidized by peroxide to a red free radical form, the reaction being catalyzed by peroxidases, including hemoglobin. The chromophore has an absorption maximum at 530 nm and Lee and Ling (1969) showed that the reagent could be used to quantitate Hb in serum. Collier (1974) confirmed these results and recommended chlorpromazine as a substitute for o-dianisidine and o-tolidine. White (1977) reported that chlorpromazine and

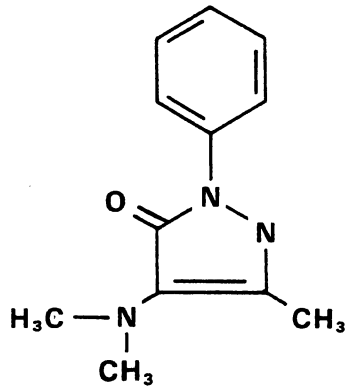


Figure 6.12 Amidopyrine

H_2O_2 detect Fe(III) mesoporphyrin IX as a microspot on filter paper.

6.6.12 Diphenylamine

In 1970, Woodman proposed the use of diphenylamine as a catalytic test reagent for blood in feces. The test was sensitive to about 1:4000 dilutions of blood in water and the oxidized chromophore is a green color. The reagent is also suitable for detecting hematin compounds on paper or cellulose acetate membranes following electrophoresis, and was recommended because it is not carcinogenic.

6.6.13 Fluorescin

Fluorescin is the reduced form of fluorescein, the latter being 9-(o-carboxyphenyl)-6-hydroxy-3H-xanthen-3-one. Fluorescein is soluble in alkali hydroxides and carbonates at room temperature, and exhibits an intense green fluorescence. In 1910, Fleig proposed using fluorescin to detect blood in the urine of patients. He prepared the reagent by reducing fluorescein in the presence of KOH, zinc dust and heat. He said that it was a more sensitive reagent than phenolphthalin, and suggested that it could be used to detect blood in dried stains as well.

We have conducted studies on the use of fluorescin as a presumptive test reagent, and have found that it is entirely satisfactory (Lee *et al.* 1979). We were unaware of Fleig's paper until quite recently. Fluorescin can be prepared from fluorescein in the same way that phenolphthalin is prepared from phenolphthalein. We find that the reagent is stable for months if kept over some zinc dust at 4°. We dilute it to about 1:60 with water for use, and the dilute solution is not as stable as the stock one. Water is preferable to ethanol as a diluent. A positive test can be obtained on blood dilutions up to $1:10^7$. It works on bloodstains on many different substrata.

6.7 Luminol Test

The luminol test is based on the fact that a number of hemoglobin derivatives greatly enhance the chemiluminescence exhibited by luminol upon its oxidation in alkaline solution. According to Proescher and Moody (1939), the compound was first synthesized by A. Schmitz in Heidelberg in 1902, under the direction of Prof. T. Curtius. Curtius and Semper (1913) then synthesized it in a different way, and referred to Schmitz's earlier work. But none of these workers noticed the chemiluminescence properties of the molecule. The intense blue chemiluminescence of the compound during its oxidation in alkaline solution was first observed by W. Lommel in Leverkusen (Germany) who brought it to the attention of H. Kautsky. Kautsky apparently interested H. O. Albrecht in looking into the properties of the phenomenon, the results of the investigation having appeared in 1928. Albrecht found that a number of oxidizing agents, which could be used in alkaline solution, brought about the luminescence, that H_2O_2 alone brought about only a feeble luminescence, and that luminescence was visible in a darkened room even at luminol concentrations of 10^{-8} M. Ferricyanide or hypochlorite greatly enhanced the luminescence obtained with H_2O_2, as did plant peroxidases and blood. Albrecht suggested a mechanism for the reaction as well, and this matter is discussed in more detail below.

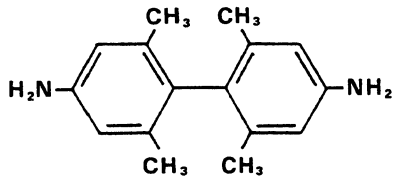


Figure 6.13 3, 3', 5, 5' - Tetramethylbenzidine

Luminol is 3-aminophthalhydrazide (Fig. 6.14). In 1934, Huntress *et al.* reported a method for the synthesis of the compound from 3-nitrophthalic acid and hydrazine sulfate, and named it "luminol". A year later, Harris and Parker, in the same laboratory, published studies on the quantum yield of the chemiluminescence. Gleu and Pfannstiel (1936) observed that crystalline hemin produced an especially intense luminescence, a fact soon confirmed by Tamamashi (1937). Specht (1937a, 1937b) did an extensive series of studies intended to design a useful medico-legal blood identification test based on luminol chemiluminescence. Old as well as recent bloodstains were examined, and able to be detected reliably using luminol reagent. Specht made two solutions, and noted that either one worked well: (1) 0.1 g luminol, 5 g $CaCO_3$, and 15 mℓ 30% H_2O_2 in 100 mℓ H_2O; (2) 0.1 g luminol in 100 mℓ 0.5% aqueous sodium peroxide. He tested a variety of substances for their ability to enhance peroxide-luminol luminescence in the same manner as did bloodstains. These included milk, coffee stains, semen, saliva, urine, feces, dyes, moldy bread, leather, fabrics, oils, varnish, wax, shoe polish, wood, grass, leaves and a number of metals, and all gave negative results. The reagent could be used in solution or sprayed onto suspected surfaces of all types using an atomizer. The spraying of luminol reagent onto bloodstain

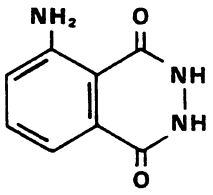


Figure 6.14 Luminol (3-aminophthalhydrazide)

material was said not to interfere with subsequent crystal or spectral tests, nor with serological tests for species or blood groups. The luminescence lasts for quite a while, at least a matter of minutes, and under proper conditions can be photographed to provide a record of the location of stains. Specht recommended the test strongly for medico-legal examinations, and believed that it was quite specific for blood. Proescher and Moody (1939) looked into the test fairly extensively, using commercial luminol at a concentration of 0.1 g in 100 mℓ 5% Na_2CO_3. This reagent solution was indefinitely stable. The test was performed by adding 15-20 mℓ 3% H_2O_2 or 1 g sodium peroxide to 100 mℓ luminol solution just prior to making the test. For the detection of fresh blood in solution, the hemoglobin was converted to hematin by the addition of concentrated HCl to the sample, which was then boiled briefly to destroy vegetable peroxidases. The test solution was then made alkaline again with sodium carbonate, and luminol reagent added. For bloodstains, the surfaces were first sprayed with 1-2% HCl, sprayed again after 10-15 min with sodium carbonate solution, and finally with luminol-peroxide reagent. Proescher and Moody regarded the test as extremely useful, but presumptive. The test could be given by hypochlorites, ferricyanide, and several other inorganic substances. Many of the substances with which Specht (1937a, 1937b) had obtained negative results were tested, and the negative results confirmed. Bloodstains were found to give more intense and longer lasting luminescence than fresh blood, and could be made luminescent many times by allowing the sprayed reagents to dry, and then re-spraying.

McGrath (1942) recommended the luminol test for use in forensic blood detection. He noted, as had others, that older stains gave more intense and longer-lived luminescence than fresher ones, because more met-Hb and hematin has formed in the older stains. He believed the test to be quite specific for blood, having obtained negative reactions with serum, bile, pus, semen, pleural fluid, earth, feces, fresh and spoiled vegetable material, various paints, metals, wood and shoe polish. He nevertheless cautioned that the test should not be used as a final, specific test for blood by itself. The main disadvantage of the test, in McGrath's mind, was that it had to be carried out in the dark. Kraul *et al.* (1941) noted that they regarded the test as a good presumptive one, but that it was not specific for blood. They also determined the wavelength of maximum chemiluminescence to be at 441 nm, with a shift to longer wavelength, 452 nm, in the presence of old blood. Schneider (1941) reported that a number of iron chlorophyll derivatives give luminescence with luminol-peroxide in sodium carbonate solution.

Grodsky *et al.* (1951) described a number of studies on the luminol test. They recommended a reagent consisting of 0.07 g sodium perborate in 10 mℓ water, to which is then added 0.01 g luminol and 0.5 g Na_2CO_3. The order of addition was important because the perborate is more soluble in water than in sodium carbonate, while the opposite is true of luminol. Reagents for the preparation of this reagent were incorporated into a field test kit recommended by the authors. The test was considered to be quite specific for blood, no false positives having been observed with a variety of materials that affected other catalytic tests. A noteworthy exception was copper salts. Grodsky *et al.* found that most brass, bronze and similar alloys which contain copper gave the reaction, a very important consideration if one is dealing with locks, door handles or other fixtures constructed of these materials. Indeed, Steigmann (1941) recommended the use of luminol for the detection of copper, as well as iron and cobalt, ions. He noted in 1942 that the reagent could be used for peroxide determinations as well.

Zweidinger *et al.* (1973) evaluated a number of types of film for the photography of bloodstains sprayed with luminol reagent. Various reagents, made with peroxide or perborate, were also tested. In aqueous solutions, the peroxide yielded a more intense, but shorter-lived chemiluminescence than the perborate. Similar results were obtained if the reagents were made up in 95% ethanol, it being necessary in this latter case to basicify the solution with 0.02 M KOH since sodium carbonate is quite insoluble in ethanol. A number of different photographic films were investigated, along with the effects of varying f/stop, exposure time and development conditions. It was possible to obtain good photographic records of bloodstains on items of evidence, and the procedure was recommended for adoption in routine practice.

One of the claims that has been made for the luminol test, particularly by those recommending sprayed reagents, is that its presence does not interfere with subsequent confirmatory blood tests or serological tests (Specht, 1937a, 1937b; Proescher and Moody, 1939; McGrath, 1942; Grodsky *et al.*, 1951). Srch (1971) reported, however, that the presence of luminol reagent on a sample may interfere with the Takayama test, the determination of ABH agglutinins by the method of Lattes, and the absorption-inhibition test for ABH agglutinogens. It did not interfere with the benzidine test, nor with species determination by the precipitin test. Schwerd and Birkenberger (1977) confirmed Srch's finding that luminol-peroxide spray can interfere with ABO grouping by inhibition technique, especially in small stains. Mixed agglutination could still be used, but did not work as well as on unsprayed controls. The precipitin test was not affected by the spray reagent.

An advantage of the luminol test is its great sensitivity. Albrecht (1928) stated that chemiluminescence was obtainable at luminol concentrations of 10^{-8} M, and Wegler (1937) reported luminescence at 10^{-10} g/mℓ luminol, or about

5.6×10^{-10} M (the MW of luminol is 177.16). The more usual way of expressing sensitivity, of course, is in terms of maximal dilutions of blood which still give positive reactions at some constant reagent concentration. Most authors have used 0.1% (w/v) luminol solutions, corresponding to about 0.056 M. Proescher and Moody (1939) said that the test was sensitive to $1:10^9$ dilutions of hematin. Grodsky *et al.* (1951) obtained luminescence lasting at least 15 minutes within 5 sec of spraying the reagent on stains made from $1:5 \times 10^6$ dilutions of blood. Weber (1966) quoted a sensitivity of $1:10^7$ dilution of blood using a photomultiplier tube detection system.

Bujan (1948) attempted to take advantage of the fact that luminol gives its intense luminescence with hematin, which is formed upon bloodstain aging. The luminescence intensity, measured photoelectrically, could be correlated with the age of the bloodstain, or with the amount of blood that was present in the stain.

The luminol reaction is somewhat more complex than those involved in the phenolphthalin, benzidine, leucomalachite green, and other catalytic tests. While it is probably not wrong to refer to the luminol test as a "catalytic test", it is not mechanistically a catalytic test in quite the same way as are the others. In dilute acid solution, luminol is relatively insoluble, and has the structure shown in Fig. 6.15(a). This compound gives a strong blue fluorescence with UV light. The tautomeric forms shown in Fig. 6.15(b) exist in alkaline solution, and it is these which produce chemiluminescence upon oxidation. Albrecht (1928) proposed a mechanism for chemiluminescence (Fig. 6.16), in which the phthalazine (I) was oxidized to form a diimide compound (II). This material would be hydrolyzed in the basic solution to yield the phthalic acid compound (III) and N_2H_2, which reacts with an additional mole of II to form nitrogen, IV and light. Compound IV, it will be noticed, is the form of luminol which exists in acid solutions (Fig. 6.15(a)), and thus presumably a partial regeneration of starting material. Tamamushi and Akiyama (1938) studied the reaction and their results were consistent with this mechanism, but Stross and Branch (1938) obtained results using fast-flow methods which could not be explained by it. Other studies were done by Sveshnikov (1938) whose results suggested a prior hydrolysis, with luminescence being due to the oxidation of a hydrolysis product. Kubal (1938) and Plotnikov and Kubal (1938) investigated the spectral changes associated with the reactions. In the presence of rhodamine or fluorescein, some of the chemiluminescent energy is apparently absorbed by the dyes, which then fluoresce at wavelengths longer than that of the luminescence. This phenomenon, they called chemifluorescence. Baur (1940) said that the decay of luminescence of luminol in the presence of hemin and peroxide followed a bimolecular rate law. Weber and co-workers carried out extensive studies on the luminescence reaction, and state, among other things, that substances which greatly increased the peroxide oxidation-dependent luminescence, such as chlorhemin, met-Hb and ferritin, do not, strictly

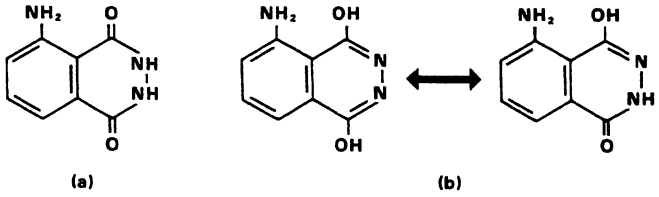


Figure 6.15 Luminol Structures in Solution

speaking, act as catalysts under all conditions (Weber, 1942; Weber *et al.*, 1942; Weber and Krajčinović, 1942). Apparently, these compounds are best thought of as "accelerators", which may act catalytically. Weber *et al.* (1942) suggested that the products of the initial oxidation reaction included O_2 and reduced accelerator. If the reduced accelerator could be reoxidized by O_2, the compound would be acting catalytically, while if the reoxidation were not possible, the accelerator would have acted as a reactant. The subject is complex, and it may be that the mechanism is not the same with every "accelerator" or catalyst. Shevlin and Neufeld (1970) studied the mechanism of the ferricyanide-catalyzed luminescence of luminol, and proposed the scheme shown in Fig. 6.17 to explain their data. It should be noted that the ferricyanide acts catalytically in this scheme. The exact role of the catalysts or accelerators is not clear from Albrecht's scheme (Fig. 6.16). White and Roswell (1970) reviewed the chemiluminescence phenomena characteristic of organic hydrazides generally, including luminol. Isaccson and Wettermark (1974) noted that the mechanism of luminol oxidation in aqueous solution has still not been satisfactorily elucidated in spite of many studies.

It may be mentioned, finally, that Weber (1966) proposed an improved reagent for blood testing. Stock solutions were: (A) 8 g NaOH in 500 mℓ H_2O, or 0.4N; (B) 10 mℓ 30% H_2O_2 in 490 mℓ H_2O, or 0.176M; (C) 0.354 g luminol in 62.5 mℓ 0.4N NaOH to a final volume of 500 mℓ with water, or 0.004M luminol. To make up testing reagent, 10 mℓ of each of these solutions is mixed with 70 mℓ H_2O. It will be noted that the Na_2CO_3 used by others is here replaced by NaOH, and that the luminol and peroxide concentrations are very much lower. This reagent works well with both fresh and dried blood, whereas the older reagents did not readily react with fresh blood because there was too little met-Hb or hematin present. The fact that this reagent serves with fresh stains is explained by the rapid conversion of Hb to met-Hb and/or hematin in the strong base. The reagent is more sensitive than the older ones because of the lowered H_2O_2 and luminol concentrations. Higher concentrations of these compounds tend to be inhibitory.

6.8 Catalytic Tests—General Considerations

There can be no doubt that most authorities have considered the catalytic tests as presumptive when used alone. Their value is ascribed to the ease and rapidity with which

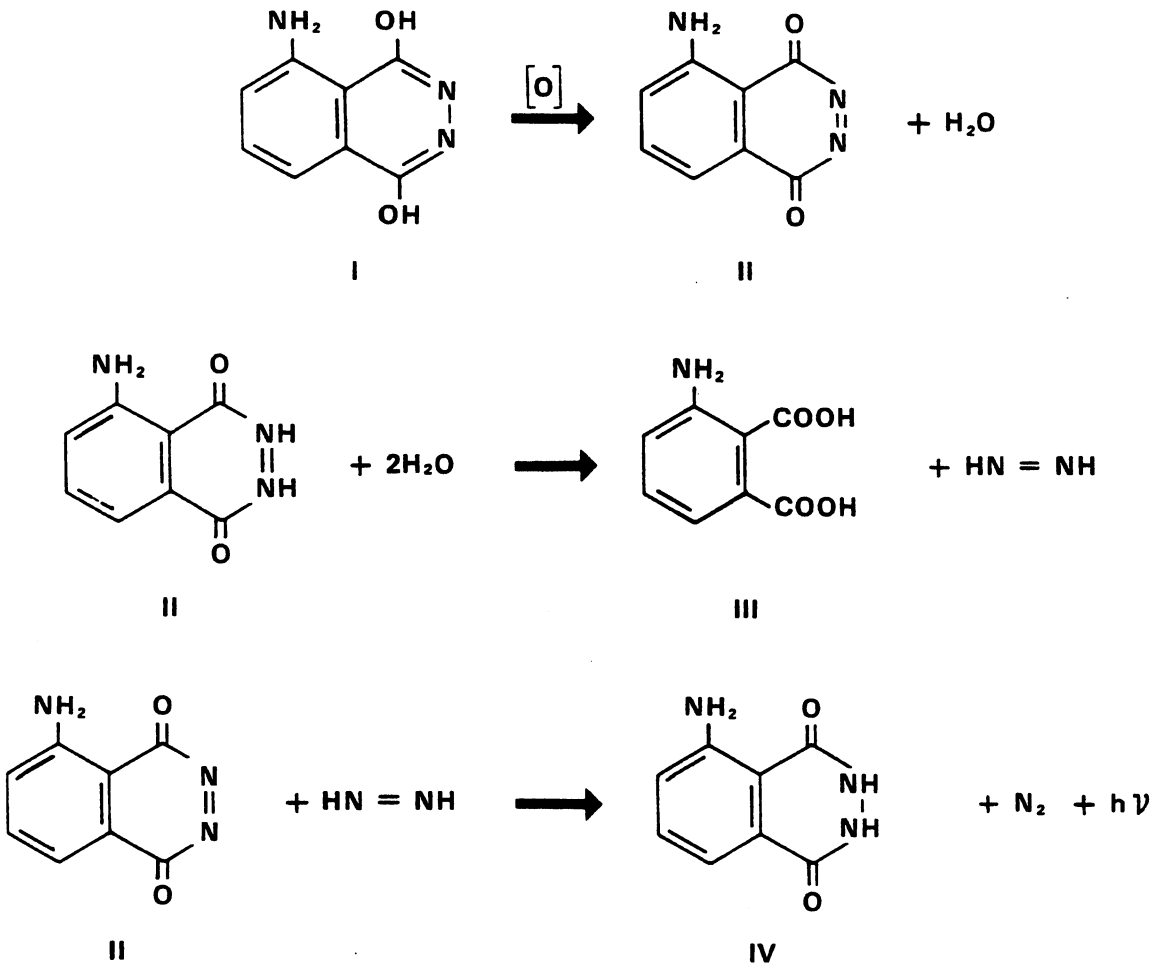


Figure 6.16 Albrecht Mechanism

they can be carried out in order to decide which exhibits should be subjected to further tests. It has occasionally been persuasively argued that in the hands of an experienced investigator, who is aware of the principles underlying the test and of the materials other than blood likely to give misleading reactions, the tests can be virtually specific. Grodsky *et al.* (1951) made something of a case for the combined use of several tests, namely benzidine, phenolphthalin, leucomalachite green and luminol, especially in cases where there was a limited amount of material to work with. These recommendations were based in part on their own studies, as well as on a set of experiments conducted by Pinker (1934). Pinker tested some 200 different biological substances and organic and inorganic chemicals for reactions with benzidine, phenophthalin and leucomalachite green reagents. A very small amount of the substance or stained material was treated directly with the reagent in a spotting plate. The reactions were carefully observed in terms of time, intensity, color produced, and other characteristic properties such as precipitate formation. Vegetable peroxidase interference could be eliminated by testing samples which had been heated, and chemical oxidants were detected by the fact that they react in the absence of peroxide. With these materials excluded, there was not found one interfering substance which would give a false positive reaction (indistinguishable from the blood reaction) with *all three* reagents. Pinker therefore argued that if positive reactions were given by all three reagents in carefully performed tests, the likelihood of error was exceedingly remote. Not all the same substances are likely to interfere with all the tests, especially with luminol as against the others. Grodsky *et al.* felt that there was no significant probability that any substance other than blood would be found which would give positive reactions in all four tests which would be indistinguishable from the blood reaction by an experienced worker. The argument was not put forth to suggest that these tests

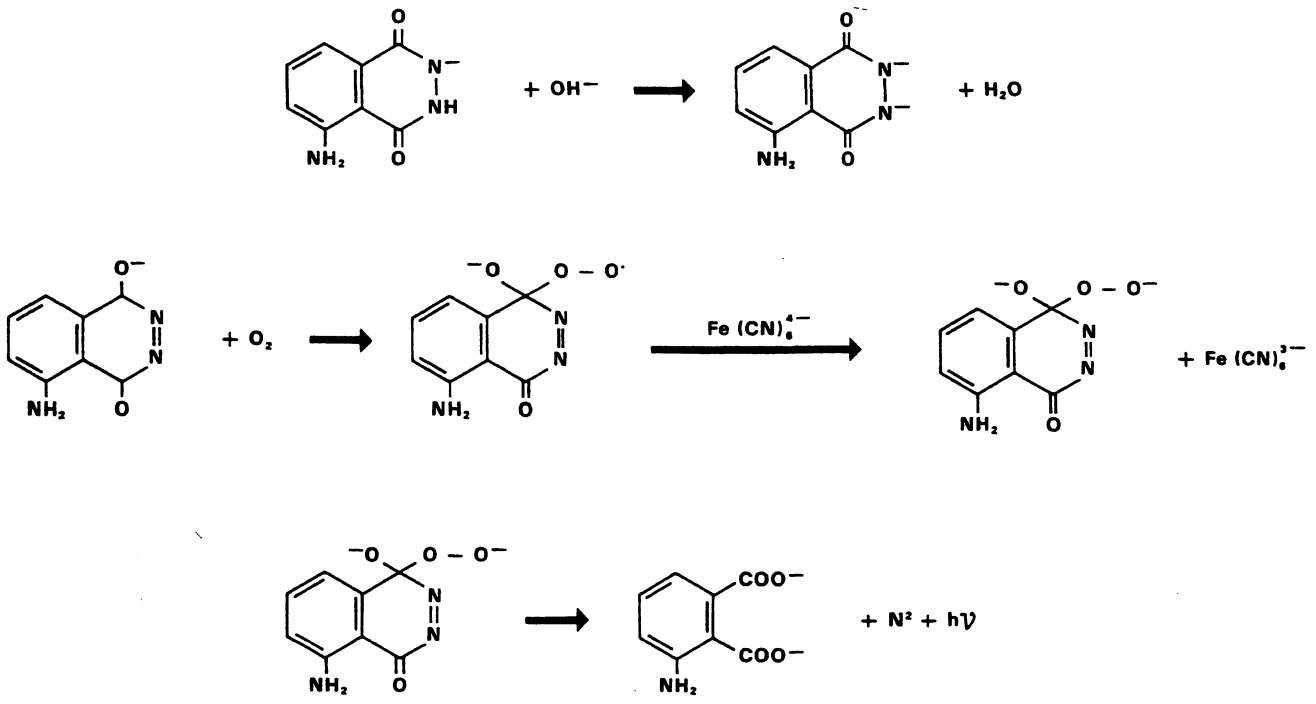


Figure 6.17 Shevlin and Neufeld Mechanism

replace crystal and spectroscopic tests; but cases do arise in which there is insufficient material for further testing. The results of Blake and Dillon (1973), discussed in Section 4.2.4, would suggest that for a number of examples of bacteria, whether in fresh culture or dried out on filter paper for 2 months, the use of several catalytic tests and/or a luminol test (Section 6.7) would not necessarily exclude false positive reactions. The ease with which suspensions of microorganisms gave the benzidine test was directly related to their catalase content, and the false positive reaction was not inhibited by exposure to high temperatures for periods of up to 30 min. Ultimately, of course, the decision concerning what proof value should be placed on the outcome of any test on a given exhibit rests with the individual expert. In the final analysis, the individual is giving his or her opinion as to what the findings mean.

A somewhat different issue, of no less concern, is raised by studies that have indicated the presence of blood on clothing and other samples that were randomly selected, and had nothing whatever to do with case material. Owen and Smalldon (1975) got positive benzidine reactions on 5 jackets of 100 tested, and on 16 pairs of trousers of 100 tested, randomly selected at a dry cleaning shop. One pair of used shoes of 100 pairs examined (50 men's and 50 women's) gave a positive reaction as well. Hunt *et al.* (1960) obtained strongly positive benzidine reactions (i.e. within 3 sec) on one of 30 used shoes randomly selected at a shoe repairer, and weakly positive reactions (i.e. within 5-10 sec) on six others. Terörde (1939) examined fingernail scrapings from 606 people in the general population for benzidine reactions. 195 samples were positive. 165 positive samples came from the 546 men in the sample, while the remaining 30 were from the 60 women. The individual's occupation or walk of life seemed to matter little, except in the case of butchers, whose scrapings were all positive. Additionally, Terörde got a positive precipitin reaction against anti-human serum with one of six of the benzidine-positive samples. The results strongly indicate that some consideration must be given to the actual evidentiary value of samples when positive results are obtained, especially in cases where little material is available for further testing.

Briggs (1978) discussed the matter of the probative value of bloodstains on clothing in connection with a case, which had required the examination of stains on a very large number of different articles. The relative occurrence of particular combinations of blood groups in the bloodstains enters into this discussion as well.

SECTION 7. OTHER TESTS

7.1 Immunological Tests With Anti-Human Hemoglobin

The first antiserum to a hemoglobin was reported by Leblanc (1901). He prepared anti-cow Hb sera in rabbits, with which a precipitin reaction could be obtained. Ide (1902) confirmed these results, although his antisera were hemolytic as well. His student, Demees (1907) was able to prepare a non-hemolytic anti-Hb precipitin antibody by using a more thoroughly purified Hb as immunizing antigen. Not all investigators obtained identical results in the early investigations, however. Gay and Robertson (1913) concluded that globin alone was not antigenic, but when combined with casein into what they referred to as "globin-caseinate", an antibody could be raised which fixed complement (see section 1.3.5.3) with the antigen as well as with casein alone. Browning and Wilson prepared antibodies to guinea pig globin in rabbits in 1909. In 1920, they enlarged these studies somewhat, noting that ox globin was antigenic as well. The anti-guinea pig serum was quite species specific, but the anti-ox globin was not. Ford and Halsey (1904) were unable to raise precipitin antisera to purified, crystalline dog or hen hemoglobin in either rabbits or guinea pigs. In 1919, Schmidt and Bennett got similar results. Unable to obtain any precipitin antisera against pure, crystalline dog hemoglobin, they concluded that hemoglobin was not antigenic. Klein (1904, 1905a) conducted extensive experiments on the immunization of rabbits with serum, whole red blood cells, and red cell extracts. Antibodies were obtained to the red cell extracts, which he called "erythropräzipitine"; these antibodies he believed to be different from the anti-serum antibodies (obtained by immunizing with serum). Klein was the first to suggest (1905b) that the anti-red cell extract serum should be employed in medico-legal investigations. He believed the antisera to be species-specific, and that their use would combine into one procedure the determination of the presence of blood and its species of origin. Leers (1910) agreed with this viewpoint. He prepared specific "erythroprecipitin" antisera of his own, and he discussed the use of these reagents in carrying out medico-legal tests for blood.

The issue of hemoglobin's antigenicity began to be settled in 1922, when Hektoen and Schulhof prepared precipitating antibodies against extracts of cow, dog, goat, guinea pig, horse, human, rat, sheep and pig red cells. Some of these were species-specific, while others showed cross reactions. The following year, they obtained many of the same antisera in species-specific form, and concluded that the red cell precipitinogen giving rise to these antibodies was in fact hemoglobin. They suggested that the antisera would be useful in solving medico-legal cases. Higashi (1923) independently arrived at the same conclusions, suggesting that the antisera be called "hämoglobinopräzipitin", and recommending that they be used in forensic practice. Heidelberger and Landsteiner confirmed these findings in 1923. They knew of Higashi's work, but had apparently not yet seen Hektoen and Schulhof's 1923 paper. Sera produced with crystalline hemoglobin, they said, reacted species-specifically with homologous antigen. The antisera worked equally well with homologous met-Hb, HbCN and HbCO. Fujiwara (1928) carried out studies on anti-hemoglobin precipitin sera in parallel with anti-serum precipitin sera. He obtained species-specific anti-Hb sera, which had titers as high as 1:40,000 against whole blood or hemoglobin.

Some years later, there was recurrent interest in antisera to hemoglobin, especially with the objective of developing immunological methods for differentiating the various human hemoglobin variants, which were coming to be known. These studies were also done as an immunological approach to discovering the differences in structure among the human hemoglobin variants. The subject of hemoglobin variants itself is involved, and will not be discussed here (see section 38).

Darrow *et al.* (1940) prepared antibodies to cord blood and to adult blood. The former, if absorbed with adult cells, could be rendered specific for cord blood hemoglobin (Hb-F; fetal hemoglobin). Antibodies to adult hemoglobin (Hb-A) reacted with both adult and fetal blood, and absorption of the antisera with either adult or fetal cells brought down all the antibodies. Aksoy (1955) prepared rabbit antibodies against cord blood, but found that in most of the preparations, absorption with adult cells precipitated all the antibody. In only one case was the serum rendered specific for Hb-F by absorption with adult cells.

In 1953, Chernoff published a pair of papers, the first of which (1953a) reported the preparation of anti-Hb-A, anti-Hb-F, anti-sickle cell hemoglobin (anti-Hb-S), and anti-guinea pig Hb, using Freund's adjuvant for the first time. The anti-Hb-F was specific for cord blood, but the antisera to Hb-A and Hb-S cross reacted. The second paper (1953b) reported the use of the specific anti-Hb-F to measure the amount of fetal hemoglobin in adult blood. Kohn and Payne (1972) described a radial immunodiffusion procedure for the determination of Hb-F, using a commercial antiserum. Goodman and Campbell (1953) showed that rabbit antisera to Hb-A and Hb-S did not differentiate them well, but that antisera prepared in chickens could be used to do so. Major

differences in reactivity between anti-Hb-A and anti-Hb-F were also confirmed. Ikin *et al.* (1953) prepared agglutinins to cord blood cells, which showed good titers only if used in the presence of 10% albumin and at 37°. Diacono and Castay (1955a) obtained antibodies from guinea pigs which could be used to distinguish between adult and fetal red cells, and they showed further (1955b) that the anti-Hb activity was not identical to the hemolysin activity. Boivin and his collaborators studied the reactions of anti-Hb-A and anti-Hb-F with a number of variant hemoglobins using Ouchterlony gel diffusion and immunoelectrophoretic techniques. They showed that hemoglobins A, F, S, C and E showed a common antigenic determinant (Boivin and Hartmann, 1958b; Boivin *et al.*, 1959). They also showed that anti-Hb-A reacted with hemoglobin-A in the hemoglobin-haptoglobin complex in gels (Boivin and Hartmann, 1958a). In 1961, Muller *et al.* obtained a rabbit immune anti-human Hb serum, which did not discriminate between Hb-A and Hb-F. But it did not react with human serum, nor with animal hemoglobins, and these workers suggested that it could be very useful in medico-legal work for confirmation of species of origin of bloodstains. Likewise, Fiori and Marigo (1962) prepared anti-Hb-A and anti-Hb-F which were species-specific for human blood and recommended their use in forensic bloodstain analysis. The method was also described by Fiori (1962) in his review.

Adult bloodstains react only with anti-Hb-A, while fetal, or newborn bloodstains react with both antisera. Stains of human body fluids other than blood may react with anti-human serum serum, but will not react with the anti-Hb sera. Hematin does not react with the anti-Hb sera, so that with old bloodstains other methods of identification must be used.

Mori (1967) prepared an anti-human Hb in goats and studied its reactivity and cross-reactivity with human and animal hemoglobins by immunodiffusion and immunoelectrophoresis. At least four precipitin lines could be observed in the reaction between crude human Hb and its antiserum. The antiserum cross-reacted with monkey, horse and dog hemoglobin as well. Absorption with monkey hemoglobin rendered the antiserum human-specific, but it was clear that human Hb seemed to share some antigenic determinants in common with monkey and several other animal hemoglobins. Ohya prepared antisera to human Hb (1970a) as well as to non-hemoglobin red cell proteins (1970b) in rabbits. The anti-Hb was found to cross react with monkey, dog and horse hemoglobin preparations. It could be shown that the cross reactivity was due not to hemoglobin, but to carbonic anhydrase in the non-hemoglobin red cell protein. The anti-human non-hemoglobin red cell protein sera readily cross reacted with many animal lysates, and it was shown that the cross-reacting protein was carbonic anhydrase. The carbonic anhydrases of humans and animals, therefore, do have antigenic determinants in common. If these are not rigorously excluded from the "crude hemoglobin" preparations used for immunization, the "anti-Hb" thus raised will contain non-species-specific anti-carbonic anhydrase antibodies as well. Ohya cautioned that great care should be exercised in using anti-Hb sera for confirmation of species of origin. Fukae *et al.* (1976) prepared antisera in rabbits against carefully purified human Hb-A and Hb-F. The antisera were then absorbed with human non-hemoglobin red cell proteins to render them species-specific. The anti-Hb-A was shown to contain approximately equal amounts of anti-α-chain and anti-β-chain antibodies, while the anti-Hb-F contained a preponderance of anti-γ-chain antibodies. This observation accounted for the superior antigenicity of Hb-F in comparison with Hb-A.

Baxter and Rees (1974a) tested a commercial anti-human Hb. They found that if the antiserum was used undiluted, it showed a titer of 1:8000 against human blood, 1:2000 against baboon blood, 1:64 against human serum and 1:16 against human semen and saliva. The antiserum could, therefore, be rendered specific for blood by moderate dilution, and further dilution would abolish the cross reaction with baboon blood (see section 16.8). These same investigators (1974b) evaluated commercial anti-human Hb-F serum in combination with anti-Hb-A for the discrimination of Hb-A and Hb-F in medico-legal cases. The anti-Hb-A reacted with cord blood, even at blood dilutions of 1:6400, but the anti-Hb-F did not cross-react with adult blood at blood dilutions as low as 1:100. Baxter and Rees recommended this method in appropriated cases (e.g. infanticide), but cautioned that there are adults whose cells may possess Hb-F in small, residual amounts, or in larger amounts because of pathological conditions. This fact must be taken into account in interpreting the results of precipitin tests using these antisera.

The use of anti-Hb sera in bloodstain detection and assessment is, of course, very closely related to the use of antiserum sera for species of origin determination. The latter is discussed in great detail in a later section (section 16.1). The advantage of anti-Hb sera is obviously that it may serve to combine the identification of blood with the determination of species of origin into a single test. This point was made emphatically by De Forest and Lee (1977). They prepared a high titer anti-human Hb and tested its applicability in medico-legal blood identification. The use of the reagent was highly recommended.

The cross-reactivity of anti-Hb antisera among different species is of theoretical interest, as well as being of considerable practical importance. Immunological relationships between the same protein(s) of different species can be used to draw conclusions about phylogeny, and various evolutionary relationships between species. This subject will not be discussed here with respect to hemoglobin. It will be discussed in somewhat more detail in the sections dealing with determination of species of origin (sections 16.8 and 16.9). The relationship between the structure and evolution of proteins and their immunogenicity forms the basis for the application of immunological techniques to identification and species determination.

7.2 Chromatographic Methods

In 1957, Fiori first suggested the use of paper chromatography for the identification of blood by separating and locating hemoglobin and/or its derivatives. Whatman No. 1 filter paper was employed, using a solvent system of 2,4-lutidine:water: :2:1, in the presence of 20–30 mℓ concentrated ammonia. Spots were detected with alcoholic benzidine (pH 4.4–4.6 with acetic acid) spray reagent. R_f values were of the order of 0.56–0.57 but were highly temperature dependent, and hematoporphyrin could be detected by its fluorescence under 366 mn light prior to spraying. Using this method, 5 nℓ of blood could be detected (corresponding to about 1 μg Hb), though best results were obtained with 30 nℓ of blood (Fiori, 1957). The disadvantages of this system were the relatively long development time required (12-14 hrs), and the fact that pre-saturation was required. In 1961, Fiori reported a modified procedure which was considerably faster. The solvent system employed was methanol:acetic acid:water : :90:3:7, Whatman No. 1 filter paper was again used, and chromatography was carried out in the ascending direction. In this system the solvent front migrated about 12 cm in 1–2 hrs and pre-saturation was not required. After a run, the paper was dried in a 100° oven to inactivate vegetable peroxidases. The chromatogram was then examined under UV light to detect fluorescent materials which differed from hematin compounds. Examination at two wavelengths, 253.7 nm and 366 nm, was recommended. Hematoporphyrin could be identified at this stage. A two-step visualization reaction was used, the first consisting of spraying with 1% (w/v) benzidine in 96% alcohol, acidified with acetic acid. Spots which developed at this stage represented chemical oxidants. The second step was the spraying of the paper with 3% H_2O_2 solution to develop the hematin compound spots. This procedure was also described by Fiori in his review in 1962. In running unknown samples, it was recommended that several dilutions of extract be run in the system in a preliminary chromatogram to determine the optimal concentration of extract to be used. A second run was then made using the optimal concentration of extract, and the proper controls. The R_f values for hematin compounds are about 0.70–0.71. Hb-A and Hb-F are not differentiated, nor are a number of animal hemoglobins. Most chemical oxidants have different R_f values. A few, such as $CuNO_3$, NiCl and $CuSO_4$, have similar R_f's but are differentiated by the two-step spraying procedure. These also give different colored spots than does blood. Rust does not interfere. Hematoporphyrin formed long "comets" in this system, and Fiori recommended use of the earlier lutidine:water system if identification of this substance was required. A control spot of 10–20 μℓ of a 1:1000 dilution of whole human blood was run with each chromatogram. If oxidants are encountered, a second chromatogram sprayed with phenolphthalin H_2O_2 reagent, which might help to differentiate them, was recommended. This procedure, Fiori said, amounted to a method for rendering the benzidine test considerably more specific than it would be if it is carried out directly; but he suggested, nevertheless, that another, equally sensitive and specific test for blood be run in parallel on case materials. An anti-Hb precipitin test in agar gels was thought to be the best choice.

Farago (1966) reported a thin-layer chromatographic method for the identification of hematin compounds from bloodstains. Kieselguhr 250 μ TLC plates were employed with a solvent system of methanol:acetic acid:water:90:3:7. 5 to 10 μℓ of water or saline bloodstain extract was spotted on the plate, with 10 μℓ of 1% whole blood as control. At 20–24°, the plates were developed for 25 min, dried at 100° and sprayed with benzidine reagent and H_2O_2 in two successive steps. The R_f of the hematin compounds was 0.79, and the method could detect 3–4 μg blood.

There were many other studies on the paper chromatography of hemoglobins prior to Fiori's (1957a) paper, but they were designed to gather information about various human hemoglobin variants and not as forensic methods of identification (Andersch, 1953; Berlingozzi *et al.*, 1953a and 1953b; Kruh *et al.*, 1952; Penati *et al.*, 1954; Sansone and Usmano, 1950a and 1950b; Schapira *et al.*, 1953; and others). There have also been studies on the separation of human and animal hemoglobins, and on the applicability of the chromatographic patterns thus obtained to determination of species of origin of bloodstains. These are described in a subsequent section (section 17.3).

Chromatography has additionally been employed as a device for concentrating diluted bloodstains and samples, and for separating blood material from various debris. Frache (1939a, 1939b, 1941) used small alumina columns to concentrate the blood substances in diluted samples in preparation for subsequent identification and species of origin tests. In 1951, Kirk *et al.* proposed the use of paper chromatography for the separation of blood from debris. It was noted that blood may be encountered mixed with a variety of materials, such as soil, leaves, wood products, and so on. It may also be extremely diluted or diffuse on surfaces from washing, or present in small quantities which occupy large surface areas, as for instance on a car in a hit-and-run case. The technique which Kirk *et al.* described is applicable to many of these situations, and involves placing a wet piece of filter paper in contact with the moistened blood-containing material. Water was used as the solvent. The capillary action of the migrating solvent carries and concentrates the blood material in a particular region of the filter paper strip, where it may then be subjected to identification, species and blood grouping tests. Obviously, different situations call for different strategies in terms of the actual experimental set-up, and Kirk *et al.* discussed a number of these in the paper. Positive identification, species, and grouping tests could be gotten from very small amounts of material from irregular surfaces and/or which had been mixed with debris. Schaidt (1958) reported a very similar technique, which he had apparently devised independently. Fiori (1962) noted a clever modification of this approach. The filter paper strip was cut so that one end was pointed (V-shaped). The pointed end

was made the top, and the chromatography allowed to proceed in the vertical direction, thus concentrating the desired blood material in the point of the filter paper. The paper could then simply be inverted, so that the V-shaped point was the bottom, and the material eluted in a small volume into any desired container, essentially by descending chromatography.

Such methods could on occasion be very valuable. Hirose (1971a) studied the extractability of blood material from soil. Acetone, acidified with acetic acid, was found to be the best extraction medium. In a test where 0.1 mℓ blood in 8.9 mℓ water was mixed with 50 g soil, it was found that only 70% of the hemoglobin was recovered if extraction began within 5 min of mixing the blood with the soil. The recovery dropped to 22% after two days, and to 7% after 40 days. Soil apparently has absorptive properties for the blood proteins which accounted for this observation. Hirose (1971b) also found that a precipitin test with anti-human-serum or anti-human-Hb serum was positive up to 2–5 days after mixing of blood with soil, if the blood material was extracted with saline. It is possible that the chromatographic techniques could yield better results than extraction methods.

7.3 Electrophoretic Methods

Electrophoretic techniques have been used extensively for the investigation of human and animal hemoglobins, but seldom for the purpose of blood identification as such. Introna and Scudier (1960) used paper electrophoresis for the identification of blood. The technique is analogous to the paper chromatographic methods, and was said to be about as sensitive and very reliable. The method took advantage of two principles: (1) serum haptoglobin (Hp) is a hemoglobin-binding protein, the complex having a particular electrophoretic mobility being detectable with the usual peroxidase reagents, such as benzidine; (2) Met-Hb will form a complex with serum albumin, as will hematin, and these have about the same electrophoretic mobility as albumin in veronal buffers at pH 8.6. Thus, if bloodstain extracts are electrophoresed, the Hb-Hp complexes, which have the electrophoretic mobility of β_1-globulins, can be detected. It is known also (Allison, 1957) that if the Hb concentration exceeds the Hp binding capacity, the free hemoglobin is converted to met-Hb, which complexes with albumin. This complex migrates to the albumin position where it can be detected as a second benzidine-positive band. Hematin does not migrate, and older stains thus present a problem. If human serum is added, hematin can be induced to migrate to the Hb-Hp position, and if hematin is in excess, the second albumin band will appear as well. Human serum is unsuitable for this purpose, however, because it may contain small amounts of Hb. It was found, however, that addition of a small amount of a 10% human serum albumin solution to the hematin would give complex formation, and a benzidine positive band at the albumin position following electrophoresis. Haptoglobin exibits polymorphism in humans, but this aspect is not discussed here, and the method above apparently did not differentiate the iso-Hp proteins.

7.4 Heating Test

In 1938, Moody *et al.* reported the results of a series of experiments in which blood had been heated to very high temperatures and the residues examined. The experiments were begun to look into methods for identifying grossly contaminated, carbonized or incinerated blood samples. Blood specimens were heated to various temperatures, from 160° to 871°. It was found that the residues took on characteristic appearances at various temperatures. Dried blood melts at around 260°. The characteristics of the residues obtained after heating blood samples to 315°, 371°, 426°, 482° and 537.8° were described in detail. It was suggested that, in some cases, heating and examination of the residue formed could itself be used as a test for blood. A number of substances were subjected to similar treatment, and none gave residues resembling those of blood. It was further shown that none of the substances interfered with the formation of the characteristic blood residue if they were mixed with the blood during the heating. There were no differences observed in residue characteristics among a number of vertebrate bloods, and results could be obtained with starting quantities as small as 1 mg. The method, however, does not seem to have been put to use or tested further by other workers.

SECTION 8. IDENTIFICATION OF BLOOD FROM PARTICULAR SOURCES

8.1 Identification of Menstrual Blood

There sometimes arise occasions in which the identification of blood as being of menstrual origin is relevant to a particular case or situation. A number of investigators have devoted their attention to this problem at various times for over a century. A number of different methods have been suggested, and all attempt to take advantage of properties of, or cellular inclusions in, menstrual blood which are not characteristic of circulating blood.

8.1.1 Microscopical and histological methods

One of the oldest methods of identifying menstrual blood is based on the fact that it contains endometrial and vaginal epithelial cells, which may be identified microscopically.

As early as 1848, however, Casanti, in a paper concerned primarily with bloodstain species differentiation, said that he could discriminate menstrual from circulating blood. The method consisted of treating a dried blood mass with concentrated phosphoric acid and carefully studying the characteristics of the resulting residue. The method did not enjoy any popularity.

In 1858, a definitive paper by Robin appeared in which it was said that careful microscopical study of the bloodstain enabled identification of characteristic exfoliated cells in menstrual blood, and thereby, identification of menstrual blood in stains.

Ewell noted this method in 1887, but said that he did not regard the finding of such cells as conclusive proof that the bloodstain was of menstrual origin. Formad (1888) also mentioned identification of characteristic epithelial cells in bloodstains as a method of diagnosing menstrual blood. He went on to note that this type of blood showed an "acid reaction". The latter observation was no doubt based on the now familiar fact that vaginal mucus is acidic. The finding of characteristic epithelial cells as a means of differentiating menstrual blood was recommended by Vibert (1911) as well. Strassman (1921) used a differential staining technique, employing a number of histological stains, which resulted in the simultaneous staining of spermatozoa and vaginal epithelial cells in samples containing both.

It has been known for a long time that characteristic bacteria occupy the normal human vagina, and these too may be found in menstrual bloodstains. For many years, these bacteria, or vaginal flora, were known as Döderlein's bacilli, after Döderlein (1892). In addition, vaginal epithelial cells are known to be rich in glycogen. There is a definite relationship between the flora, the epithelial cell glycogen, and the levels of hormones which control the menstrual cycle. This matter is discussed somewhat more fully in a subsequent section (10.2.7).

Some authorities have recommended microscopical identification of the characteristic bacterial cells, along with the epithelial cells, for the identification of menstrual bloodstains (Kerr, 1954; Smith and Fiddes, 1955). Smith and Fiddes used a methylene blue-eosin stain for the purpose, and thought that the presence of the specific bacterial cells constituted an important criterion for concluding that the bloodstain was of menstrual origin.

Wiegemann (1912) in his dissertation first established that vaginal epithelial cells characteristically contain glycogen, which can readily be detected by its reaction with iodine. This observation was confirmed by the Belgian investigator Lenger in 1911, and forms the basis of one of the most widely used techniques for identifying menstrual blood, which contains the glycogen-rich cells.

Merkel (1924) took advantage of the high glycogen content of vaginal epithelial cells, in devising a method for identification of menstrual blood and vaginal secretions. He employed Lugol's iodine solution to stain the cellular glycogen. It may be noted that the use of iodine stain for glycogen detection dates to Claude Bernard's studies of diabetes (1877). Berg (1957a) recommended Merkel's technique, using a Lugol's solution of 0.2 g iodine and 0.3 g KI in 45 mℓ water. Vagnina (1955) pointed out that he regarded the presence of glycogen-containing epithelial cells as good evidence that the stain was of menstrual blood. In 1943, Mack introduced a very simple method for the iodine staining of vaginal smears, which he called the iodine vapor technique. This consisted very simply of placing a microscope slide containing a vaginal cell smear over a container of Lugol's iodine solution with the side containing the cells toward that solution. The iodine vapors from the solution stained the glycogen in the cells. Sakamoto (1957) used this method with success in examining menstrual bloodstains. Ota *et al.* (1965) soaked fibers containing menstrual blood in a 2% bicarbonate solution, then subjected the dried smear to the Mack technique. Detectability of glycogen-containing epithelial cells in menstrual bloodstains as a function of the conditions to which the stain had been exposed was studied as well. Ota *et al.* found that some conditions had an adverse effect, the highest detection rate being with stains in dry air, and the lowest rate being with stains which had been in running water. Stains in normally and highly humid air, and in still water, showed intermediate rates of detectability.

Menstrual bloodstains do not always give positive results with the glycogen detection techniques in epithelial cells.

Furuya and Inoue (1966a) said that their rates of detection were variable. Neumann (1949) found that 23 examples of menstrual bloodstains showed negative results out of 248 examined. False positive results are apparently also possible. Oral cavity epithelial cells, which are morphologically very similar to vaginal epithelium, may contain small amounts of glycogen, although because the amounts of glycogen in the two types of cells are so different, it is not expected that this would present a practical problem (Mueller, 1953; Sakamoto, 1957). Popielski (1949), after some fairly careful investigations came to the conclusion that the finding of glycogen-positive epithelial cells was not necessarily diagnostic for menstrual blood. He showed that morphologically similar cells could be obtained from the male urethra, and that substantial numbers of them (25 to 50 percent) contained glycogen. This fraction decreased if smears were taken at times of sexual excitation, but was even higher in the cells from post-gonorrheal urethral discharge. Cells from the female urethral orifice also showed the characteristic epithelial morphology, and many contained glycogen. Thus, if any of these types of cells were to be mixed with blood, the presence of glycogen-containing cells could easily be misinterpreted. Other types of blood than menstrual also contain glycogen-positive cells. Furuya and Inoue (1966a) obtained positive results in about 54% of 133 cases of bloodstains from menstrual blood, and blood shed during labor and abortion. They also showed (1966b) that blood shed during the puerperium contained glycogen-positive cells. The puerperium is the period of approximately 40 days from birth to the complete involution of the maternal uterus. Blood shed during this time is sometimes called lochial blood. Glycogen-containing epithelial cells were found in bloodstains obtained from recently delivered women up to puerperal day 17. Thus, if nothing at all is known of the history of a bloodstain submitted for examination, some care must be exercised in interpreting the finding of glycogen-containing epithelial cells.

8.1.2 Methods based on fibrinolytic properties

That menstrual blood does not clot has been known for many years. Luginbuhl and Picoff (1966) quoted Hunter as having written in 1794 that: "In healthy menstruation, the blood which is discharged does not coagulate, in the irregular or unhealthy it does". Formad (1888) also noted the property of incoagulability as being characteristic of menstrual blood. The property is of no direct value, of course, in bloodstains. Methods have been proposed, however, to take advantage of it indirectly. To help clarify the current thinking about the reasons for the incoagulability of menstrual blood, and the forensic methods of identification based upon this property, some background on the fibrinolytic enzyme system will be presented. The fibrinolytic system is actually quite complicated, still not completely understood, and its literature is immense. In 1959, now already some 20 years ago, Sherry *et al.*, in their review, said that studies of fibrinolytic activity had resulted in a literature ". . . so vast that it would be impossible to attempt a complete survey within the confines of this report". Even at that, the review ran 40 pages and dealt only with the then current aspects. 425 references were cited. The interested reader must, therefore, be referred to the specialized literature if more detailed information on this interesting subject is wanted (Hahn, 1974; Marder, 1968; McNicol and Douglas, 1976; Sherry *et al.*, 1959; Verstraete *et al.*, 1971).

Blood clotting is an extremely complex process. Many factors are now known to be involved, and the nomenclature and terminology were most complicated and confusing until the early 1960's. At that time, a universal roman numeral designation was adopted for use by the international community, largely through the results of an international committee chaired by Dr. I. S. Wright (MacFarlane, 1976). The last step in the clotting reaction involves the conversion of fibrinogen to fibrin. The fibrinolytic system, which is of interest to the present discussion, is quite obviously related to the clotting system, and in a way, represents its physiological converse. The fibrinolytic system acts to dissociate previously formed blood clots, or to prevent their formation in the first place. The relationship between these physiological systems is still not very well understood, but their functions clearly have enormous consequences for the health and well-being of the organism. There is a fairly widely held, but unproven, hypothesis that the fibrinolytic system is in a dynamic equilibrium with the clotting system.

In its barest essentials, the fibrinolytic system is represented in Fig. 8.1. Plasminogen is a heat-stable plasma protein, the molecular weight of which has been reported to be as low as 81,000 and as high as 143,000. Its activation to plasmin, the active fibrinolysin, is a proteolytic reaction, analogous to the activation of trypsinogen to trypsin, pepsinogen to pepsin, etc. A limited number of peptide bonds are split in the activation reaction, and the reaction is irreversible. Activation is effected by so-called plasminogen activators, which may function in one of three ways:

- Acting directly on plasminogen e.g. tissue activator, and urokinase from urine
- Proteolytic enzymes acting normally e.g. trypsin and plasmin itself
- By converting normally inert proactivator to activator e.g. streptokinase (SK) and tissue lysokinase

Natural activators occur in blood plasma and in tissues. The tissues contain a soluble activator as well as a much more insoluble one. The latter may be extracted with thiocynanate and has been called fibrinokinase. Activator may be found in milk, tears, saliva, bile and semen, but not in sweat. In addition, there are known to be naturally-occurring anti-plasmins and anti-activators. The active plasmin (fibrinolysin) is responsible for the proteolytic degradation of fibrin *in vivo*.

The incoagulability of menstrual blood is apparently due to the presence of activator. Menstrual discharge has no fibrin; it contains activator, plasmin, large amounts of proactivator, and no plasminogen (Albrechtsen, 1956). Using

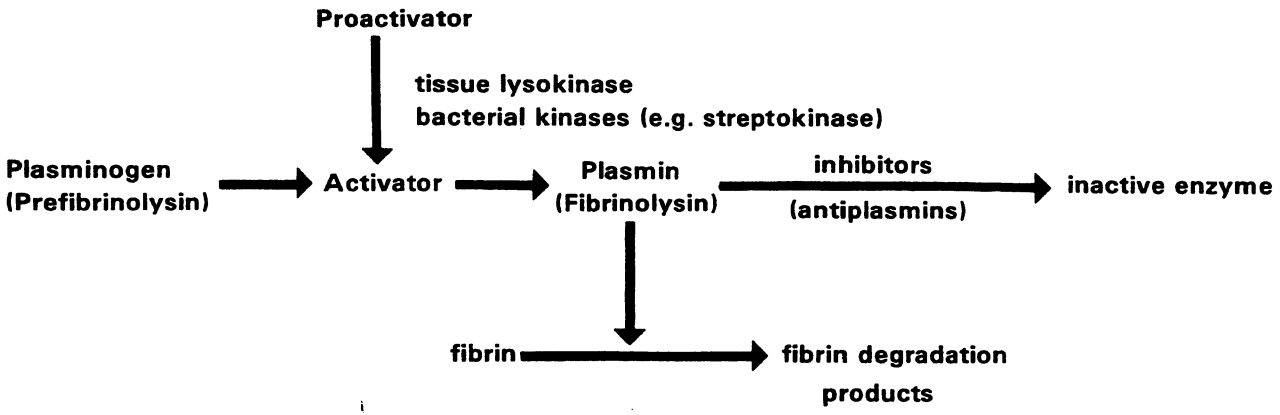


Figure 8.1 The Fibrinolytic System

histological techniques, Luginbuhl and Picoff (1966) found that proactivator was present throughout the endometrial stroma at all stages of the menstrual cycle, but that the presence of activator was confined to the superficial layers of tissue at around the time of menstruation. There was little activator present during other phases of the cycle, and virtually none at midcycle. Beller (1971) noted that the fibrinolytic breakdown products present in menstrual discharge showed the characteristics of fibrinogen degradation products, rather than of fibrin degradation products.

In 1949, Popielski had noted that the absence of fibrin threads in a bloodstain would indicate that it was not of menstrual origin, but said that this simple device could not be used if the stains had soaked into any type of absorbent substrate. Berg (1954) was apparently the first medico-legal investigator to take advantage of the fibrinolytic activity of menstrual blood for identification purposes. His method consisted of incubating suspected stain extract with fresh human fibrin at 37° for 24 hrs or so. At the end of the incubation period, a micro-Kjeldahl assay for nitrogen was carried out to assay for the fibrinolytic products. If the value was sufficiently different from a nonfibrinolytic control, menstrual blood could be diagnosed. This method was also discussed by Berg (1957b), and in this latter paper, he said that he had been using this method since 1952.

In 1959, Culliford reported an electrophoretic method based on the same principle as Berg's assay, but easier to carry out in terms of product detection. A strong extract of suspected stain was made, along with a similarly strong extract of capillary bloodstain as control. These samples (30 $\mu\ell$ minimum) were incubated with human fibrin at 37° for 24 hrs. 20 $\mu\ell$ of each sample was then applied to Whatman #1 filter paper and electrophoresed at 120–150 V for 16 hrs. The bridge buffer consisted of 33 g sodium barbital, 19.5 g sodium acetate, 205 mℓ of 0.1N HCl, all in a 3ℓ final final volume with water. The strips were dried at 105° and stained with azocarmine B. An extra band, due to the fibrinolytic products, could usually be seen in the menstrual blood samples. The presence of the band was noted to be variable, however, even in known menstrual bloodstains, and samples were encountered in which the band was absent altogether. Kamimura (1961) confirmed Culliford's observations using almost the same technique, except that he employed bromphenol blue as a protein stain. Positive results were obtained in stains up to 6 months old, and in putrefied bloodstains, but bloodstains that had been heated to 60° for 30 min, or that had been in water, gave negative results. Kamimura also noted that bloodstains from blood shed at parturition in induced abortions was indistinguishable from menstrual blood by this method.

In 1947, Permin first reported the use of what came to be called the fibrin plate method for the assay of fibrinolytic activity. The method was refined by subsequent workers (Astrup and Müllertz, 1952; and others), and can be used for the assay of any of the components of the fibrinolytic system. The method consists simply of preparing purified fibrinogen, and suspending a quantity of it in buffer in the presence of a small amount of thrombin. This mixture is placed in a Petri dish, and allowed to form a gel. The material whose fibrinolyitc activity is to be tested is placed on the surface of the plate, directly on the gel. After incubation, usually about 24 hrs at 37°, the digested fibrinogen can readily be seen. If semiquantitative results are wanted, the sample can be placed on the gel in such a way that the digested area is amenable to measurement. It has been common practice to use beef or ox plasma to prepare the purified fibrinogen. In 1962, Shiraishi utilized this method to demonstrate the fibrinolytic activity of menstrual blood, and to identify menstrual bloodstains. The fibrin plate was prepared by dissolving 1 mℓ of fibrinogen solution in 2 mℓ barbiturate buffer (0.1M, pH 7.8) and 20 $\mu\ell$ thrombin in a small (4.5 cm diameter) Petri dish, shaking gently for 3–5 sec to mix, and allowing formation of the white gel. The plate was then incubated 30 min at 37° before use. The fibrinogen was prepared from beef plasma, washed, and dissolved in the barbiturate buffer. In the first paper (1962a), Shiraishi

showed that menstrual blood serum contained large amounts of plasmin, it being able to dissolve the fibrin plate even at dilutions as high as 1:1000. The maximum dilution of menstrual blood serum which would dissolve the plate varied with the day of the menstrual period on which the sample was collected. The 1:1000 value occurred on the second day and was the highest, while the lowest dilution, 1:100, occurred in samples from Day 5. It could similarly be shown that circulating blood was always negative in the fibrin plate test unless streptokinase was added (see Fig. 8.1). In the presence of streptokinase, circulating blood serum would dissolve the fibrin plate at dilutions as high as 1:640,000. Similarly, streptokinase greatly increased the dilutions at which menstrual blood serum would dissolve the plate. The second paper (1962b) dealt with the identification of menstrual bloodstains. One thread from a 1 cm^2 stain was adequate to demonstrate fibrinolysis on the plate. The technique was extremely simple: the thread was placed onto the fibrin plate surface, the plate incubated, and examined. Stains that were kept two years at room temperature gave positive results, as did stains left a month under water and stains heated to 70–100° for an hour. Shiraishi said that neither blood shed at abortion or delivery, nor lochial blood gave a positive test.

Considerably less encouraging results were obtained by Schleyer (1963) in his study of the fibrin plate techique for menstrual blood identification. Only about half the 39 menstrual bloodstains examined gave positive results on the fibrin plate described by Astrup and Müllertz (1952). The presence of natural plasmin inhibitors was discussed as a possible reason for the false negative results. Schleyer was, therefore, less enthusiastic about the techique than Shiraishi had been, and stated that the results only had any meaning when they were positive. In 1973, Whitehead and Divall conducted experiments to determine the amounts of "soluble fibrinogen", that is, high MW fibrinogen breakdown products, in menstrual blood. They used a tanned red cell hemagglutination inhibition immunoassay (TRCHII) (Fox *et al.*, 1965; Merskey *et al.*, 1966; Hahn, 1974). It was found that menstrual blood contained significantly more soluble fibrinogen, expressed as a percentage of total protein, than did capillary blood. Mixtures of capillary blood and semen or capillary blood and vaginal secretions in stains were also higher in soluble fibrinogen than capillary blood alone, but not has high as menstrual blood stains. Whitehead and Divall continued this work (1974), conducting an immuno-electrophoretic study of the controlled degradation of fibrinogen and fibrin by plasmin, and its applicability to the identification of menstrual bloodstains. Their experiments were based on earlier work by Nussenzweig and Seligmann (1960), who carried out the first systematic immunoelectrophoretic study of fibrinogen and fibrin degradation by plasmin, and by Berglund (1962) and Dudek *et al.* (1970). The last-mentioned investigators used CM-Cellulose chromatography to separate the products. Marder (1968 and 1971) has also studied the problem extensively. The scheme for the degradation of fibrinogen which emerges from these studies is indicated in Fig. 8.2 (Marder, 1971).

Whitehead and Divall's experiments gave results in accord with the scheme in Fig. 8.2. The first stage of degradation (fibrinogen conversion to Fragment X, plus A, B and C) was reached after 4 min digestion. Fragments Y and D could be seen after 10 min of digestion, and after 45 min, Fragment Y had disappeared and only Fragments D and E were visible. The digestion of fibrin was studied as well, and followed a pattern quite similar to that of fibrinogen, except that the rate was slower. Fragments A, B and C do not react with the antiserum to fibrinogen, and thus are not detected in this immunoelectrophoretic system (Nussenzweig and Seligmann, 1960). 102 menstrual bloodstains were examined in the system. First stage degradation products were found in 56, and second stage products in 18. No third stage degradation patterns were observed, and 28 stains gave no precipitin arcs at all. The absence of precipitin arcs in 28 of the 102 stains, it was said, might be due to the low levels of degradation products in some stains. The predominance of Stage I and Stage II patterns, and the absence of Stage III ones, in the positive sample might be explained by the intervention of plasmin inhibitors present in endometrial tissue. 24 samples of circulating bloodstains were tested, and none gave precipitin arcs.

8.1.3 Immunological methods

A few attempts have been made to prepare specific antisera for the differentiation of menstrual blood. Sudo (1957) reported that he could prepare antisera to menstrual blood in rabbits or sheep, and by suitable absorptions, obtain an antiserum which detected menstrual blood in stains up to 2 years old. Domestic fowl were not suitable for making this antiserum, he said, because the anti-hemoglobin titer was too high. Harada (1960) made antisera to human and horse fibrinogen. The anti-human serum reacted with menstrual blood, and not with capillary blood, but it also reacted with the blood of victims of sudden death.

8.1.4 Methods based on menstrual blood toxicity

There have been a few reports that menstrual discharge contains various toxins, and a few investigators have indicated that these might be used as the basis for a test for the presence of menstrual blood in stains. In 1927, Böhmer noted that menstrual blood contained a substance which greatly retarded the growth of seedlings of *Lupinus mutabilis* plants (1927a). Neither cord blood nor cadaveric blood had this effect. He further indicated (1927b) that menstrual blood inhibited glucose fermentation by yeast cells, but cord serum showed this effect as well. Yamaguchi (1958) reported that menstrual blood serum contained a substance that was toxic to mice, and that the concentration of the toxin increased during successive days of the menstrual period. It does not appear that Yamaguchi was describing the same substance that had been reported earlier by Smith and Smith (1940 and 1944). The latter toxin resided not in

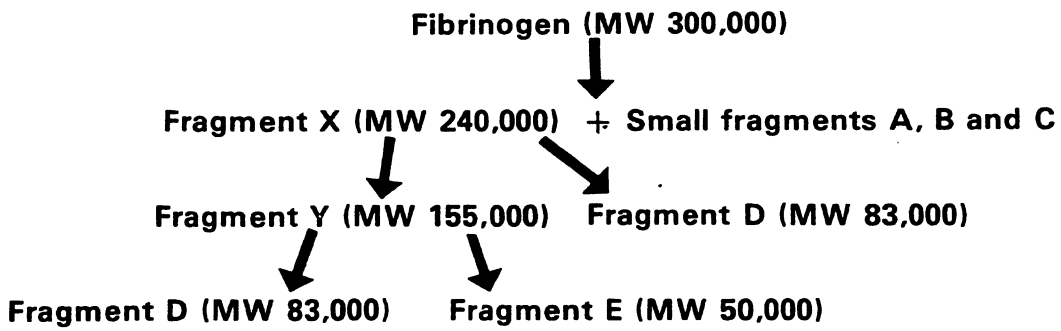
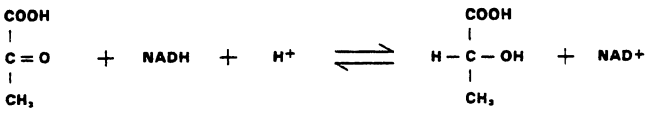


Figure 8.2 Fibrinogen Degradation by Plasmin (after Marder, 1971)

the menstrual blood, but in the endometrial cells and debris of the menstrual discharge. It had a toxic effect in rats, primarily female rats, that depended on the time of estrus of the rat at which the injections were given. There do not seem to have been many additional studies on these menstrual blood toxins. There has been very little interest in them as the basis of identification tests for menstrual blood, and hence no attempt has been made to review this leterature in detail.

8.1.5 Lactic dehydrogenase isoenzyme determination

Lactic dehydrogenase (or LDH) was the first enzyme that was recognized as exhibiting multiple molecular forms, or isoenzymes. Isoenzymes will be discussed in more detail in a subsequent unit. They comprise an extremely important category of genetic markers in blood and body fluids, and more will be said about LDH in the introductory discussion of the unit. LDH is systematically called L-lactate:NAD oxidoreductase (EC 1.1.1.27), and catalyzes the reversible conversion of pyruvic acid to lactic acid with NAD as cofactor:



LDH is an extremely important branchpoint enzyme in carbohydrate metabolism. The metabolic route followed at the level of pyruvate determines whether the cell will oxidize the starting material, glucose, to the level of lactate (or ethanol), or through the Krebs Cycle and the mitochondrial electron transport chain to the level of CO_2 and water. At pH 7, the equilibrium in the reaction as written above lies far to the right. The enzyme is a tetramer, consisting of four polypeptide chains. Two types of polypeptide chains can be present in the intact molecule. One of these, which is characteristic of muscle, is designated "M", while the other, which predominates in heart, is designated "H". Using M and H chains to form tetrameric molecules, the possible structures for LDH become: H_4, H_3M, H_2M_2, HM_3 and M_4. These five structurally different, but catalytically identical molecules constitute the five isoenzymes. LDH is found in most tissues, and all could theoretically contain the five isoenzymes. It is found, however, that different isoenzyme patterns tend to be characteristic of different tissues. In pathological states, the affected tissue sometimes synthesizes abnormal quantities of its characteristic isoenzymes, and these can be found circulating in plasma. Such altered patterns from the serum of patients can be of diagnostic value, and are used in clinical situations.

LDH isoenzymes differ sufficiently in charge at moderately alkaline pH that they are readily separated by electrophoresis on a variety of support media. At pH 8.6, the proteins migrate anodically, the fastest migrating isoenzyme often being designated LDH-1, the next-fastest LDH-2, and so forth. The most cathodic (slowest) band is called LDH-5. In 1971, Asano *et al.* reported that the LDH pattern of menstrual blood differed from that of peripheral blood, in that the LDH-4 and LDH-5 isoenzymes were markedly elevated. Electrophoretic separations were carried out on Cellogel at pH 8.6. A similar pattern, though less intense, was seen in blood shed at delivery and in cadaveric blood. Stains up to 2 weeks old could be diagnosed by this technique. Asano *et al.*(1972) thought that the source of the LDH-4 and LDH-5 isoenzymes was either the leucocytes or the endometrial tissue debris, or both. If densitometric methods were used, the increased intensity of the sum of the LDH-4 and LDH-5 was considered characteristic. These results were confirmed by Dixon and Gonsowski (1974), who carried out the separations on Sartorius cellulose acetate membranes in veronal buffer at pH 8.6.

In 1978, Stombaugh and Kearney conducted extensive studies on the use of elevated LDH-4 and LDH-5 as a means of discriminating peripheral, menstrual, cadaveric and other types of blood from one another. The LDH enzymes were separated on cellulose acetate membranes, stained for activity and the isoenzymes quantitated densitometrically. Both the range and the mean of LDH-4 and LDH-5 as a percentage of total LDH activity in peripheral, cord, cadaveric

and menstrual bloods were studied. There was considerable overlap in the ranges of activity seen in cord, cadaver and menstrual bloods, especially the latter two. Further, there was variation in the LDH-4 and LDH-5 activities in cadaver blood depending upon the cause of death. In cases of wounds with associated tissue damage, the levels tended to be higher. In four cuttings from the same menstrual bloodstain, the LDH-4 and LDH-5 activity percentages varied from 9.1 to 33.3%. Stombaugh and Kearney concluded that the use of LDH-4 and LDH-5 isoenzyme levels was not satisfactory in the discrimination of blood specimens from different sources.

8.2 Identification of Retroplacental Blood, Blood Shed at Parturition and the Forensic Diagnosis of Pregnancy in Bloodstains

As in the case of menstrual blood identification, there may be occasions on which it is desirable or necessary to try to identify bloodstains as being of retroplacental or lochial origin, of having come from a pregnant woman, or of having been shed at abortion. A number of methods and techniques have been proposed for these situations, most of which take advantage of hormones, or of various proteins present only during pregnancy and/or the puerperium. These kinds of blood are closely related, as are many of the substances thought to characterize them, so they are considered here all together.

8.2.1 Methods based on the pregnancy hormones

During pregnancy, the trophoblastic cells of the placenta secrete a hormone, similar to the pituitary gonadotropins, which is called human chorionic gonadotropin, or HCG. The hormone is believed to be physiologically important in maintaining the integrity of the conceptus during the first trimester of pregnancy, and may be found in detectable amounts in the serum and urine of pregnant women from very early in pregnancy through parturition.

One of the oldest tests for pregnancy is the so-called Aschheim-Zondek test. In this test, an ether extract is made of the urine of the pregnant woman, evaporated down, and redissolved in buffer. This material contains HCG, and if injected into immature female animals, will cause abnormal ovarian development to occur within a few days. The injected animals are sacrificed in the test, the ovaries examined at autopsy, and compared with a non-pregnant-urine-injected control. In 1932, Goroncy reported that, with minor modifications, the technique could be used to detect the presence of the hormone in extracts of blood or urine stains. Mice were used as test animals. He thought that the technique could be of value in forensic investigations on occasion, but did point out that he had sometimes observed false negatives.

In 1948, Berg employed a similar bio-assay in attempting to differentiate between blood shed at normal delivery (retroplacental) and blood shed at abortion. The assay was based on the experiments of Friedman (1932), who had noted that post-partum rabbits could be used as test animals in much the same way as immature animals. Berg obtained positive results from both types of blood, although the amounts of material required (an amount of stain containing the equivalent of 2–3 mℓ blood) were large by present-day standards. His results were apparently always positive with retroplacental blood, but variable in the case of abortion blood. The variability was a function of the progress of the pregnancy at the time of the abortion, and whether the blood being examined was shed prior to or after the expulsion of the fetus.

A more recent assay technique for HCG is hemagglutination inhibition. It is known that, under certain conditons, red cells can be sensitized with various proteins. If the cells are then incubated with specific antisera to the protein, agglutination occurs. If, however, the antiserum is incubated with an antigen containing solution prior to adding the sensitized test cells, some or all of the antibody will be bound, and subsequent agglutination will be correspondingly inhibited. This method will be discussed in more detail in a subsequent section (section 16.3). In the situation presently being discussed, red cells may be sensitized with HCG and used as test cells. An extract of the suspected stain, and a control extract, are incubated with a suitable titer of anti-HCG. Test cells are then added, and agglutination read after a suitable waiting period. Inhibition of agglutination, relative to the control, means that the test material reacted with some or all of the antisera, i.e., that it contained HCG. Semi-quantitation of the HCG may easily be had by carrying out the test on a series of doubling dilutions of the antiserum, i.e., a titration.

Abelli *et al*. (1964) utilized this procedure to try to diagnose bloodstains from terminated pregnancies. They employed a commercial test kit in which sensitized cells and antisera are provided. Stains were found to give positive reactions for short periods following pregnancy termination. In stains stored at 4°, positive results could be obtained in all samples up to 13 days old, and in some up to 18 days old. Stains stored at room temperature gave positive results only up to 8 days of age, and if kept at 60°, gave positive results only up to six days of aging. HCG is thus heat-labile in the stain. Tesar (1967), using a different kind of commercial test kit based on the identical principle, showed that positive results could be obtained from bloodstains up to 20 days old from pregnant women. He occasionally got positive results on stains as much as 3 months old. Göring *et al*. (1968) did a study of the effect of the presence of cleansing agents in textiles on the ability to detect HCG in bloodstains from pregnant women by the hemagglutination-inhibition technique. Stains deposited on fabrics that contained the washing agents were tested weekly for HCG. Two detergents were tested. In one case, detection was possible to 5 weeks, and in the other, to 7 weeks. The control stains gave positive results to 3 months of age. The agents tested were called "Fit" and "Persil", and were apparently marketed in Germany. Low-Beer and Lappas (1980) have recently described a crossed electroimmunodiffusion procedure for

detecting HCG in bloodstains representing 100–200 μl blood. The hormone could be detected in bloodstains from pregnant women from 45 days following the last menstrual period to parturition.

8.2.2 Methods based on pregnancy-associated proteins

The subject of pregnancy-associated proteins (as distinct from pregnancy-associated enzymes, which are considered in sections 8.2.3 and 8.2.4) was opened by Smithies in 1959. In some 10% of serum samples from recently delivered women, or women in late pregnancy, a protein band was observed in the haptoglobin region following starch gel electrophoresis. This observation was confirmed by Afonso and De Alvarez (1963) who noted that the protein was not equivalent to cystine aminopeptidase. They also pointed out that Giblett had independently observed the protein at about the same time as Smithies. Smithies called the region of the gel to which the protein migrated the "pregnancy zone", and the term "pregnancy zone protein" has persisted. The protein is especially characteristic of the 2nd and 3rd trimesters of pregnancy. Afonso and De Alvarez (1964) found the protein in 10% of sera from the first trimester, 69% of those from the 2nd, and more than 80% of those from the third trimester. The protein was found to be an α_2-globulin, and was not observed prior to the ninth week of gestation. It was not identical to ceruloplasmin, thyroxin-binding protein, nor transcortin.

Following the initial observations, the subject became somewhat complicated because, not only were additional pregnancy-associated proteins discovered, but many of the observations were independent, each group applying its own separate nomenclature. Some of the confusion has recently been cleared up, but the field is very active, and rapidly developing,

In 1966, Bundschuh reported a new antigen in serum, detected by variously absorbed horse immune antisera, which could be found in almost 78% of women, but in only about 22% of men. The antigen appeared to be inherited, but its expression was under the influence of sex hormones in some way. This antigen was named "Xh". MacLaren *et al.* (1966) found a similar protein, and called it Pa-1. Kueppers (1969) studied Xh further, noting that he had found it in 97% of women and 88% of men, using anti-female-serum serum absorbed with pooled male serum. He also found that the purified protein had a sedimentation coefficient of 12.2S and an isoelectric point of about 4.75. Dunston and Gershowitz (1973) studied the antigen and found that, in addition to the sex dependence of its expression, there was some age dependence of expression in females as well. They argued for the Xh designation, saying that the "Pa-1" designation implied more about the protein in terms of standard nomenclatures than was actually known.

In 1969, Margolis and Kenrick reported that "pregnancy zone protein" occurred not only in pregnant women, but also in those taking estrogenic oral contraceptives. They found that the protein had a MW of 450,000. Beckman *et al.* (1970) found that the protein was not present in cord blood, and they confirmed (1971) that women taking estrogenic oral contraceptives had the protein in their sera. Bohn (1971) reported that four proteins could be detected in pregnant serum by immunodiffusion with antisera made from placental extracts. One of these was identical to human placental lactogen (HPL). Two others were β_1-glycoproteins and one was an α_2-glycoprotein. One of the β_1-glycoproteins was pregnancy-specific, occurring in urine and in serum. The other β_1-glycoprotein and the α_2-glycoprotein could be found in non-pregnant serum but were elevated in pregnancy or in the presence of estrogenic oral contraceptives. The α_2-glycoprotein, Bohn said, was identical to pregnancy zone protein, and to Xh. In 1972, having found that the nonpregnancy-specific β_1-glycoprotein and α_2-glycoprotein were elevated in some disease states as well, he proposed to name them β_1-AP-glycoprotein and α_2-AP-glycoprotein, where "AP" stood for "acute phase". By 1973, Bohn was referring to the pregnancy-specific β-glycoprotein as "SP_1", while the non-pregnancy-specific β-glycoprotein was "SP_2" and the α_2-glycoprotein was "SP_3". "SP" in these designations stood for "Schwangerschaftsprotein", i.e., "pregnancy protein".

By 1975, the number of different names being applied to the α_2-glycoprotein by the above-mentioned and still other authors had reached such absurd proportions that a large group of workers in the field jointly agreed that this protein would henceforth be called "pregnancy associated α_2-glycoprotein" or "α_2-PAG" (Berne *et al.*, 1975). This protein is the same one that has been called "pregnancy zone protein", pregnancy-associated α_2-globulin (Hasukawa *et al.*, 1973), pregnancy-associated globulin (Horne *et at.*, 1973), alpha-2-pregnoglobulin (Berne, 1973), pregnancy-associated α-macroglobulin (Stimson and Eubank-Scott, 1972), and has been described additionally by Than *et al.* (1974) and by von Schoultz and Stigbrand (1974). The MW has been reported to be as low as 360,000 (von Schoultz and Stigbrand, 1974; Bohn and Winckler, 1976), and as high as 760,000 (Than *et al.*, 1974).

In 1972, Gall and Halbert first reported finding four antigens in pregnancy serum by immunodiffusion using anti-pregnancy-plasma serum, exhaustively absorbed with non-pregnant plasma. These were soon named "pregnancy-associated plasma proteins A, B, C and D", or PAPP-A, -B, -C and -D (Lin *et al.*, 1973). B and C were β-globulins while A and D were α_2-globulins. The MW of A, C and D were determined to be 750,000, 110,000 and 20,000, respectively. It became clear almost immediately that PAPP-D was human placental lactogen (HPL), and that PAPP-C was identical to Bohn's SP_1 protein (Lin *et al.*, 1974a, 1974b). None of the PAPP's were equivalent to the pregnancy associated α_2-glycoprotein (Lin and Halbert, 1975), and the amounts present in the placenta were shown to be, from greatest to least, D (or HPL) > B> C> A (Lin *et al.*, 1976). PAPP-A and PAPP-C, as well as HPL, are probably synthesized by the placental trophoblast cells (Lin and Halbert, 1976). Lin *et al.* (1976) showed, finally, that PAPP-B and PAPP-D (i.e. HPL) disappear within a day of delivery.

The level of PAPP-A drops rapidly within a few days of delivery and becomes undetectable at 4–6 weeks postpartum. PAPP-C levels fall rapidly too, being undetectable at 3–4 weeks postpartum.

In summary, the proteins which have had more than one name, but which are identical, are: (1) pregnancy-associated α_2-glycoprotein, with many former names; (2) PAPP-D = HPL; (3) PAPP-C = SP_1. PAPP-A is apparently pregnancy specific, and is not immunologically identical to other known pregnancy-associated proteins. SP_2, a β-glycoprotein, remains unique, though not pregnancy-specific. Curiously, Than *et al.* (1974) reported a MW of 760,000 for their protein, which they said was identical to SP_3, i.e., α_2-PAG. All other reports of the MW of α_2-PAG have been in the neighborhood of 500,000 except that Bohn and Winckler (1976) reported 360,000 for the purified protein. The 760,000 value of Than *et al.* (1974) is very close to the MW of 750,000 reported for PAPP-A although this could certainly be a coincidence. Lin *et al.* (1974b) insisted that PAPP-A is not equivalent to any of the SP proteins. Towler *et al.* (1976) showed that the levels of HPL and of the specific β_1-glycoprotein (probably equivalent to SP_1) correlate well with gestation stage. These, as well as perhaps PAPP-A, seem to offer the best prospects for application to the forensic diagnosis of pregnancy in bloodstains.

8.2.3. Methods based on leucine aminopeptidase and cystine aminopeptidase

Leucine aminopeptidases (EC 3.4.1.1) are α-aminopeptide amino acid hydrolases, which hydrolyze L-peptides, splitting off N-terminal leucine residues which have a free α-amino group. The enzymes hydrolyze a fairly broad range of substrates, and may also show esterase activity as well. Cystine aminopeptidases are very similar enzymes, except that, as the name implies, they prefer substrates having N-terminal cystine residues. These enzymes have usually been assayed using artificial substrates, L-leu-β-naphthylamide for leucine aminopeptidase, and L-cys-S-S-cys-β-naphthylamide for cystine aminopeptidase. The cystine aminopeptidase (hereinafter, CAP) enzymes do show leucine aminopeptidase (hereinafter, LAP) activity, but LAP does not hydrolyze the cystine substrate.

In 1961, Page *et al.*, using vertical starch gel electrophoresis, noted that LAP could be detected in all sera, but that two CAP enzymes in serum, CAP_1 and CAP_2, were characteristic of pregnancy. CAP was not found in nonpregnant, nor in fetal serum. The CAP is believed to function physiologically as an oxytocinase. Oxytocin is a hormone, elaborated by the mammalian neurohypophysis. A cystine-containing nonapeptide, its function is to elicit smooth muscle contraction, as of the uterus during birth, and the ejection of milk in lactating females. It is very closely related structurally to another neurohypophyseal hormone called vasopressin. The various reports of "LAP isoenzymes" in pregnancy, in addition to the enzyme found in all normal sera, may actually represent reports of the apparently pregnancy-specific CAP enzymes (Rowlessar *et al.*, 1961; Smith and Rutenberg, 1963). This is partucularly a possibility in the studies in which CAP was not separately assayed, because, as noted above, CAP possesses LAP activity. The serum LAP pattern is also altered in various pathological states, especially in hepatic and biliary diseases (Kowlessar *et al.*, 1961). Robinson *et al.* (1966) noted that the CAP enzymes were found only in pregnant serum, and not in the sera of non-pregnant women, even those on estrogenic oral contraceptive therapy.

In 1970, Gladkikh reported that bloodstains from pregnant or puerperal women could be discriminated on the basis of an additional, slow LAP band following electrophoresis. It seems probable that the slow band represented one of the CAP enzymes. The band could be seen in bloodstain extracts from women, from the 8th to 10th week of gestation until about 30 days postpartum. The enzyme could be detected in stains up to 50 days old. The slow band was not seen in bloodstains from men or from non-pregnant women. Some fetal sera, but not bloodstain extracts from fetal blood, showed a similar but qualitatively different slow band. Oya and Asano (1971) reported very similar results after electrophoresis on Oxoid cellulose acetate membranes, noting that the enzyme appeared in the last half of pregnancy, and also characterized retroplacental blood. In 1974, Oya enlarged his studies, noting that the resolution of the usual and pregnancy-specific LAP enzymes was not terribly good on cellulose acetate membranes, but that L-methionine inhibited the usual serum enzyme, but not the pregnancy-specific one, with L-leu-β-naphthylamide as substrate. By running paired samples, and staining with and without L-met, the bands could be resolved. Oya *et al.* (1975a) employed polyacrylamide disc gel electrophoresis to examine these enzymes in serum and in placental extracts. In this study, a CAP assay was incorporated, and it was clear that the two slower bands had CAP activity as well as LAP activity, while the fastest band had only LAP activity. The fast LAP band was the enzyme found in all normal sera, was heat-stable and L-met inhibitable. The CAP bands were heat-labile, not inhibited by L-met, and seemed to originate in the placental lysosomes. The bands were detectable in bloodstains after about the 4th month of pregnancy, and in stains up to about 2 weeks old (Oya *et al.*, 1975b). It was recommended that a phosphocellulose column be employed to remove the excess hemoglobin when examining bloodstains for CAP enzymes.

8.2.4 Method based on alkaline phosphatase

Alkaline phosphatases (EC 3.1.3.1) are widely occurring enzymes with broad substrate specificities. They catalyze the hydrolysis of orthophosphoric monoesters at alkaline pH optima, and are systematically named orthophosphoric monoester phosphohydrolases (alkaline optimum). Serum contains a number of alkaline phosphatase enzymes derived from different tissues, including liver, intestine, bone, and in pregnancy, placenta. Some aspects of the alkaline phosphatase enzymes are not pertinent to the present discussion,

and are not taken up here. These include the relationship between intestinal enzyme expression and the ABO blood groups and secretor loci (Schreffler, 1965; Beckman and Zoschke, 1969), and the fact that placental alkaline phosphatase exhibits polymorphism in human beings (Beckman and Beckman, 1968; Beckman, 1970).

That plasma contains alkaline phosphatase activity has been known since the work of Kay (1930a, 1930b). Fishman and Ghosh (1967) said that the French investigator, Coryn, first noted in 1934 that the activity of the enzyme is elevated in pregnancy. The enzyme in maternal circulation during pregnancy was shown to differ from the other alkaline phosphatases in its immunological properties, K_m for various substrates, electrophoretic mobility, inhibition by L-phenylalanine and heat stability (Posen *et al.*, 1969). By 1967, it had become clear that the heat-stable alkaline phosphatase of pregnancy plasma was of placental origin (Fishman and Ghosh, 1967; Posen, 1967). The placental alkaline phosphatase is stable to at least 55° for 2 hours, is almost 90% inhibited by 10 mM L-phe, has a pH optimum of 10.6 in 18 mM phenylphosphate and a K_m of 0.51 for phenylphosphate (Ghosh, 1969).

In 1973, Oya *et al.* showed that the heat stable alkaline phosphatase could be detected in bloodstains from the blood of women in the latter half of pregnancy, the blood shed at delivery, or puerperal blood. The assay of total alkaline phosphatase and heat-stable alkaline phosphatase (that which survived 56° for 30 min), using p-nitrophenylphosphate as substrate, was recommended for the medico-legal diagnosis of pregnancy from bloodstains. Stafunsky and Oepen (1977) recommended a slightly modified, but similar procedure. Results could be obtained in bloodstains stored up to 19 months. A disadvantage of the method is the relatively large amounts of sample required. Stafunsky and Oepen extracted stains from 1 to 3 cm^2 in size. Fishman *et al.* (1972) said that the level of the placental enzyme increased exponentially in maternal serum as a function of gestation time. Thus the amount of enzyme to be expected will depend on the progress of the pregnancy. In bloodstains, the age of the stain is probably inversely related to the amount of active enzyme as well. Older stains, or stains from persons whose pregnancies have not progressed very far, or a combination of these circumstances, might, therefore, require prohibitively large amounts of sample in order to obtain unequivocal results with the usual spectrophotometric assay technique.

There is another consideration, which is of enormous importance in the interpretation of results of heat-stable alkaline phosphatase assays in bloodstains of unknown origin. Fishman *et al.* (1968a, 1968b) first described an alkaline phosphatase in the serum of a patient named Regan with a bronchiogenic carcinoma. This enzyme in every way resembled the placental enzyme, and was found in serum, in primary tumor tissue and in its metastases. The enzyme in the plasma of patients with neoplastic disease came to be called the Regan isoenzyme, and is immunologically and biochemically indistinguishable from the heat-stable alkaline phosphatase of the placenta (Fishman, 1969). Fishman has recently (1974) reviewed this entire subject excellently.

8.3 Identification of Fetal and Blood from Children

8.3.1 Fetal hemoglobin

Fetal hemoglobin (Hb F) is best distinguished from the hemoglobin of adults (Hb A) by electrophoresis. The test for Hb F is probably the simplest way of diagnosing fetal, or early childhood, blood. Before an electrophoretic method had been worked out, it was common practice to discriminate Hb F from Hb A on the basis of their differential sensitivity to alkali denaturation. Hb A is quite alkali-labile, whereas Hb F is relatively resistant, the denaturation usually being detected spectrophotometrically. This method was applicable to bloodstains (Huntsman and Lehmann, 1962; Culliford, 1964; Watanabe, 1969). Pollack *et al.* (1958) successfully separated Hb A from Hb S (sickle-cell hemoglobin) in a medico-legal case in Massachusetts using paper electrophoresis, and suggested that it would be very desirable to have such a method for the separation of Hb A and Hb F as well. Wraxall provided such a method in 1972, which was simple, reliable and was performed on Sartorius cellulose acetate membranes. Wilkens and Oepen (1977a) fully confirmed the usefulness of Wraxall's method with bloodstains from 160 cord blood specimens on glass, wood, paper and textiles. The technique is also fully described by Culliford (1971). Immunological methods, using anti-Hb F, have been employed as well (Baxter and Rees, 1974b), and were fully discussed in Section 7.1. It must be noted that there are a few adults whose red cells contain abnormal amounts of fetal hemoglobin, and this fact must be kept in mind in interpreting the results of tests for Hb F.

8.3.2 Methods based on α_1-fetoprotein

In 1956, Bergstrand and Czar reported that they had found a high concentration of a protein in fetal serum that did not occur in maternal serum. This protein eventually became known as α_1-fetoprotein, or α-fetoprotein. Nishi and Hiraki (1971) reported that the protein had a MW of 64,600 and gave its amino acid composition. Masopust *et al.* (1971) said that the MW was 76,000, the isoelectric point 5.08, and that the protein had no associated sialic acid or lipid. The protein occurs normally in fetal serum, but is found in the serum of adult patients suffering from malignant tumors, especially hepatomas. It is clinically useful in the latter regard as a diagnostic tool (Abelev, 1971).

Patzelt *et al.* (1974) detected α-fetoprotein in bloodstains by what they referred to as "immunoelectroosmophoresis" (crossed-over electrophoresis), using a specific rabbit immune serum. The test was positive in bloodstains stored up to 3 months, and was carried out on 1% agar gels in veronal-sodium acetate buffer at pH 8.1. Thomsen *et al.* (1975) noted that there was a definite, inverse relationship between gestational age and the serum concentration of α-fetoprotein, and that this fact could be used as an aid to the

estimation of fetal age in medico-legal investigations. Wilkins and Oepen (1977b) showed that α-fetoprotein could be detected in fetal bloodstains up to 8 months old by crossed-over electrophoresis on Biotest cellulose acetate membranes. Stains on glass, wood, paper and textile substrates all gave positive results.

Pietrogrande *et al.* (1977) have described a quantitative immunoelectrophoresis (rocket electrophoresis) procedure for α-fetoprotein with a high sensitivity at serum concentrations of about 50 $\mu g/\ell$. Breborowicz and Majewski (1977) gave a direct radio-immunoassay procedure which detected 2 to 500 ng/mℓ of the protein in 20 $\mu\ell$ serum samples. Katsumata *et al.* (1979) used a commercially available RIA assay to detect alpha-fetoprotein in bloodstains. They said that this assay would detect from 21 to 320 ng per 9 mm^2 stain, and that the protein was detectable in stains on filter paper 1 month old. Tomasi (1977) has recently reviewed the subject of alpha-fetoprotein.

8.3.3 Miscellaneous methods

It is not intended to discuss the serum Gc (group specific component) polymorphism here, but it may be mentioned that Forster and Joachim (1968) reported that there were occasional differences in the Gc 2-1 immunoelectrophoretic patterns between the blood of adults and that of newborn or young children. The differences were such that the "variant" pattern could be mistaken for a Gc 1-1. They thought that antisera raised in goats were the best for discriminating the patterns. Occasionally, differences were observed in the Gc 2-2 patterns between adult and children's blood as well, but no differences were detected in the Gc 1-1 types.

In 1975, King and Whitehead reported that it was possible to identify with reasonable confidence the blood of persons over 15 years of age by virtue of natural antibodies that were present in sera. These antibodies reflected the individual's acquired immunities, and were expected, therefore, to be age-dependent. They tested for antibodies to *Mycobacterium tuberculosis, Vibrio cholerae, Candida albicans* and *Treponema pallidum,* using an indirect fluorescent antibody technique. The antigen was fixed on a slide, and incubated with bloodstain extract. If the serum of the bloodstain extract contained antibodies to the antigen being tested, binding would occur. The samples were then washed, and fluorescent-labelled antihuman IgG was added. This labelled antibody would bind to any IgG antibody which was bound to antigen being tested, and which would have to have been present in the bloodstain. After washing away excess antihuman IgG, the slides were examined in a fluorescence microscope. Good correlations with the age of the bloodstain donor were found for *M. tuberculosis* and *V. cholerae*, but not with *C. albicans* or *T. pallidum.*

SECTION 9. DETERMINATION OF THE AGE OF BLOODSTAINS

Aging bloodstains tend to undergo characteristic changes, and various attempts have been made to devise methods for estimating the elapsed time between the deposition of a bloodstain and its analysis in the laboratory. Although this information would be exceedingly valuable in some instances if it could be reliably obtained, there are many difficulties with all the methods. The major factor causing interpretive difficulty, regardless of the method chosen, is that a variety of different environmental factors, including heat, light, humidity, washing, putrefaction and the presence of contaminants, influence the rate at which the changes occur.

The majority of methods that have been suggested have to do with the transformations of hemoglobin into its derivatives, and the changes in color and solubility which accompany them. Tomellini (1907) devised a color chart with which unknown stain color could be compared to get an approximate estimate of stain age. Leers (1910) used solubility as a guide, noting that fresh stains dissolve readily in water and show an HbO_2 spectrum, while day old stains dissolve slowly and show the spectra of metHb. Older stains become water-insoluble but will dissolve in 2% KOH (week-old) or 33% KOH (month-old). Rauschke (1951) used water and 2% KOH solubility as guides to age. Minette (1928) tested for solubility in saliva, water and glycerine:water::1:4 at 37°, and correlated the solvation time with stain age. Bujan (1948) studied the Hb-metHb transformation in dried stains, noting that it could be complete within 2–3 hrs at ordinary temperatures, and even faster at higher temperatures. He tried to correlate the luminescence intensity of luminol in NaOH and Na_2CO_3 (in the presence of H_2O_2) with the age of the stain (see Section 6.7). Patterson (1960) showed that reflectance measurements carried out on experimental stains on filter paper, using a standardized color index, correlated with the stain age. The greatest rate of change in color occurred in the first few hours, decreased somewhat up to about 72 hours age, and was fairly gradual after that time. He noted that the role of environmental influences to which an unknown stain may have been subjected was a complicated matter, and that a variety of "standard curves" would have to be constructed if the method were to be employed in actual practice.

Schwarzacher (1930) proposed a method wherein stains were artificially aged under UV light. A third of the stain was irradiated for 30 min, the other ⅔ being covered. A second third was then irradiated along with the first third, the last third being non-irradiated. Color comparisons could then be made between the artificially aged and non-irradiated zones, and curves constructed as standards to be used with unknown stains. Rauschke (1951) said that the method was fairly precise for stains that were quite fresh, but that information about environmental influences would be needed.

Weinig (1954) applied a method to blood and semen stains that had been used by Metzger *et al.* (1931) to determine the age of inks. Bloodstains on paper and cloth can be examined in this way. The age of the stain is correlated with the progressive diffusion of Cl^- around the stain, which can be fixed as AgCl, and upon reduction forms a black border around the stain. Fiori (1962) gave the complete details of this method. A border forms around stains which are more than 2 months old, and its size increases in small increments as an approximate function of the age of the stain up to about 9 months. Humidity is a factor in this method, but aging results are independent of temperature and sunlight effects.

Schwarz (1936) determined catalase and "peroxidase" in bloodstains as a method of determining age. The "peroxidase" was determined using guaiacum, and was undoubtedly a determination of the amount of hemoglobin remaining in the stain which would catalyze the guaiacum bluing reaction with H_2O_2. In this sense, the study was very similar to that of Bujan (1948). The guaiacum blue color was said to vary inversely with stain age, as would be expected. Leucocytes possess peroxidase activity as well, and Undritz and Hegg (1959) carried out a microscopical study of peroxidases in the various leucocyte types as a function of bloodstain age (see Section 5.3).

Direct spectral determination of metHb is not very valuable in assessing stain age, because the hemoglobin is so rapidly converted to metHb (Bujan, 1948). Somewhat more involved spectral methods have been proposed, in which relationships between changes at several wavelengths have been studied. Funao and Maeda (1959) monitored the changes in optical density at 544 and 577 nm in the visible region, 345 nm in the near UV and 275 nm in the UV region in aging bloodstains. The visible bands decreased steadily up to 2 months of age. The UV bands decreased, and shifted to shorter wavelengths up to about 2 months of aging. The 345 nm band continued to shift for up to 3 years. These changes took place more rapidly in a lighted room than in the dark. In 1967, Kleinhauer *et al.* reported two methods of determining bloodstain age. In one, differential extractability in water as against ammonia solutions was measured, along with differential extractability in ammonia solution as against "transformation solution." The last mentioned consisted of 200 mg KCN and 200 mg potassium ferricyanide in 1ℓ H_2O, and effected the transformation of hemoglobin the stain to metHbCN. The differential extractabilities were expressed as ratios which they called "quotients," and these could be related to stain age. Both

quotients were higher in fetal bloodstains than in adult bloodstains. In the other method, the ratio of the OD of the 540 nm peak to the 500 nm trough in metHbCN, obtained by treatment with "transformation solution," was correlated to stain age up to 15 weeks. Kind *et al.* (1972) proposed a method whereby the spectrum of the stain was determined in the visible region from 490 to 610 nm. Vertical perpendicular lines were then dropped at wavelength values of 490, 560, 578 and 610 nm, and the points of intersection of the perpendiculars with the spectral trace at 490 and 610 connected by a straight line (as shown in Fig. 9.1). Points of

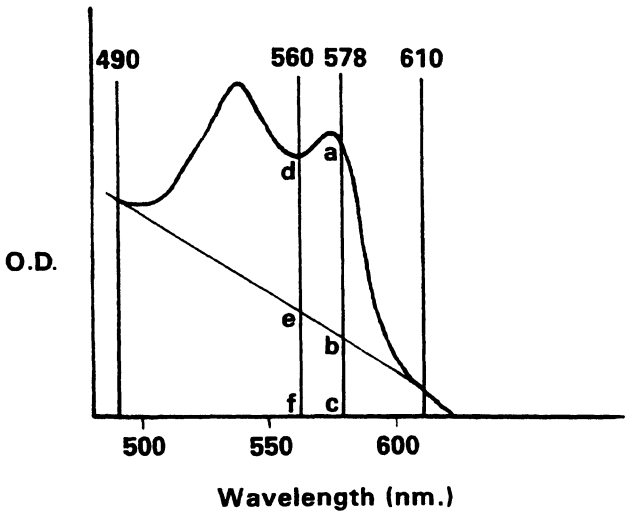


Figure 9.1 Scheme for Determination of α- ratio (after Kind et al., 1972)

intersection were identified by letters as shown, and a quantity called α could be calculated, according to:

$$\alpha = \frac{OD_{ac} - OD_{bc}}{OD_{df} - OD_{ef}}$$

where OD_{ac}, OD_{bc}, etc., represented the changes in optical density represented by line segments ab, bc, etc. The α ratio was found to decrease in some 200 bloodstains, which had been stored at room temperature in the dark for up to 8 years. The ratio underwent a change of from about 1.50 to about 0.80 over the course of some 10^5 hours of aging. There was considerable scatter in data points from samples of the same age group, however. An improved ratio, called α_s, was proposed by Kind and Watson (1973). α_s is calculated in precisely the same way as is α, except that two of the wavelengths at which the perpendiculars are dropped are changed as shown in Fig. 9.2. The spectra were determined in ammoniacal extracts of bloodstains, rather than directly in stains mounted in paraffin, as had been done in the 1972 experiments. The α_s ratio was seen to decrease in a consistent way for bloodstains aged up to 15 years, and there was less scatter in samples of the same age group. Köhler and Oepen (1977) reported a lengthy series of experiments on 85 blood-

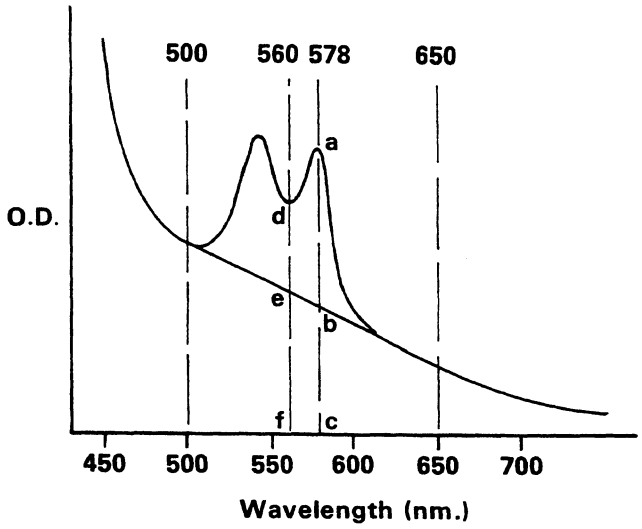


Figure 9.2 Scheme for Determination of α_s - ratio (after Kind and Watson, 1973)

stains on various substrates aged up to 5 years in which the methods of Kind *et al.* (1972) and of Kleinhauer *et al.* (1967) were tested. It was found that the variations of the parameters defined by the previous workers within an age group exceeded those between samples of different age groups. Köhler and Oepen concluded, therefore, that neither of these methods was suitable for reliable estimation of bloodstain age in practice.

Nuorteva (1974) pointed out that in decaying samples, which are found to be maggot-infested, an estimate of the age of the material could be made on the basis of knowledge of the length of the life cycle stages of the insects whose larvae were present.

Rajamannar (1977) looked at the serum protein profile by immunoelectrophoresis in stains as a function of their age, from 15 days to one year. The presence of precipitin arcs representing γ-, β_2M-, β_2C-, β_2B-, β_1-, α_1- and α_2-globulins and albumin was followed with time. A characteristic pattern of disappearance of various proteins at test points along the time line could be constructed. Albumin, α_1- and α_2-globulins were absent at 15 days of age. At 30 days, β_2M-globulin disappeared, with β_2C-globulin disappearing at 60 days, β_2B-globulin at 150 days, and β_1-globulin at 300 days. All the proteins were undetectable at 365 days of age. It should be pointed out that Sensabaugh *et al.* (1971) found albumin to be detectable by its immunological reaction in a dried blood sample eight years old.

The apparent disappearance of albumin in a comparatively fresh bloodstain in Rajamannar's (1977) experiments can be accounted for on the basis of a change in the electrophoretic mobility of albumin in aging bloodstains. Beginning almost immediately when blood dries, and progressing steadily with time, the amount of albumin which appears at the "albumin position" on electrophoretic support media de-

creases, while there is an apparent increase in the amount of "gamma globulin." Using monospecific precipitating sera, it can be shown that albumin does not denature; rather it migrates differently, and appears at the γ-globulin position. Quantitation of the amount of protein detectable with precipitating antisera in the "albumin" position and the gamma globulin "position" indicates that the ratio is approximately proportional to the age of the stain from which the extract was obtained. A preliminary account of this work has appeared (Lee and De Forest, 1978). Further studies are in progress in our laboratory, using two-dimensional immunoelectrophoresis (Laurell electrophoresis technique), to gather additional data about this interesting change in the albumin molecule (Lee, H.C., R. E. Gaensslen, B. Novitch and R. Fossett, in preparation). The alteration in electrophoretic mobility of albumin, and perhaps of other serum proteins, does not appear to be restricted to aging bloodstains. We have noticed that it can occur in aging serum samples as well, and others (e.g. Heftman *et al.*, 1971) have reported the same effect. Antisera specific for a particular serum protein must be used in examining bloodstain extracts because one cannot use the electrophoretic mobilities of these proteins in fresh serum as a guide to identity.

Shinomiya *et al.* (1978) used immunoelectrophoresis to estimate the age of bloodstains. They found that stain age could be correlated to the number of precipitin arcs detectable in stain extract following immunoelectrophoretic analysis.

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§ 1 *Hoppe-Seyler's Zeitschrift für Physiologische Chemie.* Began 1877, and published as *Zeitschrift für Physiologische Chemie* (*Z. Physiol. Chem.*) from volume 1 to volume 21.

§ 2 *Japanese Journal of Legal Medicine* (*Jpn. J. Leg. Med.*) has Japanese title *Nippon Hoigaku Zasshi*

§ 3 *Archiv für Experimentelle Pathologie und Pharmalkologie* (*Arch. Exp. Pathol. Parmakol.*), published 1873–1925, volumes 1–109 under above title. Volume 110–263 as *Naunyn-Schmiedeberg's Archiv für Experimentelle Pathologie und Pharmakologie,* often cited as *Naunyn-Schmiedeberg's Archiv.* Volume 264–271 as *Naunyn-Schmiedeberg's Archiv für Pharmakologie,* and from Volume 272 as *Naunyn-Schmiedeberg's Archives of Pharmacology.*

§ 4 *Archiv für Pathologische Anatomie und Physiologie und Klinische Medizin* (*Arch. Pathol. Anat. Physiol. Klin. Med.*), published 1847–1902, volumes 1–170, after which title changed to *Virchow's Archiv für Pathologische Anatomie und Physiologie und Klinische Medizin.* Often cited as *Virchow's Archiv,* or as *Arch. Path. Anat.*

§ 5 *Journal of the Kurume Med. Assoc.* (*J. Kurume Med. Assoc.*) has Japanese Title *Kurume Iggakai Zasshi*

§ 6 *Schweiz Wochenschrift für Chemie und Pharmacie* (*Schweiz Wochenschr. Chem. Pharm.*) has alternative title *Journal Suisse de Chemie et de Pharmacie.*

§ 7 *Vierteljahrschrift für Gerichtliche Medizin und öffentliches Sanitätswesen* (*Vierteljahrschr. Gerichtl. Med. Oeff. Sanitaetswes.*) began 1852, vol. 1 through new series vol. 15, 1871, published as *Vierteljahrschrift für gerichtliche und öffentliche Medizin.* Ended 1921, superceded by *Deutsche Zeitschrift für die gesamte gerichtliche Medizin.* Sometimes cited in the old literature as *Casper's Vierteljahrschrift.*

§ 8 *Annalen der Physik und Chemie* (*Ann. Phys. Chem.*) sometimes cited in the old literature as *Poggendorff's Annalen* (*Pogg. Annalen*).

§ 9 English edition of Nippon-no-ikai

§ 10 *Acta Criminologiae et Medicinae Legalis Japonica* (*Acta Crim. Med. Leg. Jpn.*) has Japanese title *Hanzaigaku Zasshi.* Began 1928, and had alternative German title *Archiv für gerichtliche Medizin und Kriminologie* from 1928–1944.

¶ Symbol indicates that a translation of the paper appears in Unit IX of this book

UNIT III
IDENTIFICATION OF BODY FLUIDS

SECTION 10. Identification of Semen and Vaginal Secretions

10.1 Introduction

Identification of seminal fluid is an old problem in legal medicine, although it was not perceived as such for most of recorded history. Florence (1896) gave an interesting review of the procedures used in examining alleged victims of sexual assault prior to the 19th century (see in Unit IX, Translations). Even though the spermatozoan was discovered in 1677 (see below), acceptance of the facts that the cells were unique to seminal fluid and that the sperm cell was the fertilizing factor in reproduction were not recognized for some time. Consequently, sperm cells were not sought as evidence of the presence of semen in sexual assault cases. It was apparently not until the early years of the 19th Century that any effort was made to try to identify seminal fluid in such cases at all. That no suitable methods were available for the purpose undoubtedly accounts for that situation in part, but perhaps more importantly, examinations of alleged victims were carried out by matrons, and other reputable members of the community, rather than by physicians or scientists.

The first systematic efforts to identify seminal fluid relied on chemical tests, just as was the case with the first efforts to identify blood in medico-legal investigations (see section 3). In 1826, Ollivier d'Angers and Barruel reported on a sexual assault case in which they had been consulted. The suspect claimed that the stains on his clothing were made by fat from uncooked animal meat. The experts examined stained areas of the clothing along with cloth controls, and compared them with respect to wettability with water, the nature and color of the aqueous extract, and the behavior of the extract with absolute alcohol. The extract had a "spermatic" odor, was alkaline, and its residue after drying was sticky. They concluded that the stain could not have been made by animal fat, and that it was a seminal stain.

In 1827, Orfila reported on a series of chemical tests for the identification of seminal fluid. He was one of the most respected medico-legalists of the time (see section 3). He had been consulted in a case in which a 13 year old girl had allegedly been molested. A physician saw the victim nine days later, and issued a report of his findings stating that he thought the victim had been sexually assaulted, this based on the fact that he had recovered semen from the vagina. Orfila objected to the findings on two grounds: first, he said that it was highly improbable that semen would persist in the vagina of the victim for nine days, especially since she was suffering from a mucus discharge; and second, he said that no systematic chemical methods had been used to insure that the identification of seminal fluid was correct. The tests he devised for identifying semen were based on the appearance of the stains, changes in color and consistency upon heating and immersion in water, odor emitted by the moistened stain, and the behavior of the aqueous extract toward a number of reagents and treatments (see in Unit IX, Translations). Seminal stains were compared by these criteria with a number of other types of vaginal discharges, and with nasal mucus and saliva stains. Orfila said in this memoir that, while he had had no difficulty in finding spermatozoa in fresh seminal samples, and even in an 18 year old sample of dried semen, with the microscope, he did not have any confidence in microscopical technique for seminal stains on fabrics. He said that it was very difficult, if not impossible, to find intact cells in stain extracts, and that the chemical procedures should always be employed.

In 1834, Chevallier reported on his examinations in a sexual assault case, the methods employed being essentially those which had been described by Orfila. He did not use a microscope. In 1839, Devergie wrote a paper on the signs of death by hanging (Devergie, 1839a). One of these signs was the finding of spermatozoa in the urethral canal of the victim, accomplished by microscopical examination. He noted that he had found sperm cells in 10 month old seminal stains, and thought that the confirmation of spermatozoa in a stain was a more certain criterion for diagnosing seminal stains than the chemical methods. Orfila (1839) disagreed with Devergie on a number of accounts, and Devergie (1839b) responded to him in print.

In 1837, Rattier had published a paper suggesting that sperm cells could be identified in seminal stain material, and recommending the procedure for medico-legal investigations. Rattier said that he asked the microscopist Charles Chevalier about previous work on the subject, and that he was told that Lebaillif had identified a seminal stain by the microscopical detection of spermatozoa some years before in the Contrafato case, but had not published it. Chevalier himself mentioned this fact indirectly in his book in 1839, and Florence (1896) said that Chevalier had said the same thing to Lassaigne in 1827.

In 1839, Bayard published his extensive paper on the use of the microscope in examining seminal stains for spermatozoa. Careful procedures were set forth for carrying out the technique, and the method began to be widely accepted not long afterwards. The earlier chemical methods were gradually abandoned by many workers. Attempts to find non-microscopical methods for the differentiation of body fluid stains persisted though. Lassaigne, in 1858, indicated a series of reagents which were said to give different sets of reactions with seminal stains and other kinds of stains which might resemble them. Brouardel reviewed the techniques for identifying seminal stains in 1879, emphasizing microscopy

as the principal technique. In the absence of spermatozoa, however, one could not conclude that semen was absent, he said, because it was known that a certain number of men are azoospermic. In 1880, Boutmy and Brouardel were charged by the Society of Legal Medicine with evaluation of a technique which had been proposed by Petel and Labiche for seminal stain identification. Petel and Labiche had noted that many body fluid and other stains took up color from the dye, carmine, but that these decolorized at different rates in a sodium carbonate solution. Seminal stains required 12 hours to decolorize, while other stains they had looked at needed much shorter times. Boutmy and Brouardel could not accept the test as sufficient unto itself for identification, but noted that it might be useful in providing additional evidence in some cases.

None of the non-morphological tests used during most of the 19th Century has survived. Most authorities began to rely upon sperm cell detection for seminal stain identification around 1840. The Florence test for seminal stain identification was introduced in 1896 (see section 10.4.1).

A number of the early papers cited in this introductory section may be read in their entirety in the translations set (Unit IX).

10.2 Detection and Identification of Spermatozoa

The famous Dutch scientist van Leeuwenhoek first described the morphology of the spermatozoan three centuries ago. In a letter written in November of 1677, and published in the Philosophical Transactions of the Royal Society of London, he credited a medical student from Leiden, named Ham, with having made the actual discovery (Kemper, 1976; Schierbeek, 1959).

> I have divers times examined the same matter [human semen] from a healthy man, not a sick man, not spoiled by keeping for a long time and not liquefied after the lapse of some time; but immediately after ejaculation before six beats of the pulse had intervened; and I have seen so great a number of living animals in it that sometimes more than a thousand were moving about in an amount of material of the size of a grain of sand. . . . These animalcules were smaller than the corpuscles which impart a red colour to blood; so that I judge a million of them would not equal in size a large grain of sand. Their bodies which were round, were blunt in front and ran to a point behind. They were furnished with a thin tail, about five or six times as long as the body, and with the thickness of about one twenty-fifth of the body. They moved forward, owing to the motion of their tails like that of a snake or an eel swimming in water; but in the somewhat thicker substance they would have to lash their tails eight or ten times before they could advance a hair's breadth.
>
> (from van Leeuwenhoek's 1677 letter; after Schierbeek, 1959.)

Identification of sperm cells in a stain is not the oldest method for the medico-legal identification of seminal stains, but may still be the most reliable one. The techniques are relatively simple, and the finding of spermatozoa constitutes incontrovertible proof that the stain was of seminal origin. For a long time, until around 1900, there were really no other reliable methods for identifying semen. Reese (1891) made this point in his text. Menger (1887) gave an account of a case in San Antonio, Texas, in which an older man was accused of raping a child. In some 30 slides prepared from the stains on the victim's underclothing, however, he could find no sperm cells, and said that he could not testify to the presence of semen.

10.2.1 Isolation and identification of spermatozoa from seminal stains

Dozens of different procedures have been recommended for examining seminal stains for spermatozoa. All may be classified into one of the following categories (Pollack, 1943): (1) separation of cells from the supporting material or substratum and microscopical identification; (2) partial or complete destruction of the supporting material; and (3) identification of sperm cells *in situ,* almost always with various biological stains (dyes).

Separation of the cells from the supporting material, and examination of the extract, is the oldest method (Bayard, 1839). Often, the stain has been soaked in one of a variety of solutions, including acetic, hydrochloric, and nitric acids, KOH, ammonia, saline, sodium carbonate or mercuric chloride, glycerin, alcohol or Paccini's solution (see section 5.3). Some authorities have called for soaking, followed by filtration (Bayard, 1839), or soaking, followed by centrifugation (Corin, 1907), or soaking, followed by squeezing (Schmidt, 1848; Koblanck, 1853). Hamlin (1883) said that the squeezing technique, originated by Schmidt, and advocated by Koblanck, was extremely destructive of the cells, and was to be avoided. Scraping of the stain has also been suggested. Hamlin (1883) scraped the dry stain, and then examined the moistened material. Others (e.g. Williams, 1937b) have moistened the stain first, and then scraped material from the surface of the stain for examination as was first done by Robin and Tardieu (1860). Ellis (1960) recommended collection of the cells from stain extracts on Millipore filters. One of the oldest soaking agents is ammonia (Bayard, 1839; Mezger, 1857), and Eungprabhanth (1973) said that cells could be eluted from cotton and filter paper better in the presence of ammonia than with saline alone. Kivela, in 1964, first suggested the use of sonic oscillation as a means of recovering sperm cells from seminal stains on fabrics. The material was first soaked in distilled water for at least 10 minutes, and then shaken briefly by hand. This procedure did not remove many cells, but did extract some extraneous material from the stain. The piece of fabric was then removed to another tube containing water, and exposed to the sonication bath for 1 minute. The cloth was removed, and the tube centrifuged. Most of the supernatant fluid was removed, and the pelleted material dried onto a slide for examination. Kivela said that the method was a considerable time saver,

although there was some breakage of the cells by the exposure to sonic oscillation. Marcinkowski and Przybylski (1966) suggested exposure of seminal stains to sonic oscillations as a means of detaching sperm cells from fabric substrata, and they credited Lukači as being the originator of the method. After sonication, the material was centrifuged and the pellet examined. Gluckman (1968) found the method to be quite successful, using 30 min. sonication times in saline. After centrifugation, the pelleted material was fixed and stained with hematoxylin-eosin. Hueske (1977) compared sonication with mechanical vibration in terms of sperm cell yields from seminal stains. Sonication yielded more cells than mechanical vibration, but more of them were decapitated. He tried a combination of the methods as well, exposing the stain first to mechanical vibration in saline, and then to sonication. Yields were good, but some cells were broken by the sonication step. It is to be noted that the exposure to sonic oscillations was not carried out directly in these studies, i.e., a sonic probe was not placed into the saline covering the stained material. Exposure was by means of a sonic cleaning bath, or similar device, into which tubes containing bits of stained material and saline had been placed.

Destruction of the fabric support has been carried out both mechanically and chemically. Teasing apart the threads in the fabric is the simplest technique, and is nearly always done after soaking. It can be done after staining of the material too, which may make the spermatozoa easier to visualize. Mezger (1857) used this technique after soaking the stains in ammonia water. Hamlin (1883) thought that this was the best method with thinner, more transparent fabric materials which could then be examined directly under the microscope. Hamlin used no biological dyes. Mueller (1926) examined material which had been soaked in pepsin and HCl, and the fabric threads then teased apart. Bohné and Dieckmann (1956) recommended a somewhat similar procedure using an extracting solution which was 0.01% in pancreatin followed by centrifugation. Optimal extraction time was 24 hours at 37° and the cells were not damaged by the procedure. Mezger (1857) recommended examining the center of the stain, in which it was expected to find the highest density of cells. Others have agreed with this view (Longuet, 1876; Mueller, 1926; Liethoff and Leithoff, 1965b). The supporting fabric may be completely destroyed using chemicals. Sulfuric acid has been commonly employed to destroy all the organic material except the sperm cells, which are relatively resistant. The sulfuric acid technique was first introduced by Vogel in 1882, and recommended by Grigorjew (1902) as well. Greene and Burd (1946) suggested a cuprammonium reagent for the destruction of cellulosic substrata, such as cotton. The reagent was prepared by precipitating CuOH from $CuSO_4$ with NH_4OH, filtering, washing the filtrate with water, and redissolving the material in NH_4OH. It was suggested that the reagent be freshly prepared. Kirk (1953) agreed that this was a good method in some cases.

Some workers have examined stains for spermatozoa *in situ* without destruction of the fabric or supporting material. Bayard (1839) used this technique with thin materials, but in most cases preferred filtration. The nature of the fabric, its thickness, texture, transparency, etc., must all be taken into account in deciding upon the applicability of such methods. Usually, biological stains have been employed in conjunction with these methods, as indeed they have been with the other methods. Hamlin (1883) was an exception, carrying out microscopical examinations without staining. Gabbi (1914) described a technique in which a thin layer of gum acacia may be applied to the stain on cloth or any other substratum, and then transferred to a slide. Cells are transferred with the gum acacia, and the slide may then be stained with Baecchi's acid fuchsin and methylene blue (see below). De Bernardi (1959) describes a technique for transferring spermatozoa from substrate to microscope slides utilizing "Scotch" cellophane tape.

A large variety of biological stains have been recommended for sperm cells, sometimes with prior fixation, and sometimes not. Roussin (1867) used iodine-KI staining to render sperm cells from seminal stains more visible under the microscope. Florence (1896) said that he thought Roussin was probably the first to use a stain for the visualization of spermatozoa. The stains employed appear to be largely a matter of personal preference. Some dyes and stains and their properties are given in Table 5.3. Longuet (1876) used an ammoniacal carmine solution for sperm cell staining. Carmine is a very old biological stain, its use dating to 1770 (Lillie, 1969). It is only slightly soluble in water and is used as an acid (e.g. aceto-carmine), or alkaline aqueous solution, or as an alcoholic solution. The active principle is carminic acid, an anthroquinone derivative. Weigert (1887) used carmine as a Gentian violet counter-stain for bacteria. Best (1906) noted that carmine in potassium carbonate solution stained glycogen, a fact which could be useful in examining vaginal swab smears for sperm cells. Baecchi (1909) originated a sperm cell staining mixture of methylene blue and acid fuchsin which was recommended by Strassman (1921). Tsunenari *et al.* (1971) said that methyl blue could be substituted for methylene blue in Baecchi's stain. The heads of the sperm stain red, while the tails and midpieces are blue. Methyl blue is a triaminotriphenylmethane derivative, structurally related to the fuchsin dyes, but not very similar structurally to methylene blue. Corin and Stockis (1908) used erythrosin in ammonia solution for staining cells after fixation. Fixation and staining could be carried out in one step using an erythrosin solution made up in ammoniacal potassium dichromate and sodium sulfate. Tsunenari *et al.* (1971) got good results with Corin and Stockis' method, as did Ponsold (1957). De Rechter (1914) noted that ammoniacal erythrosin was not very stable; he prepared the erythrosin solution in methanol, diluting it 1:1 with ammonia just prior to use. Erythrosin is a fluorescein dye, and is very closely related to eosin structurally, the only difference being that the four Br atoms of the latter are replaced by I atoms in the former (see Fig. 5.4). Florence (1896) noted that eosin was introduced by Prof. Renaut in Lyon in the 1870's, although its introduction was apparently attributed to Schmitter in 1883 by some writers. De Domincis (1909)

recommended the use of 0.01 g eosin in 6 mℓ ammonia for staining spermatozoa in smears. Nickolls (1956) said that hematoxylin-eosin stain worked well. Casarett (1953) recommended a one-solution stain for smears, consisting of 2 volumes 5% aniline blue, 1 volume 5% eosin B and 1 volume 1% phenol. Eosin B is a close relative of eosin, except that two of the Br atoms are replaced by $-NO_2$ groups. "Aniline blue" is apparently a mixture of methyl blue and water blue I (Lillie, 1969).

Mueller (1926) used May-Grünwald or iron-hematoxylin staining, following the pepsin-HCl soaking treatment of the fabric. May-Grünwald stain is equivalent to Jenner's stain. Alum hematoxylin, with eosin as counterstain, was suggested by Rentoul and Smith (1973). Raitzin (1928) used Giemsa stain after alcohol fixation of the smear. Holbert (1936) used gentian violet with a rose bengal counterstain. Paulsen and Varnek (1953) used a "carbol-gentian violet", or gentian violet in a phenol-glycerin solution. "Gentian violet" is a mixture like "methyl violet", and it is best to substitute crystal violet (see Fig. 6.5), a pure compound, in techniques calling for these stains (Lillie, 1969). Macaggi (1925) used an ammoniacal extraction solution, followed by a crystal violet-tannic acid staining mixture. The tannic acid is said to have a protective effect on the cells. Hankin (1904) placed seminal stain in a boiling solution containing 0.5% tannic acid and 1:1000 dil H_2SO_4. He then treated the stain with dilute ammonia and 2% KCN prior to staining with gentian violet. Williams (1937b) employed a seldom-used biological stain called wool black, with a methylene blue counterstain for spermatozoa. Heads stained a golden yellow, with tails and background remaining a gray color. It is not clear which of the several "wool black" dyes (Gurr, 1960) was actually used. Baima-Bollogne (1968) said that he had been able to identify cells in seminal material that had seeped into wood by means of the Feulgen reaction using basic fuchsin. The Feulgen technique stains nuclei, and could be used for stains on fabrics as well. Döllner (1913) used so-called Ruthenium Red stain for seminal spots. This staining solution is prepared from $RuCl_3$ dissolved in ammonia. Greene and Burd (1946) used a safranin stain, which could have been Safranin O, a mixture of dimethyl- and trimethylphenosafranins, or Methylene Violet. Rentoul and Smith (1973) noted that Papanicoleau stain could be used for examining smears. The staining procedure is somewhat involved, but the solutions required (Clark, 1973) are commercially available. Oppitz (1969) described a staining procedure using Nuclear Fast Red (calcium red), or "kernechtrot", with indigo carmine in saturated picric acid as counterstain. Material is eluted in saline and collected by centrifugation, or scraped off and saline-moistened. Heads are stained red, midpieces, pink to green, and tails green with this procedure. Stone (1972) described the procedure in detail, and provided a translation of some of Oppitz's original observations. A number of the biological stains mentioned in this section were discussed in section 5.3.

The material in section 10.2.1 was comprehensively and excellently reviewed by Pollack in 1943. The older literature was reviewed by Florence (1895 and 1896) and by Lecha-Marzo in 1907 and again in 1918. A complete review of the pre-1943 literature did not, therefore, seem warranted, and the discussion has been an attempt to cover major points. Pollack (1943) carried out a large series of experiments on the methods as well. He recommended moistening stains with water or alcohol, and teasing the fabric into fine fibers. Staining was carried out with erythrosin and iron hematoxylin, or with Giemsa stain. Alternatively, the material could be placed into concentrated H_2SO_4 at 50° and checked every hour for chemical destruction of the substratum. Still a third technique involved boiling a portion of the stain in water, and passing the material through a fixation process before embedding in paraffin, and sections being cut, stained and examined. Hankin (1904) had used boiling water as a fixation device for seminal stains. Gültingen (1961) compared the recovery of spermatozoa from seminal stains using the methods of Corin and Stockis (1908), Bohné and Dieckmann (1956) and the sulfuric acid technique of Pollack (1943). Best results were obtained with Pollack's method, relatively good results with Corin and Stockis' method, cells being recovered from an 11 year old seminal stain. Bohné and Dieckmann's method was not very satisfactory, failing to yield cells in a number of stains from 1 month up to 11 years old. Pollack noted especially the tendency of cells to adhere to the fine fibers of fabric. Failure to find large numbers of cells in a normal stain was explained by the frequent failure of the method used for separating them from the substratum to actually do so. Kirk (1953) made the same point. Walther (1967) studied the effect of washing of seminal stains on the subsequent detection by sperm cell identification and acid phosphatase methods. Even after an hour of washing in 20° detergent solution, sperm cells, or at least heads, could still sometimes be found after a careful search. Janssen and Kiesling (1967) noted that motile sperm survive only a short time in seminal stains. Only about 10% were motile after 5 mins. and all were non-motile in 50 min. There was some individual variation as well.

It may be mentioned that more sophisticated types of microscopy have been recommended in looking for spermatozoa. Mueller *et al.* (1966) got good results with phase contrast microscopy, as did Dérobert *et al.* (1966) with fluorescence microscopy using specific fluorescent staining agents. Cortner and Boudreau (1978) recently discussed the value of phase contrast and differential interference contrast microscopy in searching for sperm cells, and the situations in which one or the other of these might prove advantageous.

10.2 ? Survival of spermatozoa in the vagina

The question of the length of time that sperm cells survive in the vagina following ejaculation can sometimes be of importance. Demonstration of the presence of sperm cells in the vagina may not be adequate evidence in itself in a sexual assault case, if sufficient time has elapsed between sexual contact and examination to leave open the possibility that the sperm cells found are unrelated to the alleged assault.

The information available has come primarily from the fertility-sterility literature, and from data collected by medical examiners in cases or in controlled experiments. A number of factors appear to be involved in determining survival time. The number of sperm present in the ejaculate may have an effect. Shorter survival times have been reported if the initial count is low. There is some dependence on the cycle stage at which coitus occurs, and there is quite a bit of individual variation as well.

Menger (1887) mentioned a case in which sperm cells were recovered from a child victim 14 days after sexual contact had occurred. In living, sexually mature women, time estimates are generally considerably shorter. The manner of collecting the sample swab may make some difference to the conclusions, for it is known that sperm survive longer in the cervix than in the vagina *per se*. Sharpe (1963) said that motile sperm persist for 30 min and up to 6 hrs in the vagina, but may persist in the cervix from 7 hrs to over 5 days. Non-motile sperm may be found in the vagina from 7–12 hrs after coitus, much less often up to 24 hrs, and exceptionally as long as 3 to 4 days later. In the cervix, however nonmotile sperm may be found after 17 days. Sperm cells could be recovered in anal swabs up to 24 hrs following an episode of sodomy. Enos and Beyer (1977) said that rectal swabs could be sperm positive up to 20 hrs after attack. They also noted that in cases where oral sex has been reported by the victim, oral swabs must be examined. Sperm could be identified by Papanicolaou staining in oral swabs up to 6 hrs after the incident, and could survive the use of a toothbrush, mouthwash or the ingestion of various liquids. In 1978, Enos and Beyer said that the presence of spermatozoan heads in the rectum or anal area should be interpreted with great caution. They found heads on rectal or anal swabs in some cases where no history of anal sex was reported, and they thought that the findings were due to contamination from the vagina. Sperm identification in vaginal swabs was normally not a problem for up to 3 days following coitus. Nicholson (1965) studied 85 patients, and said that sperm survived in the cervix up to 8 days, and that motile sperm could be found sometimes even after six days. Rupp (1969) noted that studies on a series of 84 rape case samples indicated that there was an approximately equal chance of finding motile and nonmotile sperm within 8 hrs of coitus, and that nonmotile sperm were found in vaginal aspirates for up to 14 hrs after intercourse. Morrison (1972) thought that sperm cells survived longer following coitus in the first 14 days after menstruation. In 104 subjects studied, the ability to find sperm dropped markedly after 48 hours postcoitus. Sperm could be found in the vagina up to 9 days after intercourse which had occurred on the 5th postmenstrual day. In one case, sperm was found on a cervical smear 12 days after coitus which had taken place on the 8th day after menstruation. This case was exceptional, no other cervical smears being positive after 10 days following intercourse. In women who are pregnant, sperm could survive up to 7 days in the vagina. Morrison noted that, in rare instances, sperm could be found on cervical smears taken after the end of a menstrual period where coitus had occurred during menstruation. Georgiades and Schneider (1972) reported that motile sperm could be recovered from the cervical mucus up to about 8 days postcoitus, and that nonmotile sperm persisted for up to about 10½ days. Davies and Wilson (1974) studied the persistence of spermatozoa and of other seminal fluid constituents in the vagina following single coitus. Cells could ordinarily be identified on swabs for up to 3 days, less often up to 6 days. However, swabs with no cells could be found in some cases at 28 hours after coitus. Wallace-Haagens *et al.* (1975) studied the numbers and motility of sperm in 22 subjects throughout a complete menstrual cycle. Motility declined rapidly at about 12 hours postcoitus, and only 6% of the samples showed spermatozoa 48 hrs after intercourse. In most samples, the number of sperm was very small in the washings as compared with the number in a single ejaculate. Brown (1977) reported that studies on 22 subjects indicated that motile sperm may survive up to 9 hrs, while sperm could be found up to 72 hrs after intercourse. Breen *et al.* (1972) said that motile sperm survive up to 28 hrs in the vagina, while nonmotile sperm may be found up to 48 hours postcoitus. Evrard (1971) said that motile sperm could survive up to 96 hrs in the vagina. Duenhoelter *et al.* (1978) reported their findings in 288 cases of women examined because of alleged sexual assault. Almost all of them had been examined within 24 hours of the attack. Motile sperm were found in about a third of the cases seen within 6 hours; nonmotile sperm were identified in a larger percentage. The value for motile sperm decreased significantly for cases examined from 7 to 24 hours after the incident. Dahlke *et al.* (1977) reported results on 500 patients who had been examined in connection with alleged sexual assault. Semen was identified by the finding of sperm in about 60% of the cases. The longest known interval between the attack and the examination in a case where sperm was found was 48 hours.

Sperm apparently survive longer in the vaginas of women who are dead. Wilson (1974) reported a case in which sperm were recovered from a rape-murder victim 16 days after the incident. Although the environmental temperature was low for most of this time, it was not always below freezing (the body was outside, in a mountainous area). The conditions to which the body is subjected undoubtedly play a major role in determining sperm survival. Willott (1975) mentioned that sperm had been found in one of the Christie victims after she had been dead between 3 and 4 months.

These considerations have implications for the doctors who carry out the actual examinations of the victims. In most cases, the person carrying out the physical examination and the person examining the evidence collected are different, the latter having not much control over, and sometimes not much knowledge of, the actions of the former. While this situation is not the most desirable from a medico-legal standpoint, it persists in many places as a matter of practicality and/or lack of communication.

Much of what can be concluded from the detailed examination of the evidence is determined not only by what is

found, but by the manner in which it was collected. A number of authorities have addressed this question, recommending procedures that should be followed (Enos *et al.*, 1972; Vitullo, 1974; Paul, 1975; Enos and Beyer, 1975). Paul (1977) described special procedures that should be employed in the case of children who are victims of sexual assault. Paul (1975) made the point that contamination of the samples during the physical examination is entirely possible, and renders the evidence useless. An example would be accidental contamination of a vaginal swab with sperm traces from the perineum. Since there are differences in the time of survival of spermatozoa in the vagina as against the cervix, the manner in which the swab is taken, or washings collected, may make a difference to the interpretation of the findings. Pollack (1943) said that only findings from the vagina should be used to try to assess elapsed time since last coitus. He said that, although there may be occasional exceptions, one may expect to find sperm from about 30 min to 24 hours after intercourse in most situations. Most authorities have agreed that it would be desirable to have physicians familiar with medico-legal practice carry out the physical examinations of victims, but it is recognized that this is seldom practical.

Some authorities have noted that the finding of isolated parts of the sperm cell may suffice for seminal stain diagnosis, especially the finding of heads (Willott, 1975), but Hektoen and McNally (1923) and Pollack (1943) said that no conclusions should be drawn unless intact cells are found. Fibers, certain bacteria and molds could suggest tails, while yeast, certain Monilia or various other cells could suggest heads in a stain extract preparation.

10.2.3 Spermatozoan morphology—medico-legal implications

For a number of years there has been an interest in the relationship between variable spermatozoan morphology and male infertility. The subject was opened by Moench in 1927. Prior to his work, it was fairly generally accepted that male fertility had mainly to do with sperm count, usually expressed as the number of cells per mℓ semen. It was usually said that a sperm count of about 60×10^6/mℓ was the minimum for fertility. Moench, however, began to examine the cells themselves in detail, noting that there were quite a number of morphologically variant sperm cell types which could occur (1927a) and that studies of the numbers and kinds of these cells in particular individuals might provide an alternative explanation for some cases of infertility (1927b). In addition to establishing profiles for variant morphological types, measurements of sperm cell heads were made on a large number of cells, and this plotted as a function of the numbers of each size observed. The curves thus obtained were called biometric curves, and were fairly characteristic of individuals over the course of time. By 1934 it was clear that the morphological and biometric profiles were fairly characteristic of individuals. At least 300 cells had always to be examined, and up to 500 were often included. Moench (1934a) noted that the characteristic individual patterns were obtainable not only in smears prepared from fresh semen, but also in "reconstituted" semen, i.e. from dried material. He thought that this finding might have particular applicability in rape cases, and published a paper in the medico-legal literature (1934b) suggesting the possibilities. It may also be noted that his initial inclination for taking up the studies, the relationship of aberrant sperm morphology to male infertility, proved to be well founded (Moench, 1944 and 1955; Joel, 1953).

Williams (1937a) published a classification scheme for morphological variants of spermatozoa, and suggested (1937b) that individualization of the sample might be possible in some medico-legal cases. MacLeod took up seminal cytological studies in 1951, in connection with impaired male fertility (MacLeod, 1951; MacLeod and Gold, 1951). He confirmed that the seminal cytological profile exhibited individuality, and that there was a definite relationship between sperm quality and fertility (MacLeod and Gold, 1953). There was also a definite correlation between increased numbers of aberrant morphological types of sperm, and various kinds of stress (MacLeod, 1967). Infections, allergic reactions and varicocele all affect the morphological type of the sperm produced. Psychological and behavioral stress are apparently factors as well, there being fewer morphological variants in seminal samples from prison populations, in which "stress" is minimized by the managed environment, than in comparable samples from the general population. Drugs of the bis-dichloroacetyldiamine family cause increases in the numbers of morphologically aberrant sperm cells, as does an elevation of 17-ketosteroids (MacLeod, 1962). Hartmann *et al.* (1964) said that they believed that the degree of individualization which could be achieved by studies of sperm cell morphology was of the same order as that of fingerprints.

It should be noted that there is, as yet, no general agreement on exactly what constitutes a morphologically aberrant sperm cell, nor on a classification scheme for structural variants. In some cases, of course, the variance and interpretation are obvious, such as in the case of two-headed cells, but in other cases, the differences are considerably more subtle. The role of the observer is very critical in that different observers may well obtain different "profiles" after examination of the same specimen. MacLeod and Gold recognized this problem in 1951, and noted the importance of having the same observer carry out all examinations in which results were to be internally compared. MacLeod reiterated this view in 1967. Freund (1967) underscored the point by conducting a "blind study" wherein photomicrographs of 500 cells were sent to many laboratories engaged in this sort of work for determination of normal vs aberrant cell numbers, and for classification of the variants. There were enormous discrepancies in the results from various observers. Pollack (1943), who presented a detailed classification scheme for the cell types within an ejaculate, noted that material recovered from the female reproductive tract was not suitable for profile determination or biometry. Contamination of the sample with vaginal and/or cervical

material and cells and alterations of the sperm cells by the vaginal environment might give a completely different picture than that which would be obtained from a smear made from a semen sample. There is also the possibility that more than one individual's semen is present. Fredericsson *et al.* (1977) mentioned that in freshly obtained semen samples, a better profile can be obtained through the use of supravital staining with eosin Y in phosphate buffer at pH 7.4. Living cells may be distinguished in this way from nonliving ones, and information about each population of cells separately determined. Fraas and Soldo (1977) were involved in a medico-legal case involving aberrant sperm morphology. Dr. MacLeod examined a sample from an alleged rapist, and compared it with the pattern on the vaginal smear recovered from the victim at the time of the incident. Because there were only about 10 intact cells on the smear slide, however, the court found that no reasonable conclusions could be drawn as to exclusion of the defendant.

Fredericsson and Björk (1977) examined the morphology of spermatozoa in postcoital samples (10 hrs) from the vagina and from the cervix. Vaginal samples exhibited a morphological profile comparable to that of the deposited semen, but the cervical samples showed a much lower fraction of aberrant types of cells. They suggested the existence of a kind of "cervical barrier" to the morphologically aberrant types, especially those with abnormal heads.

10.3 Seminal (Prostatic) Acid Phosphatase and Vaginal Acid Phosphatase

10.3.1 Introduction

It has been known for a very long time that semen may lack spermatozoa. There are many different reasons for this circumstance, including congenital defects, pathologies, and vasectomy. Examination of physical evidence in sexual assault cases is more difficult if the identification of semen lacking sperm cells is required. For many years, forensic scientists have been concerned about this problem, and a number of methods have been offered as solutions to it. Most of the remainder of Section 10 is taken up with discussions of these techniques.

Pollack (1948) drew a distinction between "aspermia" and "azoospermia". The former condition is characterized by a total lack of testicular elements in the ejaculate, only accessory gland secretions being present. In the latter, spermatozoa are absent, but early cells in the spermatogenesis series are usually present. The ejaculate from a person suffering from aspermia, Pollack said, should not be called "semen". Eliasson (1975) gave virtually equivalent definitions for the terms. These distinctions are important in evaluating fertility, but probably matter little in medico-legal examinations in cases of sexual assault. That said, the term "azoospermic semen" will be used in all subsequent discussions to refer to any seminal sample in which mature spermatozoa are not present.

10.3.2 Seminal acid phosphatase detection for medico-legal identification of semen

The "acid phosphatase" test, as it is usually called, is one of the best known and most widely employed techniques for semen identification, apart from sperm cell identification itself. It is based, in its many variations, on the presence in human semen of high levels of a non-specific phosphohydrolase with acid pH optimum. This acid phosphatase (EC 3.1.3.2) is of prostatic origin, and is sometimes abbreviated in what follows as "AP".

In 1935, Kutscher and Wohlbergs reported that male ejaculate contained an enzyme which hydrolyzed various phosphate esters at an acid pH optimum. Kutscher's earlier (1935) studies on phosphatases in urine had prompted the investigation. The seminal enzyme hydrolyzed phenylphosphate better than α-glycerophosphate, and this better than β-glycerophosphate. It hydrolyzed hexose diphosphates slowly, and pyrophosphate almost not at all. The pH optimum for the hydrolysis of phenylphosphate was 4.65, and the enzyme was inactivated by exposure to 60° for 5 minutes. Its origin was established as being in the prostate gland, and the enzyme was called "prostate phosphatase". Kutscher and Wörner (1936) showed that the enzyme had a fairly broad pH optimum with β-glycerophosphate as substrate, and that it was inhibited by fluoride but not by cysteine. It had no demonstrable Mg^{++} requirement. The prostatic origin of the enzyme was confirmed by Gomori in 1941 using histochemical staining techniques.

In 1936, Gutman *et al.* noticed that acid phosphatase activity was greatly elevated in osteoplastic skeletal metastatic tissue in patients suffering from prostatic cancer. They suggested that the metastasizing prostatic tumor cells retained their ability to synthesize the enzyme described by Kutscher and Wohlbergs (1935). In 1938, Gutman and Gutman went on to show that there was a significant increase in acid phosphatase activity in the serum of 11 of 15 patients with metastasizing prostate carcinoma. Sera of 88 other patients having other diseases did not show the high AP values, except for that of one woman suffering from widespread Paget's disease. They assayed the enzyme in pH 4.9 buffers using phenylphosphate as substrate; a unit of activity was defined as that amount of enzyme which liberated 1 mg phenol from phenylphosphate per hour at 37° at pH 4.9. Levels in excess of 4U/100 mℓ serum were considered to be high. The assay was a slight modification of the classical assay devised by King and Armstrong (1934), except that a King-Armstrong unit was originally defined (for alkaline phosphatase) as that amount of enzyme which liberated 1 mg phenol from phenylphosphate at 37.5° in 30 min at pH 9.1. The Gutmans suggested that the assay of serum AP might be valuable in helping to diagnose prostatic carcinoma, an idea which rapidly gained favor in clinical circles (Cf. Benotti *et al.*, 1946; Walker *et al.*, 1954; Woodard, 1959; Südhof *et al.*, 1964).

In 1945, Lundquist, in Copenhagen, suggested that the extraordinarily large amounts of prostatic acid phosphatase

present in human semen be used as the basis for the identification of semen in medico-legal situations. Studies on this possibility were carried out in Denmark by Hansen (1946), Riisfeldt (1946) and Rasmussen (1945).

Rasmussen (1945) did not cite Lundquist's paper, and the publication of his paper seems in fact to have preceded Lundquist's by a matter of months. It would be correct, therefore, to credit the origin of the medico-legal utilization of seminal AP as an identification technique to both of these investigators. Rasmussen carried out quantitative determinations of AP in semen, saliva, urine and vaginal secretions in stains, using units of activity per mg dry weight as a basis of comparison. A unit of activity was that amount of enzyme which liberated 1 mg phenol from phenylphosphate per hour at 37° and pH 5.9. The lowest values observed in seminal material exceeded by nearly 200 times the highest values seen in other materials. Decline of AP activity in stains at ordinary temperatures was fairly rapid at first, i.e., in the first few days, but levelled off thereafter. Different individuals had different levels of seminal AP and these remained relatively constant in the absence of disease, infection, etc. The enzyme activity in a stain was reduced by about 50% by exposure to 74° for an hour, indicating a considerably higher heat stability than had been noted earlier by Kutscher and Wohlbergs (1935) (see above). Rasmussen recommended the test as a useful means of identifying seminal stains in legal medicine, especially azoospermic samples.

Hansen (1946) looked at the AP activity in a substantial number of seminal samples, and of samples of other bodily secretions. Measurements were done in liquids, where units of activity could readily be related to volume, as well as in stains. In the stains, the units of activity were related to the area occupied by the stain in order to be able to compare different materials. Knowledge of the volume of liquid material that might be maximally absorbed on any given substratum area allowed him to relate the units of enzyme activity per volume to units of activity per area, referred to as "voluminal potency" and "areal potency", respectively. Different fractions of urine, including urine collected after ejaculation, gonorrheal discharge from men, vaginal and cervical secretions, gonorrheal discharge and urine from women, feces, blood, saliva, bile and pancreatic juice were all tested for activity. Plant materials were treated as well. Seminal fluid invariably contained higher concentrations of the enzyme than the other materials. Hansen employed the same units of activity as had Rasmussen (1945) (see above), and said that AP activities in excess of 2 units/cm^2 stain were definitely diagnostic for seminal stains. A kind of "qualitative" test was arranged, in which the sensitivity of the assay was simply adjusted such that only seminal materials (i.e. materials with activities of 2–4 units/cm^2 stain) would give positive reactions. It was noted that only human and monkey prostatic secretions contained large AP concentrations, and that this test could therefore be of value in discriminating animal semen should the need arise. The obvious value of the test lay in the identification of azoospermic semen. Hansen noted that the first part of the ejaculate was particularly rich in prostatic AP. This fraction contained minimal spermatozoa. Later fractions are richer in cells and have less AP. In medico-legal exhibits, no cases were found in which sperm was present, but AP was negative. Hansen felt that the test was specific for semen, but nevertheless recommended that it be used in conjunction with, and not as a replacement for, the search for an identification of sperm cells.

The third extensive study was carried out by Dr. Ove Riisfeldt in 1946. High values of acid phosphatase activity were found in all ejaculates except those from prostatectomized subjects. Most samples contained 1500 to 3500 units of activity/mℓ semen, the lowest value noted being 400 units/mℓ. A unit of activity in these studies was defined as that amount of enzyme which liberated 1 mg phenol from phenylphosphate per hour at 37° and pH 4.9, and the enzyme concentration was expressed as "per mℓ semen". Vaginal secretions and cervical mucus from normal women and patients with gonorrhea and salpingitis, urine from men, women and children, serum, feces, male gonorrheal discharge, milk, saliva, gastric juice, tears, sweat, pus and a number of beverages, including coffee, tea, cocoa, beer, wine and distilled spirits were all tested for AP activity. None contained the enzyme at levels higher than 10 units/mℓ, well below the minimum value of 400 observed with seminal fluid specimens. Heating the sample to 60° for 5 min abolished enzyme activity, as did exposure to 37° for 14 days. A sample kept at room temperature for a month showed a 30% reduction in activity. A few chemicals that could conceivably be found as components of vaginal deodorant preparations or spermicidal contraceptives did inhibit the enzyme.

For stains, the assay system was adjusted such that material had to contain at least 20 units of activity to be detected. This device served to exclude all the non-seminal substances tested in the study which could have given a "false positive" result. Riisfeldt was convinced that the method was specific for semen, and believed that when it yielded positive results, one could safely conclude that semen was present. Situations did occur, however, in which the AP test was negative but sperm cells were still found. In the case of a negative AP test, therefore, the search for cells had to be undertaken. If no enzyme was present, and no spermatozoa found, the conclusion that semen was absent would be warranted.

In 1947, Kaye recommended the acid phosphatase test for seminal stain identification. He employed the Gutman and Gutman (1938) technique, as modified by Benotti *et al.* (1946) with phenylphosphate as substrate. Seminal fluid stains would contain a minimum of 30 King Armstrong units of AP activity, he said, and any stain containing that level of activity or higher could be considered as being of seminal origin. He recommended that a search for sperm cells be conducted as well. In 1949, Kaye reported that a wide variety of substances, including vaginal secretions, urine, serum, blood, menstrual blood, saliva, perspiration, pus, nasal mucus, gastric juice, feces and a number of foods and beverages contained less than 5 King Armstrong units of activity

per mℓ, or per cm^2 of stained cloth. Any value in excess of 25 K-A units/cm^2 stain, he thought, could be considered positive for semen. Stains up to 6 months old gave strongly positive reactions. The only possibility of obtaining an inordinately high value for AP from another body fluid would be in serum from a patient suffering from metastasizing prostatic carcinoma. In 1951, Kaye noted that stains kept at room temperature for up to 3 years still gave a strongly positive AP test, well in excess of 25 K-A units/cm^2 stained cloth. Faulds (1951) said that he regarded 5 K-A units/cm^2 stain as suspect, and that 10 K-A units/cm^2 definitely indicated that the stain was seminal. Fisher (1949) advocated the test, saying that the finding of $\geqq$ 100 units AP/mℓ original fluid volume was a positive test. A unit was that amount of enzyme which liberated 1 mg of phenol from phenylphosphate per hr at 37° at pH 4.9, and the area of a stain could be related to the original volume of fluid by an empirically determined factor.

In 1950, Lundquist reviewed the experience of the University Institute of Legal Medicine in Copenhagen with the AP test. More than 2000 stains from 346 cases were examined over a period of several years. Careful examinations for spermatozoa were conducted in many of the cases, in addition to the acid phosphatase test. It was found that the AP test could be negative even when sperm were present. Similarly, the test was sometimes positive in the absence of sperm cells. The test, therefore, has no useful negative value, but Lunquist said that appropriately controlled positive AP tests leave no doubt about the presence of semen, even if no sperm cells be found.

It will be noted that phenylphosphate was used as substrate in the phosphatase assays in many of the above studies. The liberated phenol was then determined colorimetrically by means of phenol reagent (Folin-Ciocalteu reagent) (Folin and Ciocalteu, 1927). In 1948 and 1949, a somewhat different assay, or histochemical demonstration technique, for phosphatases was introduced (Manheimer and Seligman, 1948; Seligman and Manheimer, 1949). The principle of the method lay in the use of naphthylphosphate substrates, and subsequent coupling of the naphthol liberated in the reaction with a diazonium compound to form an insoluble colored product (see in Table 5.3). The original method employed a freshly prepared α-naphthyldiazonium chloride reagent, but it was soon found that a stabilized α-naphthyldiazonium derivative could be prepared, which was stable for months, and which reacted readily with β-naphthol to form a purple-red insoluble product (Fig. 10.1). The stabilized diazonium compound in Fig. 10.1 is also known as Fast Garnet B. Similarly, a stabilized diazonium compound was described which reacted with the α-isomer (Fig. 10.2). The stabilized diazonium compound in Fig. 10.2 was commercially available as Naphthanil Diazo Red AL, and diazonium compounds of o-dianisidine and 2-amino-4-chloroanisole were commercially available as Naphthanil Diazo Blue B and Naphthanil Diazo Red RC, respectively (See Fig. 10.3 and 10.4). There are a variety of other stabilized diazonium coupling salts available as well.

In 1950, Walker applied this technique to medico-legal identification of seminal stains. The test could be performed directly on fabric, or on filter paper to which stained material had been transferred. Blood was also colored by the reagents, but the color was very different, and the characteristic seminal AP color soon replaced the blood color in blood-semen stains. Walker noted further that the test reagents did not stain spermatozoa, and in no way interfered with their detection using a carbolfuchsin dye. The phosphatase activity of stains was observed to be concentrated more in the periphery, whereas the sperm cells tend to concentrate more in the center of the stain (see Section 10.1.1). This fact was quite clearly established by Leithoff and Leithoff (1965b). The use of α-naphthylphosphate substrate and a coupling diazonium salt for the acid phosphatase was soon adopted in the medico-legal AP tests for seminal stains and semen. Berg (1955) recommended this method, incorporating aqueous lauryl sulfonate into the reagent. He said (1957) that only the blue-purple color characteristic of seminal AP should be used to judge the result, and that he had always obtained the test when spermatozoa were found, but never in their absence. Several minor modifications of Berg's method appeared (Schiff, 1969; Ponsold, 1957), and Kempe (1958) said that he found no false positives with urine or with vaginal smears from pregnant or non-pregnant women, provided only the deep blue color was used to judge whether the reaction was positive. Kind (1958) described stable acid phosphatase test papers based on the azo dye coupling principle. Hazen (1955) used an inorganic phosphate assay to estimate AP activity with β-glycerophosphate as substrate, and said that values in excess of 18μg P_i released/mℓ extract could be regarded as being of seminal stain origin. Boltz and Ploberger (1956) recommended a technique using the complex salt of phenolphthalein diphosphate with pyridine. The method was recommended for darkly colored fabrics or fabrics whose weave did not readily allow a direct test. The stained material was pressed with moistened filter paper, and the filter paper then tested. Gültingen (1961) got good results with that technique in relatively fresh stains, but the test was sometimes negative with older stains whereas Berg's (1954) method with Ca α-naphthyl phosphate as substrate gave almost uniformly positive results. Phenolphthalein diphosphate was first proposed as a substrate for AP by Huggins and Talalay (1945) (see Section 10.2.4).

Not all authorities have agreed that a positive acid phosphatase test alone, in the absence of other evidence, should be taken as conclusive proof of the presence of semen. Hauck and Leithoff (1959) reviewed this subject at length, concluding that an AP test alone should not be considered conclusive for semen. They showed that a number of materials of plant origin gave high AP values. The value of the test could be improved not only by carrying out the test quantitatively, but at several different pH as well, and comparing the pH-activity curves of unknown materials with those of semen and various potentially interfering substances. The search for sperm cells should be conducted, they said, regardless of whether the test gives positive or negative results. Kind

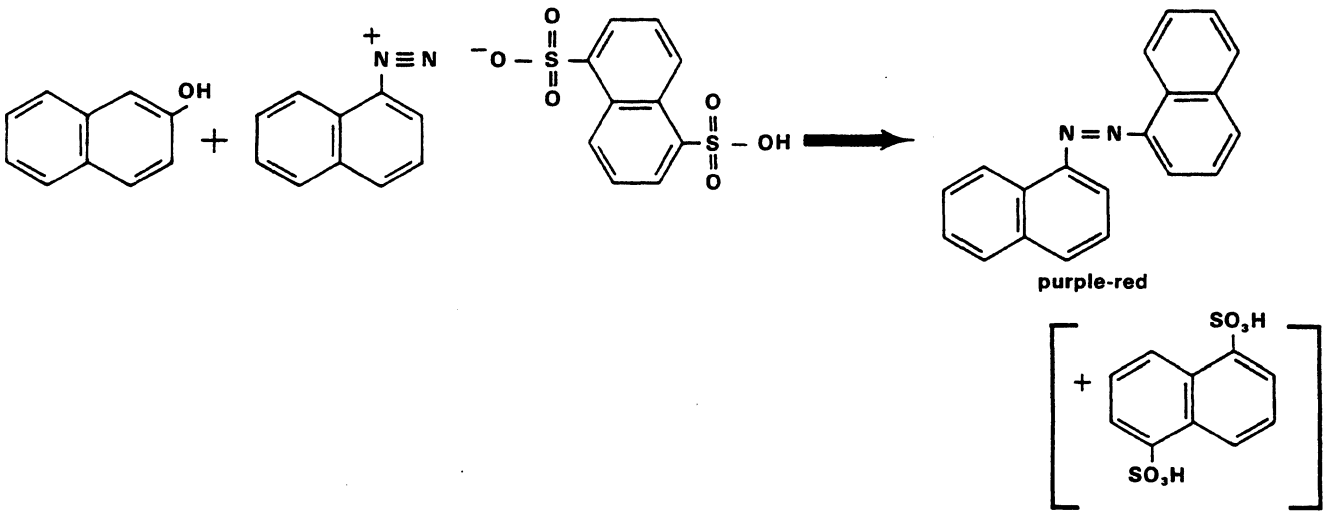


Figure 10.1 Reaction of β-Naphthol with Stabilized Diazonium Compound (Fast Garnet B)

(1964) reviewed the acid phosphatase test as well, and agreed in part with Hauck and Leithoff. Some of the objections to the test employing azo dye coupling technique were based on the fact that a number of amines and phenolic compounds would react with the azo dye to yield colored products. Not too many compounds, however, will react at pH 5, and fewer still would yield exactly the same color as α-naphthol. In any case, such interference can readily be diagnosed by applying the azo dye reagent separately from the phosphatase substrate reagent (Kind, 1964; Brackett, 1957). Formation of colored products in the absence of phosphatase substrate is an immediate indication of contamination by a compound capable of reacting with the azo dye. Such a two-step procedure is quite analogous to the use of a two-step procedure in the catalytic tests for blood, in which application of the reagent in the absence of H_2O_2 detects interfering oxidizing agents (see section 6). There are materials of plant origin, however, such as cauliflower juice and, as Kind established, extracts of gorse (*Ulex europaeus*) seeds, which have relatively high AP levels. Kind (1964) described the application of the phosphatase assay using p-nitrophenylphosphate as substrate, first reported by Ohmori (1937). In this technique, the p-nitrophenylate anion, which is resonance-stabilized in basic solution, is determined by its absorption at 400 nm. Kind also discussed the AP test reaction as a searching tool, for locating seminal stain on garments and surfaces, and Konzak (1977) recommended this technique as a useful one.

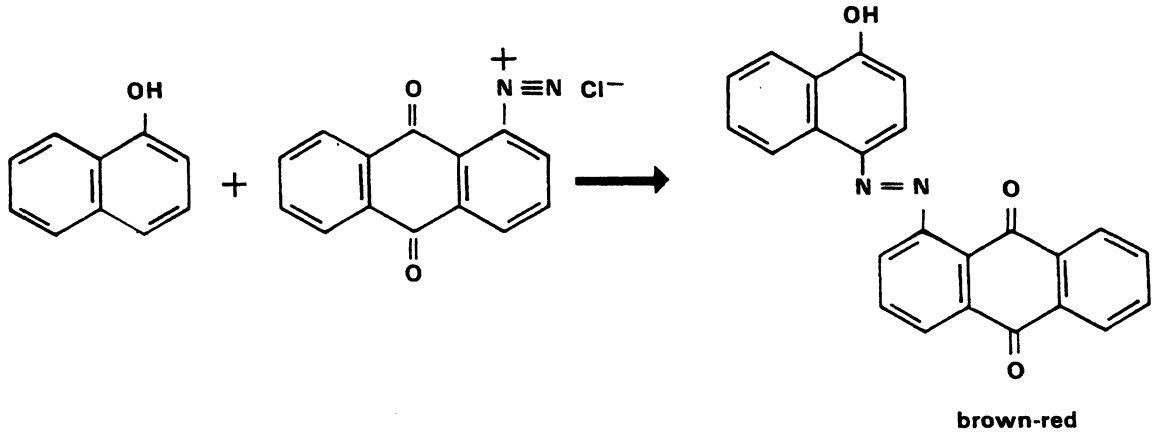


Figure 10.2 Reaction of α-Naphthol with Stabilized Diazonium Compound (Fast Red AL)

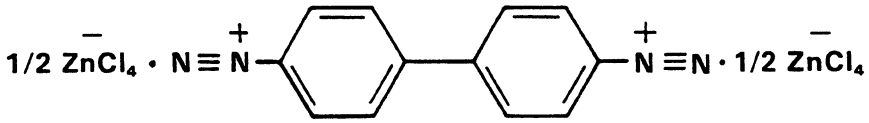


Figure 10.3 $ZnCl_2$ Double Salt of the Tetrazonium Chloride of Diazo Blue B (Fast Blue B; Dianisidine Blue)

A number of authorities have advocated quantitative AP assays for medico-legal determinations of semen and seminal stains, insisting that it is the large amount of AP and not its mere presence that characterizes seminal plasma (Rasmussen, 1945; Hansen, 1946; Riisfeldt, 1946; Hauck and Leithoff, 1959; Kind, 1964; Nakamura *et al.*, 1959; Kaye, 1947; Hazen, 1955; Walther and Höhn, 1971; Gomez *et al.*, 1975; Davis and Gomez, 1975). A number of investigators who have employed quantitative assays have recommended "cut-off" values, in units per volume, or area; samples which contained AP values in excess of these values, they thought, could definitely be regarded as being of seminal origin. In many cases, these values were empirically established by measuring the AP content of a large number of substances, and choosing values above those observed for substances which were non-seminal. Some of the recommendations are given in Table 10.1.

Walther and Höhn (1971) measured quantities of AP in some plant materials, with o-carboxyphosphate as substrate, which were as high as in some samples of semen. The AP enzymes could be differentiated by disc polyacrylamide gel electrophoresis, and this procedure would be necessary to eliminate the possibility of adventitious AP (Walther, 1971). Pinto (1959) had suggested that a control sample, heated to 145° for 30 min could be incorporated in answer to this problem, at least for the bacterial phosphatases, since the prostatic enzyme is quite heat-labile while the bacterial enzymes are not. Kind (1964) said that he would not report the presence of semen in a stain based on an acid phosphatase alone; corroboration of identification by finding sperm cells, or a positive Florence test would be required. Pinto (1959) said that he would report a high AP value even in the absence of spermatozoa or corroboration, with a suitable explanation, and let the prosecutorial or judicial authority judge its merit. Schiff (1978) disagreed with this idea. He thought that the expert witness should give an opinion on a scientific measurement which he thought would be too complex for nonscientists to be able to evaluate properly. He reviewed the AP test, and was convinced of its reliability as an indicator of the presence of semen, even in the absence of sperm. He favored a qualitative test in the hands of an experienced worker. If a quantitative test is used, he said, more time is required and an arbitrary "cut off" point must be chosen. He said that his experience with the test over many years had indicated that it was a reliable one for azoospermic semen.

Brown and Brown (1974) tested a large number of douche preparations, creams and foams used to treat vaginal infections, contraceptive creams and foams, vaginal deodorant sprays and foams, diaphragm lubricants, a number of detergents and several miscellaneous materials for positive AP reactions using two commercially available test kits, one by American Monitor and Warner-Chilcott's Phosphatabs-Acid. They could show that four products, Triva douche powder, V.A. douche powder, Langeen vaginal jelly (spermicidal) and Acinjel Therapeutic Vaginal Cream, gave positive reactions with the Warner-Chilcott Phosphatabs-Acid kit. Quantitative retesting could eliminate the false positive reactions but some problems were encountered (precipitation). The American Monitor kit reacted only with dilute Clorox solution, but not with fabric soaked in dilute Clorox

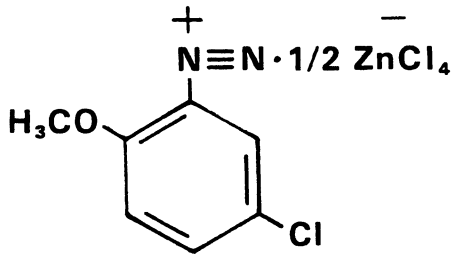


Figure 10.4 $ZnCl_2$ Double Salt of Diazo Red RC (Fast Red RC)

Table 10.1 Recommended Concentrations of Acid Phosphatase to be Observed for the Diagnosis of Semen

Acid Phosphatase Concentration	Units Used★	Reference
2 units/cm²	King-Armstrong	Hansen, 1946
20 units/mℓ original material	King-Armstrong	Riisfeldt, 1946
30 units/cm²	King-Armstrong	Kaye, 1947
25 units/cm²	King-Armstrong	Kaye, 1949
20 units/mℓ original material	King-Armstrong	Fisher, 1949
10 units/cm²	King-Armstrong	Faulds, 1951
2 units/mg difference in dry weight before & after extraction	—	Gilli & Fallani, 1952
20 units/cm²	—	Perez de Petinto y Alfonzo Martinez, 1953
18 units/mℓ extract	Modified Bodansky	Hazen, 1955
50 units/mℓ vaginal aspirate	King-Armstrong	Breen et al., 1972
300 units/dℓ (swab in 3 mℓ saline)	King-Armstrong	Schumann et al., 1976
300 units/ℓ (swab in 3 mℓ saline)	International	
300 units/ℓ (swab in 2 mℓ saline)	International	Findley, 1977

★ Note that not all units are necessarily identical because of variations in pH, substrate concentration, etc. (See Section 10.2.4)

and allowed to dry. Walther (1967) said that stains which had been washed in detergents and water hotter than 40° no longer showed AP activity.

Owen & Smalldon (1975) raised the issue of the evidentiary meaning of positive AP test findings. This question was discussed in Section 6.8 in connection with catalytic tests for blood. In examining 100 men's jackets and 100 pairs of men's trousers selected at random from a dry cleaner, it was noted that 44 pairs of the trousers showed areas of significant AP activity. 37 of them were sufficiently high to indicate possible prostatic origin. This sort of finding goes to the question of interpreting the evidentiary value of positive results, rather than to any technical considerations involved in actually obtaining them.

It should be noted that negative results can have value in using the AP test as well. In the absence of any acid phosphatase activity, the probability of semen being present may not be very great, and this fact should not be overlooked.

10.3.3 Persistence of acid phosphatase

There are two separate issues to be considered under this heading: (1) the persistence of AP activity in the vagina as a function of elapsed time after semen deposition; and (2) the persistence of the enzyme in a dried seminal stain.

As with sperm cells, there has been an interest in establishing the time of persistence of AP activity in the vagina following coitus, and in relating the residual activity to the elapsed time if possible. Pinto (1959), using the α-naphthylphosphate-Naphthanil Diazo Red AL assay method, constructed a color chart which showed that color intensity gradually decreased as a function of elapsed time since intercourse from 6 to 48 hrs. The chart could be used to obtain an estimate of elapsed time in unknown samples. In a series of 34 specimens, in which the time estimate from the color chart was compared with the elapsed time reported by the examining physician, 15 were in close agreement. In 16 cases, there were moderate discrepancies amounting to a few hours. In 12 of these 16 samples, the color chart overesti-

mated the elapsed time, while in 4 others, it underestimated the time. In 3 of the cases, there were large discrepancies, amounting to a matter of days. In these cases the chart greatly underestimated the elapsed time since coitus. Pinto recognized that this approach provided only a crude estimate in some cases, and said that a quantitative assay correlation with time might be more informative. Rupp (1969) noted that AP activity survived well beyond 24 hrs postcoitus. Davies and Wilson (1974) noted that AP activity may be present up to 3 days following semen deposition, but that the test is most useful on swabs taken within one day, and rarely useful after two days. McCloskey *et al.* (1975) said that all specimens which showed at least 25 K-A units of activity were taken within 48 hrs of intercourse. Samples showing at least 50 K-A units were all collected within 24 hrs. Some samples collected within the first 24 hrs following coitus, however, showed activities of less than 25 K-A units. If 25 K-A units were used as a "cut-off" value, therefore, some of these latter samples would have been called "negative". Brown (1977) reported that AP was detectable on swabs up to 24 hrs following intercourse.

Gomez *et al.* (1975) assayed AP activity in vaginal washings from 41 women, using several different methods. Two of the methods were qualitative, and were based on the azo dye coupling assay with α-naphthylphosphate as substrate. One of these made use of the General Diagnostics Phosphatabs-Acid kit, while the other was a modification of several published assays using α-naphthylphosphate as substrate in citrate buffers at pH 5.6 with tetrazotized o-dianisidine as coupling dye (Fig. 10.3) Two quantitative assays were used as well. One employed α-naphthylphosphate as substrate and diazotized 5-nitro-o-anisidine (Fast Red Salt B), and was described by Babson and Phillips (1966). The other employed thymolphthalein-phosphate as substrate, which generates its own chromophore upon addition of base. Thymophthalein can be determined at 590 nm, and the procedure was initially described by Roy *et al.* (1971). Patients were divided into two groups: those with no history of recent intercourse, and in whose washings no sperm were found, and those with histories of recent coitus and/or in whose washings sperm could be identified. It could be shown that the Phosphatabs quantitative test was more sensitive than the author's modified qualitative test. The highest values measured quantitatively in the first group of patients (no history of recent coitus) overlapped with the lowest values in the second group (recent coitus). For example, one person whose washings were sperm positive, and who was alleged to have been raped 5 hrs previously, showed a lower AP activity than another patient who said she had not had intercourse for 60 hrs. The qualitative test would have been negative in both cases. There was a fairly good correlation between the qualitative and quantitative tests in most cases. There was sufficient endogenous vaginal AP activity and sufficient individual variation in its levels, even in the few people studied here, to suggest that errors would sometimes result using a qualitative test or a quantitative test in which a particular "cut-off" point had been chosen. In many cases, when an enormous amount of enzyme is present, there will be no doubt that vaginal AP can be excluded as a possible source. But it would be necessary to be employing a quantitative test to know that a relatively large amount of enzyme was present.

Enos *et al.* (1963) reported their results in 36 cases of sexual assault. They used so-called Bodansky units for quantitation of the AP activity. A Bodansky unit was originally defined as that amount of enzyme which liberated 1 mg P_i from glycerophosphate per hour at 37° (Bodansky, 1933). In samples taken from 1½ to 5 hrs postcoitus, the activity varied from 8.2 to 78 units. In several control patients, it was noted that the AP activity declined to zero activity within 12 hrs of coitus. Enos and Bayer (1977) correlated the number of Bodansky units found in vaginal material with the elapsed time since intercourse. 100 units corresponded to 1 hr, 30–50 units to 2–3 hrs, 10 unit to 6 hrs, 5 units to 12 hrs and zero units to $\geqq$24 hrs. Rupp (1969) said that AP activity could be detected in vaginal aspirates for periods exceeding 24 hrs after intercourse. Schumann *et al.* (1976) studied the time decay characteristics of AP in a number of subjects, using a manual quantitative assay with phenyl phosphate as substrate and an automated assay with thymolphthalein phosphate as substrate. They were of the opinion that a definite cut-off point could be established for determining positively the presence of semen (see Table 10.1), and they found the AP measure to be more certain than the finding of sperm cells. The decay of activity could be used as a fair measure of time elapsed since coitus. Findley (1977) did very similar studies and came to similar conclusions. His threshold value for interpretation of the presence of semen is slightly lower than that of Schumann *et al.*, however, because of dilution factors (see Table 10.1). Davies (1978) and Allard and Davies (1979) presented their studies on a large number of cases, in which acid phosphatase activity had been determined on vaginal swabs quantitatively using p-nitrophenyl phosphate as substrate. These workers thought that measured levels of AP not very much above 20 Sigma units per swab provided quite convincing evidence that semen was present.

Dahlke *et al.* (1977) and Duenhoelter *et al.* (1978) reported the presence or absence of acid phosphatase in the series of materials they examined. A qualitative test was used by Duenhoelter *et al.* (1978). They reported the surprising result that AP was undetected in a higher percentage of the cases examined within two hours than in those examined in the 3 to 12 hour interval. Dahlke *et al.* (1977) employed a commercially available assay kit, based on the thymolphthalein phosphate method of Roy *et al.* (1971). Their data indicated that values in excess of 50 units per liter vaginal eluate were suggestive of semen. This level was seen in 40% of the study population.

Sensabaugh (1979) carried out a detailed statistical analysis of endogenous and post-coital AP levels using his own data, and a number of sets of published data. Endogenous

AP levels were found to be lognormally distributed. The percentage of postcoital AP values which would fall into the "endogenous" range increased with time after semen deposition. This complex but important paper should be read by those interested in the interpretation of quantitative AP results.

Standeffer and Street (1977) determined the post-mortem stability of AP activity. They said that samples collected at autopsy should be analyzed within 48 hrs, and stored at 4° in the interim. Longer storage was possible if samples were kept at −20°. AP activity could be detected for up to 7 days postmortem in vaginal samples, for up to 36 hrs in the oral cavity, and for up to 24 hrs in the rectum. They noted that temperature could be a major variable in postmortem survival, the enzyme tending to be more stable in a body that was in a colder environment. AP was determined quantitatively in these studies.

The other issue which is of importance here is the survival of AP activity in stains as a function of drying and elapsed time. Faulds (1951) studied this matter in some detail. Stains were prepared and kept at different temperatures for a number of months. Over the course of about 5 months, the stains declined in AP activity by as much as 78% and by as little as 48%. The decline in activity was as great in the stain kept at −14° as in the stain kept at 37°. He also noted that the semen could lose up to 50% of its AP activity simply upon drying. Perez de Petinto y Alfonso Martinez (1953) indicated that semen having about 2,000 units of activity/mℓ showed only about 90 units/cm^2 in 6 hr old stains. At 3 months of age, the remaining activity was 20 to 25 units/cm^2. As noted above, Kaye (1951) showed that stains kept at room temperature for 3 years retained AP activity. Schiff (1975) agreed with this estimate. Kerek (1972) indicated that seminal stains kept at room temperature retained activity for at least 14 months, and could still be active after 4½ years if stored at −20°.

10.3.4 Acid phosphatase assay techniques and activity units

It will be clear from the foregoing discussion that a number of different substrates and assay techniques have been employed in studies on phosphatases, and that different authors have made use of many different activity units. Naturally, the enzyme is not expected to exhibit the same affinity for all substrates so the sensitivity of assays using different substrates will be different. Many of the apparent discrepancies in estimates of survival can probably be explained in this way. Moreover, because different units tend to be defined for different substrates, it is often impossible to compare the results of one author with those of another. And even if comparable units have been used, they are not always related to the material being assayed in the same way. For example, one author might relate units of activity to stain area, while another relates the same units to the protein concentration in the stain extract. The relationship between a cm^2 of stain area and a particular protein concentration will be neither obvious nor comparable. A brief discussion of phosphatase assay methods, and expressions of activity units, has therefore been included here.

The acid phosphatase enzyme, as noted, is nonspecific, i.e., it will hydrolyze a variety of phosphate esters. Substrates that have been used for AP determinations include α- and β-glycerophosphate, phenylphosphate, p-nitro-phenylphosphate, α- and β-naphthylphosphates, phenolphthaleinphosphate, thymolphthalein phosphate, phosphate esters of 3-hydroxy-2-naphthanilides, flavone-3-diphosphate and 4-methylumbellifrylphosphate. Since the reaction has two products, either one of them could be determined in assessing the progress of the reaction. Substrate disappearance could be used as well, but has not been common. Phosphate determinations have been used by a number of authors, this method being more popular some years ago than it is now. Most methods of P_i determination are based on the fact that orthophosphate will form a complex with ammonium molybdate in strong (10N) H_2SO_4 and this complex may be reduced to yield a blue chromophore (Taussky and Schorr, 1953). The optical density may be read in the 700 nm region of the visible spectrum. There are, in addition, extraction procedures for separating P_i from organic phosphates in the reaction mixture prior to assay (Lindberg and Ernster, 1956). Since any substrate yields orthophosphate as one of the products, this method can be employed with all of them.

Most assay procedures have concentrated on the other product. Substrates have been chosen because they generate a chromophore upon hydrolysis, or else a compound that is readily convertible to a colorimetrically determinable chromophore. Phenylphosphate yields phenol, which is determined with phenol reagent (Folin and Ciocalteu, 1927). Para-nitrophenylphosphate generates p-nitrophenol. This method was first employed by Ohmori (1937). The p-nitrophenol readily forms a resonance-stabilized p-nitrophenylate anion in basic solution which absorbs at around 400 nm. This substrate is hydrolyzed a little faster than phenylphosphate (King and Delory, 1939) and has been used to assay serum phosphatases (Bessey *et al.*, 1946; Andersch and Szczypinski, 1947) and seminal AP (Kind, 1964). The α- and β-naphthylphosphates have enjoyed extensive use as substrates. The naphthols may be determined directly by their absorption at 335 nm (α) and 345 nm (β) (Moss, 1966). The α-compound has a higher extinction coefficient than does the β-compound. Alternatively, the naphthols may be allowed to react with a variety of azo coupling dyes. These methods are especially valuable for qualitative assays, histochemical localization of the enzyme (Kupcsulik *et al.*, 1970), and for detecting enzymes in gel electrophoretic media, since the brightly colored products are frequently insoluble. Quantitative assays can be carried out using these methods, however, by selecting azo compounds which form soluble colored products (Babson and Phillips, 1966). Phenolphthaleinphosphate (Huggins and Talalay, 1945) and thymolphthaleinphosphate (Roy *et al.*, 1971) generate their own chromophores upon hydrolysis if the solution be made basic. The latter was employed by Gomez *et al.* (1975) and Konzak (1977), and Willott (1975) said that he found the

sensitivity to be of the same order as that of p-nitrophenylphosphate.

Fluorimetric procedures have been employed to increase the sensitivity of the assays. Both α- and β-naphthol fluoresce and their phosphate esters may be used as fluorogenic substrates in solution (Campbell and Moss, 1961), or for localizing enzymes in gels with UV light (Moss *et al.*, 1961). Vaughan *et al.* (1971) studied a series of so-called naphthol AS phosphates as fluorogenic substrates for acid and alkaline phosphatases: Naphthol AS (3-hydroxy-2-naphthanilide); Naphthol AS-BI (6-bromo-3-hydroxy-2-naphthyl-o-anisidine); Naphthol AS-D (3-hydroxy-2-naphthyl-o-toluidine); Naphthol AS-GR (3-hydoxy-2-anthryl-o-toluidine); Naphthol AS-LC (4′-chloro-3-hydroxy-2′,5′-dimethoxy-2-naphthanilide); Naphthol AS-MX (2′,4′-dimethyl-3-hydroxy-2-naphthanilide) and Naphthol AS-TR (4′-chloro-3-hydroxy-2-naphthyl-o-toluidine). Naphthol AS-BI-phosphate was found to be the best substrate for phosphatases. It had a K_m for alkaline phosphatase of 2.2×10^{-5}M and detected alkaline phosphatase at a concentration of 5×10^{-5} units, where a unit was 1 μmole substrate hydrolyzed/min at 25°. Land and Jackson (1966) used flavone-3-diphosphate as a substrate, and noted that it was more stable than 3-O-methylfluoresceinphosphate, and more sensitive than β-naphthylphosphate. Ortho-carboxyphosphate may be used as a fluorogenic substrate (Brandenberger *et al.*, 1967), the reaction product being salicylic acid. It can also be employed as a substrate and salicylate detected by its absorption properties, as was done by Walther and Höhn (1971). Neumann (1948) described three fluorogenic substrates for phosphatases: fluoresceinphosphate, eosinphosphate and 4-methyl-7-oxo-coumarinphosphate, the last of which is better known as 4-methylumbelliferylphosphate, and has been used by Adams and Wraxall (1974) to detect seminal, vaginal and fecal AP in acrylamide gels following electrophoresis. The substrate is in use in a number of laboratories for detection of the isoenzymes of erythrocyte AP in gels as well (Wraxall and Emes, 1976). For a review of fluorimetric enzyme assays, including the assay of phosphatases, see Roth (1969).

The other matter is that of units of activity. Units are always defined as mass units of substrate hydrolyzed (or product formed) per unit time at some defined temperature and pH. Some of the unit definitions in the phosphatase field have acquired the names of their definers, and may still be encountered in the literature in those terms. Some unit definitions that have appeared over the years are summarized in Table 10.2. Since the enzyme exhibits different affinity for, and different rates of hydrolysis with the various substrates, the units are not always readily comparable. The international unit is based on the recommendations of the International Union of Biochemistry (IUB) for all enzyme units. In expressing enzyme concentration, the IUB recommended the use of units/mℓ solution. In clinical chemistry, it is sometimes preferred to express concentrations in units/ℓ. The international unit is denoted by the symbol "U". In order to obtain convenient numbers, it is permissible to express international units in logarithmic multiples using the usual metric prefixes, e.g. milli-units, mU; kilounits, kU; etc. It was common in the older literature to express serum phosphatase concentrations in terms of units/100 mℓ serum. Babson and Read (1966) said that 1 μmole α-naphthol/min (international unit) was equivalent to 0.865 Babson-Read units. A Babson-Read unit is equivalent to about 0.18 Bodansky units, and a King-Armstrong unit/100 mℓ is equivalent to 1.8 I.U./ℓ at the same temperature and pH (Bowers and McComb, 1970).

10.3.5 Specificity of the acid phosphatase test and the problem of vaginal acid phosphatase

In sexual assaults, the seminal stains may well be contaminated with vaginal secretions. In swabs taken from victims, there is no question that they are so contaminated. Because vaginal secretions contain endogenous, though variable, acid phosphatase activity, and because seminal samples may show low AP activity, there has been some concern about the possibility of vaginal AP leading to "false positive" reactions. As was discussed above, it matters little to this problem whether qualitative or quantitative tests are being used. In a quantitative test, some judment has to be made as to where the "cut-off" point is going to be, in terms of units per volume or area, and above which the material will be regarded as being of seminal origin. A qualitative test has an internal, or built-in, "cut-off" point, as it were, namely the lower limit of the sensitivity of the assay. The problem has been one of attempting to discover whether the upper limits of endogenous vaginal acid phosphatase (VAP) activity may overlap the lower limits of seminal acid phosphatase (SAP) activity.

The studies of Gomez *et al.* (1975) indicated that endogenous VAP activity could be higher than some examples of SAP activity measured in vaginal washings, and that misinterpretation was therefore possible using AP alone as a criterion for the presence of semen. The meaning of the term "vaginal secretions" is not very clear, and the term does not describe a well-defined bodily secretion whose origins are known. In medico-legal discussions, the term is usually taken to refer collectively to any material which is recovered on vaginal swabs or in vaginal washings in the absence of semen. As such, it may consist of substances from the vaginal mucosa, cervix, endometrium, bacterial flora, lymph or tissue fluids, or even urine. In pregnancy, substances from gestational tissues might be found. Moreover, if an infection is present, substances or cells characteristic of yeast, fungi, gonorrheal or leucorrheal discharge may be present. No one would suggest that materials characteristic of vaginal pathologies constitute "vaginal secretions", but the fact is that unless a pathological condition has been diagnosed by the examining physician, and the evidence examiner informed, these types of adventituous substances might be present in the sample and no tests conducted to determine their possible presence.

Table 10.2 Some Units of Phosphatase Activity

Name of unit	Substrate	pH	Temperature	Definition of Unit	Reference
King-Armstrong (for alkaline phosphatase)	phenylphosphate	9.1	37.5	1 mg phenol/30 min	King & Armstrong (1934)
King-Armstrong (for acid phosphatase)	phenylphosphate	4.9	37	1 mg phenol/hr	Gutman & Gutman (1938), Riisfeldt (1946)
	phenylphosphate	5.9	37	1 mg phenol/hr	Rasmussen (1945), Hansen (1946)
Bodansky (for alkaline phosphatase)	β-glycerophosphate	8.6	37	1 mg P_i/hr	Bodansky (1933)
Bodansky (for acid phosphatase)	β-glycerophosphate	5.0	37	1 mg P_i/hr	Bodansky (1972), Enos et al. (1963) Enos & Bayer (1977)
	β-glycerophosphate	6.0	37	1 mg P_i/30 min	Hazen (1955)
Babson-Read	α-naphthylphosphate	5.2	37	1 mg α-naphthol/hr	Babson & Read (1959)
Huggins-Talalay	phenolphthalein-diphosphate	5.5-6.0 (acid phosphatase)	37	0.1 mg phenolphthalein/hr	Huggins & Talalay (1945)
		9.1-9.6 (alkaline phosphatase)		0.1 mg phenolphthalein/hr	
International (I.U.)	any	defined	defined	1 μmole substrate/min	Bowers & McComb (1970)

Fishman and Mitchell (1959) showed by histochemical techniques that AP is present in the middle and superficial layers of vaginal epithelium. Moursi *et al.* (1971), studying exfoliated vaginal mucosal cells by histochemical techniques, indicated that AP was present in cytoplasmic granules, but not in nuclei. Activity was lower in most patients in the follicular phase of the menstrual cycle than in the luteal phase. Gregoire *et al.* (1972) measured the AP activity of cervical mucus during different cycle phases in patients using no contraceptive devices as well as in patients using an IUD or hormonal contraceptive therapy. AP activity was expressed in units/100 mg protein where a unit of activity was defined as that amount of enzyme liberating 1 mmole p-nitrophenol from p-nitrophenylphosphate in 30 min at 37°. With no contraceptives, AP was relatively low in the proliferative phase (roughly the same as the follicular phase), increased slightly in the ovulatory phase (midcycle), and then increased dramatically in secretory phase (roughly corresponding to the luteal phase). With an IUD, AP was highest in proliferative phase, decreased significantly (by about 80%) at midcycle, and then increased again in secretory phase, but only to about 60% of the initial (proliferative phase) value. With hormonal contraceptives, proliferative and ovulatory phases showed similar activities, but they were of the same order of magnitude as the highest values seen in the other two groups. A slight increase was observed in secretory phase. The actual values observed ranged from 3.0 to 20.7 units/100 mg protein. The only relatively comparable data on SAP is that of Kind (1964) who used a similar assay, but whose units were defined as μg p-nitrophenol liberated/min at 37°. There would be no difficulty in converting mmoles to μg, but Kind expressed the concentration of the enzyme not in terms of a mass value of protein, but in terms of that amount of seminal stain extract which gave an OD of 1.0 at 270 nm with a 1 cm path length. The OD_{270} is a rough measure of protein concentration to be sure, although it is more usual to determine either OD_{280} or the ratio of absorbancies at 260 and 280 nm. The trouble is that every protein has a different extinction coefficient, and Kind did not say what this was in his paper.

A quite definitive study of the problem was carried out by Godwin and Seitz (1970), using a commercial kit, based on the assay of Babson and Read (1959), for quantitative AP determination. Measurements were made on swabbed material eluted into 1.5 mℓ saline. The highest value seen in a non-pregnant patient in the absence of semen was 64 units, while in a pregnant patient, it was 85. Where intercourse had occurred within 12 hours, the AP activity was usually >300 units, and spermatozoa were usually found. In one patient, AP activity was 905 units, but no sperm cells could be found 11 hours after coitus. In 9 cases in which sperm were found, however, the AP activity was 85 units. In samples collected up to 36 hours after intercourse overall, AP activity varied from 10 units to 4,100 units. Godwin and Seitz concluded that the AP activity was not as reliable an indicator of semen as the presence of sperm cells, as is evident from the data, but that AP activities might be useful in diagnosing azoospermic semen, presumably where the activity is very high, and well above any endogenous VAP level that has been observed. Sensabaugh (1977) has shown that the total acid phosphatase activity in 8–12 hour postcoital samples of the vaginal pool is only about 1% of that present in the ejaculate. In addition, the activity in the recovered samples varied from 0.16 to 32.4 units, where a unit was 1 μmol p-nitrophenyl phosphate hydrolyzed/min at 22°. Endogenous VAP activity varied from 0.12 to 0.68 in the same units, a significant overlap between the range of endogenous activity and the range of the activity in samples recovered 8–12 hours post coitus in the undoubted presence of semen. Sensabaugh (1979) has extended his analysis of this problem, using data collected in a number of different laboratories. The paper, which must be read by those interested in the significance of quantitative AP results, places the entire problem into a statistical perspective.

It is quite clear that some other approach is required if the AP test by itself is to be rendered specific for semen. Quantitative determinations alone do not necessarily exclude VAP, although most authorities agree that they are preferable to a qualitative test. One way of avoiding the difficulty, of course, is simply to side-step the issue and regard the AP test as presumptive. In the absence of a corroborative finding (sperm, Florence test, immunological test, etc.), therefore, the results would be reported as "negative for semen", or as "negative for spermatozoa, but with such-and-such an AP result", depending on the individual expert's feelings about the matter. There are, however, other approaches which may be taken to meet the problem. Two of these, which have been tried, are: differential inhibition, in which an inhibitor is used which inhibits either SAP or VAP, but not the other; and separation of SAP from VAP by a protein separation technique, such as electrophoresis, isoelectric focusing, etc.

In 1970, Sivaram reported that he could take advantage of the fact the L-tartrate inhibited SAP to distinguish this enzyme from other acid phosphatases in a qualitative test using α-naphthylphosphate as substrate and Brentamine Fast Blue B as the coupling dye (Table 5.3). In 1971, Sivaram and Bami enlarged these studies, carrying out a quantitative AP assay with phenylphosphate as substrate, and showing that L-tartrate completely inhibited SAP at a concentration of 0.04M. That L-tartrate inhibits prostatic AP had been known since the work of Abul-Fadl and King (1949), who noted that L-tartrate did not inhibit erythrocytic AP. This fact was soon put to use in clinical chemistry, because in assaying serum AP, the circulating enzyme of prostatic origin could be assessed based on the fraction of "tartrate-inhibitable" AP present (King and Jegatheesan, 1959; Fishman and Lerner, 1953; and many others). King and Jegatheesan (1959) observed complete inhibition of the prostatic enzyme in serum by 0.025M tartrate. Unfortunately, the possibility that an inhibitor had been found in L-tartrate which could differentiate between SAP and VAP was soon laid to rest by Willott (1972). He showed that

L-tartrate inhibited endogenous VAP to the same extent as it did SAP at the same concentrations. Gomez *et al.* (1975) fully confirmed Willott's findings, as did Brown (1977). Thus, while L-tartrate inhibition may be useful in differentiating SAP from plant, vegetable or other adventitious acid phosphatases, it will not differentiate SAP from VAP.

The other approach, that of devising a separation method for SAP and VAP, was much more successful. Walther and Höhn (1970) using polyacrylamide disc gel electrophoresis had noted two bands of AP activity were observed with semen, but only one with vaginal secretions. Höhn *et al.* (1971) confirmed that two AP active bands could be separated by polyacrylamide disc gel electrophoresis of semen, and that a "storage band" developed after about 4 weeks of storage. VAP was not studied, but the system was useful in differentiating SAP from the AP of plant origin. The discreet banding patterns of the plant source AP enzymes tended to become diffuse, however, after relatively short storage periods.

In 1964, Anzai showed that SAP and VAP could be separated electrophoretically on agar gels in veronal buffers at pH 8.5, the SAP migrating anodically while the VAP migrated cathodically. This observation clearly established that SAP and VAP differ at least in respect to net charge at pH 8.5. Not much notice appears to have been taken of this work in the Western literature for nearly 10 years, perhaps because the paper was published in a rather obscure journal. In 1974, Adams and Wraxall drew attention to this work in proposing a somewhat different electrophoretic method for the separation of SAP from VAP. Their method was carried out on 1 mm polyacrylamide gels with a starch gel insert (Parkin, 1971) with separation taking place in veronal buffers at pH 8.5. Sites of activity were detected using 4-methylumbelliferylphosphate (Cf Section 10.2.4). SAP and VAP could be differentiated from one another using this method, and from fecal AP, buccal epithelial AP, several animal seminal AP, and a number of AP enzymes of plant origin including vaginal yeast type organisms, as well. The activity could be detected in swabs up to 72 hrs post coitus, provided that the swabs were kept in a dry storage environment. Stolorow *et al.* (1976) confirmed the value of this technique. They showed, however, that seminal and fecal enzyme could not always be readily distinguished, particularly in cases where SAP activity was relatively weak. The substitution of α-naphthylphosphate for 4-methylumbelliferylphosphate as substrate in the system did not substantially improve the results. They cautioned, therefore, that great care should be exercised in the diagnosis of azoospermic semen on anal swabs by this technique. In 1975, Sutton and Whitehead reported that seminal, vaginal and fecal AP could be separated by isoelectric focusing in a pH 5-7 gradient on polyacrylamide gels. Seminal AP could be separated readily from either vaginal or fecal AP by this method, but because the isoelectric points of the fecal and vaginal AP bands were very close or identical to one another, fecal and vaginal AP activities could not be distinguished from one another. It was said that this technique had the advantage of speed in comparison to the polyacrylamide gel technique. Isoelectric focusing required only about 3 hrs whereas the polyacrylamide gel system was electrophoresed for 17 hrs. Linde and Molnar (1980) have recently described a procedure in which SAP and VAP can be electrophoretically discriminated, and the PGM_1 isozymes can be typed simultaneously. PGM typing is discussed in section 27.

There is little question that the separation and identification of SAP and VAP presently represent the best method available for assessing vaginal secretion contamination of seminal fluid containing material, and for ensuring that the AP activity in an unknown sample is in fact due to the presence of seminal material.

Blake, Lofgren, Inman and Sensabaugh (see in Sensabaugh, 1977) have made the very important point that any realistic expectation of devising methods for the differentiation of SAP from vaginal and other tissue AP must be based ultimately on molecular specificity. The answer to this problem rests finally on a determination of the relationships between the genetic loci which code for the various enzymes. In comparative biochemical studies, they could show that the catalytic properties and MW of SAP and VAP were similar to one another and to those of kidney or liver lysosomal AP. Further, antisera prepared against SAP cross-reacted not only with VAP, but with the A, B & C isoenzymes of placental acid phosphatase as well. These isoenzymes are discussed in Unit VI, but the results are consistent with SAP and two of the tissue isoenzymes being coded for at a common genetic locus (see in section 29.3). If this turns out to be the case in fact, theoretical limitations would be imposed on the degree of specificity which can be expected from any AP test. Blake *et al.* (see in Sensabaugh, 1977) could show that neuraminidase treatment of SAP and VAP resulted in a change in their electrophoretic mobility on acrylamide gels, treated SAP running with the same mobility as untreated VAP. The result suggests that the two may differ only in respect to sialic acid residues attached to the protein. It could also be shown that vaginal mucus from women using IUD contraceptive therapy frequently showed multiple AP bands on acrylamide, perhaps related to uterine leucocyte build-up in these patients and the leakage of white cell AP into the vaginal pool.

10.3.6 Purification, properties and molecular heterogeneity of prostatic acid phosphatase

The prostatic (seminal) acid phosphatase is systematically called an orthophosphoric monoester phosphohydrolase (EC 3.1.3.2), and has been studied extensively. In the biochemical literature it is often referred to as acid phosphomonoesterase, and abbreviated as "acid PME". In 1946, Lundquist presented preliminary evidence that phosphorylcholine was the natural substrate for SAP. He established this fact firmly the following year (1947a), and carried out a number of biochemical studies on the enzyme as well (1947b). Fluoride, Zn^{++}, oxalate and maleate inhibited SAP to varying degrees, while citrate acted as an activator with phosphorylcholine or glycerophosphate as substrate in

the absence of inhibitors. Systematic studies on the enzyme were carried out by Abul-Fadl and King (1949). They showed, among other things, that the enzyme is not activated by a variety of metal ions, while Fe^{3+} strongly inhibits the enzyme in acetate buffers, but not in citrate buffers. Glycine, alanine and stilbestrol had no effect, whereas cysteine slightly activated and L-tartrate strongly inhibited the enzyme. London and Hudson (1953) purified the enzyme about 5000-fold. Davidson and Fishman (1959) purified the enzyme by different techniques, taking advantage of its stability in Tris-ammonium sulfate solutions at acid pH. An approximately 50-fold purification could be obtained from filtrates of homogenized frozen prostate glands. Nigam *et al.* (1959) studied the properties of the enzyme purified in this way. The K_m for phenylphosphate, p-nitrophenylphosphate and glycerophosphate were found to be different and were not the same in acetate as in citrate buffers. The K_i for tartrate in acetate buffers was 9.5×10^{-6}M with phenylphosphate, 4.5×10^{-5}M with p-nitrophenylphosphate and 2.4×10^{-5}M with glycerophosphate. Oxalate inhibited the enzyme with any substrate, but pyruvate, maleate, glutamate, malonate and glucuronate inhibited only with β-glycerophosphate as substrate. In 1961, Ostrowski and Tsugita reported a purification procedure which yielded a prostatic AP preparation devoid of appreciable phosphodiesterase activity. Kuciel and Ostrowski later (1970) purified the prostatic phosphodiesterase and examined its properties. Ostrowski and Rybarska (1965) reported that the purified prostatic acid phosphatase enzyme had a MW of 95,000. Greenberg and Nachmanson (1965) studied the effect of DIFP on the protein, and could show that it did not irreversibly inhibit the activity. These data, along with the results of the ^{32}P incorporation studies, indicated that, unlike the situation in other phosphatases, O-phosphorylserine did not form as an intermediate in the reaction, and that an amino acid other than serine must be involved in the active site. Ostrowski and Barnard (1971 and 1973) confirmed these results, but said that their kinetic and ^{32}P incorporation studies indicated that a phosphoryl-enzyme intermediate did form. It was of a quite different nature than the intermediate formed by alkaline phosphatase (i.e. O-phosphorylserine), however.

The first suggestion that prostatic AP might exhibit molecular heterogeneity came in 1962, when Sur *et al.* reported that prostatic tissue extract could be separated into multiple bands with AP activity by starch gel electrophoresis. That there are indeed a number of molecular species of prostatic AP came to be recognized through several different lines of evidence, both biochemical and immunological. In 1964, Shulman *et al.*, employing immunoelectophoresis and a specific anti-prostatic AP serum, found that there were two bands with AP activity. The bands could not be observed in extracts of a number of other organs and tissues, except in some sera from patients with prostatic carcinoma. In 1970, Pfeiffer *et al.* confirmed that rabbit immune anti-prostatic tissue extract serum reacted with the serum of patients with prostatic tumors, malignant or benign, and that the precipitate complex had AP activity. Vernon *et al.* (1965) isolated and partially purified two different AP enzymes from prostatic tissue homogenates, and called them P_1 and P_2. Their apparent molecular weights were similar, and were estimated to be about 120,000 to 150,000 by sedimentation equilibrium, and 110,000 to 130,000 by calibrated Sephadex columns. Two enzyme activities could be isolated from seminal plasma as well, but their apparent molecular weights were not only different from one another, but considerably smaller than the prostatic tissue enzymes. Lundin and Allison (1966a and 1966b), in their studies of tissue acid phosphatases from a number of different species including human, noted multiple AP bands from human prostate tissue using polyacrylamide disc gel electrophoresis. Ostrowski and Rybarska (1965) had noted that two fractions of AP activity could be observed from prostatic tissue by CM-cellulose chromatography. In 1968, Smith and Whitby purified two AP activities from prostate gland on DEAE-cellulose and noted that the biochemical properties of the two enzymes were identical. Treatment of the material with neuraminidase abolished the apparent molecular heterogeneity of the enzyme, and indicated that the different fractions of activity being observed were due to the attachment of different numbers of sialic acid residues to one and the same protein. Ostrowski *et al.* (1970) confirmed these results using isoelectric focusing. However, the two fractions obtainable by chromatography had different kinetic and biochemical characteristics, and the "desialo" enzyme, obtained by neuraminidase treatment, had somewhat different characteristics than either of the initial fractions. Among other things, the "desialo" enzyme has a greater substrate affinity, and was more strongly inhibited by L-tartrate (Ostrowski, 1971, Dziembor *et al.*, 1971). Derechin *et al.* (1971) purified to a high degree one of the two fractions obtained from DEAE-cellulose chromatography of prostatic tissue homogenate. The enzyme had a MW of 102,000 and was found to be dissociable into two subunits without disulfide bond cleavage. It was found to contain 13 neutral sugar residues, 10 hexosamine residues and 6 N-acetylneuraminic acid residues per molecule. Lam *et al.* (1973) estimated the MW of what was probably the same enzyme from prostatic tissue as 100,000. Either of the two fractions obtained from DEAE-Cellulose chromatography exhibits a number of bands of activity upon polyacrylamide gel electrophoresis or isoelectric focusing (Ostrowski, 1971).

In 1973, Ueno and Yoshida said that they could obtain multiple molecular patterns of AP activity on starch gels from seminal plasma which were reproducible from a given person. Three different patterns were apparent in 95 subjects. Vaginal acid phosphatase showed no heterogeneity in the system. An underlying genetic mechanism was implied, but no genetical hypothesis was presented, nor confirmatory family studies carried out. Pfeister *et al.* (1975) looked at 75 seminal samples, and confirmed that there was molecular heterogeneity, but they observed considerable individual variation and said that they could not confirm Ueno and Yoshida's observations. Suyama *et al.* (1976a and 1976b)

fractionated seminal plasma by Sephadex chromatography and by polyacrylamide disc gel electrophoresis, obtaining two fractions of AP activity. From their results, it is pretty clear that these differed in carbohydrate content, although the neuraminidase experiment was not done. It was worthy of note, however, that only two fractions were obtained from seminal plasma, whereas multiple bands had been obtained by others from prostatic tissue homogenate or extract (Lundin and Allison 1966a and 1966b; Smith and Whitby, 1968; Paris *et al.*, 1974). It would appear that seminal plasma may not contain as many molecular species of AP as does the parent prostate gland. Ohya *et al.* (1976) showed that multiple AP bands could be detected in seminal fluid by isoelectric focusing. There were three distinct patterns in 37 different people, very likely corresponding to Ueno and Yoshida's (1973) results. Following neuraminidase treatment, however, AP migrated to a single isoelectric point indicating that the differences could be fully accounted for on the basis of variable numbers of sialic acid residues on the protein.

The immunogenicity of prostatic AP has been studied by a number of workers, and deserves brief mention. As previously discussed, Shulman *et al.* (1964) utilized an antiserum to the enzyme in their immunoelectrophoretic studies, which suggested the presence of more than one enzymatically active molecular entity. Abe (1968) noted that some preparations of anti-AP cross-reacted with serum, and that the cross-reacting serum protein was transferrin. Some transferrin contamination was apparently present in the "purified" acid phosphatase which had been used for immunization. Repurification of the enzyme to remove the contaminant, or absorption of the cross-reacting antisera with partially purified transferrin, rendered the antiserum specific. Mukherjee and Ghosh (1970) showed conclusively that the antigenic determinant and the active site of the enzyme were independent. The antibody from rabbit or guinea pig did not inhibit enzyme activity at all. Kulhanek and Pernicova (1971) confirmed these findings. The fact that the enzyme is still active in an antigen-antibody complex was clear from the work of Shulman *et al.* (1964). If antibody had inhibited the enzyme activity, the precipitin bands could obviously not have been "activity stained". Milisauskas and Rose (1972) could prepare active anti-AP from ammonium sulfate fractions of urine. Moncure and Prout (1970) prepared antibodies in goats to azoospermic semen and to hyperplastic prostatic tissue extracts. By suitable absorption, the antisera could be rendered specific for prostatic proteins. Four prostatic-specific precipitin lines were obtained upon immunoelectrophoresis, two of which had acid phosphatase activity.

10.3.7 Identification of vaginal secretions

It was previously mentioned that "vaginal secretions" do not constitute a well defined body fluid. A brief discussion of the biology and physiology of the vagina, cervix and uterus is included here because the composition of the secretions of these tissues and organs and their cyclic variations have a bearing upon the tests for identifying menstrual blood (section 8.1.1) and on the assessment of results obtained when examining vaginal swabs in cases of sexual assault (sec-10.3.5). Vaginal acid phosphatase has already been discussed in connection with the acid phosphatase test for semen (section 10.3.5).

One of the techniques of identyfying menstrual blood (section 8.1) is based on the identification of glycogen containing epithelial cells (Strassman, 1921; Merkel, 1924). The presence of glycogen in the vaginal epithelium is closely related to ovarian endocrine activity. This relationship has been clear at least since the extensive studies of Cruickshank and Sharman in 1934. Glycogen is present in the vaginal epithelial cells from menarche to menopause. Cruickshank and Sharman suggested (1934a) that glycogen deposition is under the control of estrogens. The fact that glycogen-containing epithelial cells can be found in newborns up to about 3–4 weeks of age is explained by the hormonal influences of maternal circulation. Rakoff *et al,* (1944) agreed with this view. They could also show that the glycogen content of vaginal epithelial cells was cycle-dependent, increasing in the superficial epithelial layers throughout the cycle until just prior to menstruation, when it decreased. Maeyama *et al.* (1977) showed by means of a sensitive enzymatic assay that a similar pattern could be observed in endometrial tissue. Furthermore, fertile patients showed a large increase in endometrial glycogen (about 5-fold) at midcycle, whereas infertile patients showed a much smaller increase (about 1.7-fold). Willson and Goforth (1942) showed that some glycogen was present in the vaginal epithelial cells of postmenopausal patients, and that dietary carbohydrate had no effect on the amount. Administration of 1 mg doses of diethylstilbestrol for 12 days led to significant increases in glycogen levels in these patients. Diethylstilbestrol is used for therapeutic estrogen replacement.

The glycogen content of epithelial cells is closely related to the presence of the natural vaginal flora, which are found during the fertile period of life. The flora consist of a form of bacterial life known for a long time as Döderlein's vaginal bacillus, after Döderlein (1892), who did extensive studies on it. The presence of Döderlein's bacillus follows the same pattern as the presence of glycogen in epithelial cells throughout life (Cruickshank and Sharman, 1934b). The bacteria ferment the glycogen to lactic acid (Cruickshank, 1934) which, in turn, accounts in large part for vaginal acidity. Rakoff *et al.* (1944) showed that there tends not only to be an intravaginal pH gradient, the pH being lower closest to the vaginal orifice and about the same as that of vaginal secretions in the mid-vaginal region, but that there is cyclic variation of the pH as well. Intravaginal pH is lowest at midcycle, and highest at the premenstrual stage. Moreover, the pH determines the type of bacteria which characterize the vaginal flora. Lower pH tends to favor Döderlein's bacillus as the exclusive resident flora, while higher pH (around 5) allows the growth of various staphylococci, streptococci, coliforms, yeast and yeast-like organisms. pH values of 5.5 and upward tend to inhibit

Döderlein's bacillus, and favor the other organisms (Rakoff *et al.*, 1944). More recently, it has become clear that Döderlein's vaginal bacillus is not a single organism, but a mixture of a number of different strains of *Lactobacillus acidophilus* (Hunter *et al.*, 1959). Yeasts and yeast like fungi which may be cultured from the vagina consist of species of *Saccharomyces, Cryptococcus* and *Candida* (Monilia). *Saccharomyces* infections are relatively infrequent, while *Crytococcus* infections are more common and *Candida albicans* is a common cause of mycotic vaginitis (Carter *et al.*, 1959). The gram-negative *Hemophilus vaginalis* has been linked to non-specific vaginitis (Heltai *et al.*, 1959) but not everyone agrees that it is the specific etiological agent (Gardner and Dukes, 1959). The incidence of mycotic infection is related to ovarian hormone function, hormone administration and pregnancy (Carter *et al.*, 1959) as is so-called leucorrhea of non-infective origin (Cruickshank and Sharman, 1934a).

Cervical mucus may be a component of "vaginal secretions", depending upon how the sample is collected. The properties of cervical mucus undergo cyclic changes, which are believed to be related to sperm penetrability and survival, and ultimately, therefore, to fertility. The quantity of cervical mucus secretion increases from the beginning of the menstrual cycle to midcycle, and then decreases again steadily in the secretory phase to menstruation.

Cervical mucus viscosity is more or less inversely proportional to quantity, midcycle mucus being least viscous (Moghissi, 1967). Cervical mucus can be fractionated into two major components, one of which is largely glycoprotein and is responsible for the rheological properties. This material, sometimes called the "high viscosity component" (Davajan and Nakamura, 1975) is thought to consist of a polypeptide backbone with various carbohydrate residues, including N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid, attached by way of O-seryl and/or O-threonyl linkages (Gibbons and Mattner, 1967). The other component, sometimes called the "low viscosity component" (Davajan and Nakamura, 1975), consists in part of serum proteins, albumin and γ-globulins which exhibit cyclic variations as well (Moghissi, 1967). As previously noted (section 4.2.5), cervical mucus contains an endogenous AP activity which exhibits cyclic variation, and the variation is altered by hormonal or intrauterine contraceptive therapy (Gregoire *et al.*, 1972).

Vaginal secretions may also contain esterase, alkaline phosphatase, β-glucuronidase and DPNH-diaphorase which originate from the epithelial linings (Fishman and Mitchell, 1959) and smaller molecular weight carbohydrates, such as glucose, maltose and maltotriose (Sumawong *et al.*, 1962). Recently, Raffi *et al.* (1977) identified albumin, α-antitrypsin, α_2-haptoglobin, α_2-macroglobulin, β-lipoprotein, orosomucoid, ceruloplasmin, γ-chains, γG.K. (Bence-Jones) and IgG, IgA and IgM in vaginal fluid as well as in cervical mucus. The absence of α_2-haptoglobin, α_2-macroglobulin, β-lipoprotein, orosomucoid and IgM in a population of hysterectomized women led to speculation that the presence of these proteins in the vaginal fluid of normal women may in fact be due to cervical mucus contamination. Not all the proteins were found in all the normal subjects with the single exception of albumin, which was universally present.

It is worthy of note that Thomas and Van Hecke (1963) proposed that recent intercourse by males could sometimes be diagnosed by demonstrating vaginal epithelial cells on the glans penis. The iodine vapor method for glycogen containing epithelial cells was employed (section 8.1.1). obviously, the suspect man would have to be examined quite soon after coitus, and prior to any cleansing, for this method to be of value. And too, Popielski's findings would have to be considered before interpreting the results (section 8.1.1). Rothwell and Harvey (1978) have recently questioned the validity of "vaginal" epithelial cells being identified on penile swabs. Their studies indicated that false positive findings can be obtained, and that this approach to determining whether a male has recently had intercourse may not be a reliable one.

10.4 Immunological Methods for the Identification of Semen

10.4.1 Precipitin tests

The development of an immunological test for semen identification followed closely upon the development of the immunological test for species determination of blood and bloodstains by Dr. Uhlenhuth. This latter material is discussed in Unit IV (section 16.1.1). Once it became recognized that more or less specific antibodies could be prepared against blood, organ and body fluid proteins, the preparation of anti-seminal plasma antisera and the exploration of their applicability to medico-legal evidence logically followed. It must be kept in mind too that, at the time, around 1900, essentially the only medico-legal tests available for the identification of semen were the finding of spermatozoa and the Florence test (section 10.5.1). There was not universal agreement as to the proof value of the crystal test, and the unequivocal identification of azoospermic semen in forensic cases was a significant problem. What is now generally called the "precipitin test" was then often referred to as the "biological test" or the "serological test". The term "serological test" would be less preferred today, since it could easily be confused with tests designed to determine the ABH groups in semen. The blood groups were only discovered in 1900, however, and it was not until 1926 that the presence of soluble ABH substances in semen came to be recognized.

Farnum (1901) was the first investigator to prepare antibodies to human, bull and dog semen and to testicular extracts. The antisera were species-specific within the domain of the species under investigation, and the anti-bull semen did not cross react with goat semen. The anti-human semen serum reacted with the filtered extract of a 34 day old human seminal stain, and did not cross react with human serum. Farnum suggested that the medico-legal applicability of such antisera be pursued. Strube (1902) prepared antisera to human semen and testicular extracts in rabbits, but there was some cross reaction with serum. Pfeiffer (1905) looked

at the immunogenicity of bull semen, and found that the crude antiserum cross reacted with homologous serum and organ extracts, but that it could be absorbed by the cross-reacting materials and rendered specific. Dunbar (1910) studied immunological reactions of antisera prepared against the pollen of various plants, and the semen of several species of fishes. Dervieux (1921 and 1923) prepared anti-human semen serum, and found that it was species-specific. Antisera could be rendered semen-specific by suitable absorption. Dervieux thought that the antiserum cross-reacted more strongly with male than with female serum, and that this fact might be useful in sexing bloodstains. He said further that the reaction was stronger with the semen of the individual which had been used for immunization, and that the antiserum might, therefore, be individual-specific. Although the presence of soluble ABH substances in seminal plasma had not yet been recognized, it is possible that the stronger precipitin reaction he obtained with the semen against which the antiserum was actually prepared, as compared with that from different individuals, could be explained on this basis (see in Section 19.8.1).

In 1922, Hektoen carried out extensive experiments on the preparation and applicability of antisera against seminal plasma to human semen detection. The antisera obtained reacted with semen as well as with serum, though absorption by serum rendered the antibody preparation semen-specific. Absorbed antisera had titers of about 1:256 against the supernatant liquor obtained by centrifuging seminal plasma, although with different samples of semen, it could be as low as 1:8. Seminal and prostate extract stains reacted, but not stains of blood, serum, saliva or pus. Extracts of stains soaked in soap solutions gave non-specific precipitin reactions. Antisera to several animal semens were prepared and tested as well. Hektoen did not agree with Dervieux's assertion that the antisera possessed any individualizing specificity, nor with his finding that anti-serum serum did not cross react with homologous semen. Hektoen and McNally (1923) regarded the applicability of the precipitin test to forensic cases for semen stain identificaiton as being promising, but not clearly demonstrated. In 1928, Hektoen and Rukstinat noted that their anti-human serum serum, which had a titer of about 1:10,000 against serum, had a titer of about 1:20 against semen. Conversely, they could prepare anti-semen serum with a titer of about 1:1000 against semen, and this reacted with serum to dilutions of around 1:20.

There does not seem to have been a great deal of interest in the subject for a number of years following Hektoen's investigations, although the immunological test was mentioned in textbooks, and was certainly in use in some laboratories. Some authorities indicated that the technique was primarily of value for species determination, in cases where there was suspicion that non-human semen might be involved, rather than for the identification of semen as such.

In 1963, Coombs *et al.* prepared rabbit antiserum to human seminal plasma, which could be rendered semen-specific by absorption with serum and with boiled saliva. The antiserum did not cross react with extracts of stains of sweat, blood, or several animal semens. There was a weak cross reaction with pig semen, which could be removed by absorption. This work, which brought about a renewed interest in the subject, was apparently prompted by Hermann's immunoelectrophoretic studies (1964) of human seminal plasma, showing that while semen shared some proteins in common with serum, there were a number of seminal-specific proteins as well. Mischler and Reineke (1966) recommended the immunological method. They could show that the reaction occurred with seminal stain extracts even after the stains had been washed with soapy tap water or heated. Culliford (1964 and 1967) confirmed many of the findings of Coombs *et al.*, noting the importance of using antisera prepared against the specific body fluid one wants to identify in any immunological identification test. He also showed that the test could be carried out using crossed over electrophoresis. The antihuman semen serum, prepared according to Coombs *et al.* (1963), could have a titer as high as 1:8000 against human seminal plasma. Černov (1971) reported preparation of an anti-human semen serum in rabbits which did not cross react with capillary or menstrual blood, saliva, urine, nasal mucus, horse radish extract or vaginal secretions. It was species-specific as well. The problem of cross reactivity with vaginal mucus is obviously very critical if the antiserum is to be used for the identification of semen. Kerek (1972) got positive immunological reactions with seminal stain extracts after storage of the stains at room temperature for up to 14 months and at -20° for up to 4-1/2 years. Thornton and Dillon (1968) showed that the immunological test for semen could be carried out by immunodiffusion of cellulose acetate membranes. They got no cross reactions in this system with blood, saliva, urine or vaginal secretions using a commercial anti-human semen serum. Tröger and Jungwirth (1974) said crossed over electrophoresis was more sensitive than immunodiffusion (in gels), an antiserum they tested being able to detect a 1:1500 dilution of whole semen by the former, and only a 1:600 dilution by the latter method. In 1963, Suyama and Sawada reported that they had prepared a specific anti-seminal acid phosphatase antibody by immunization of rabbits with prostatic tissue homogenates and subsequent absorption with serum. The antiserum could be used to identify stains up to 4 years, 2 months old in an immunodiffusion test, but was negative with a 9 year, 4 month old stain. The antibody did not react with saliva, nasal mucus, urine or plant acid phosphatases, and with serum only in patients suffering from metastasizing prostatic carcinoma.

It is to be noted that most of the procedures mentioned in the foregoing discussion relied upon antisera raised against pooled human seminal plasma. Studies on the antigenic composition of human semen have indicated that the fluid may in fact contain several specific antigenic proteins. This matter is discussed in detail in section 10.13.2. If a seminal plasma-specific protein could be isolated, and specific antisera raised against it, the basis would be provided for a

specific immunological test for the identification of the material. This approach has been taken by the Japanese workers in their studies on γ-seminoprotein (see section 10.10) and by Sensabaugh (1977) in his studies on the p30 protein (see section 10.13.2). In the latter study, it could be shown that several commercial anti-human semen reagents contained a preponderance of antibodies against lactoferrin (see section 10.13.1) which occurs in milk, sweat and tears as well.

10.4.2 Other immunological tests

Weil *et al.* (1959) utilized an immunological test different from the precipitin reaction for detecting seminal fluid residues on female genital swabs. The test relied on the detection of the reaction between seminal plasma antigens and specific antibodies, but a complement fixation assay was employed. Vaginal swabs were soaked in saline to elute any seminal antigens. After centrifugation, serial dilutions of the supernatant fluid were incubated with antiserum and complement for a time, and the solutions then tested with sheep red cells and anti-sheep red cell homolysin. Absence of hemolysis indicated that complement had been fixed, and that the swab had contained seminal antigens. Inhibition of hemolysis at a 1:4 dilution of vaginal swab eluate was required before the test was regarded as being positive, and an antiserum had to be used which did not cross react with vaginal mucus serum proteins. While the test is exceedingly sensitive, it does not appear to have been very widely adopted.

Baxter (1973) tested a number of immunological techniques for detecting the antigen-antibody reaction, and their applicability to semen identification. A pooled, unabsorbed, hyperimmune serum was found to be species specific, but to be cross reacting with a number of other body fluids, including vaginal secretions. One cross reacting antigen was common to semen, tears and saliva, two others to tears and semen, and at least two more to semen and vaginal secretions. The serum contained an anti-A activity as well. No practical means could be found to remove all the unwanted cross reactivities. Attention was turned, therefore, to the anti-acid phosphatase activity in the antiserum. As noted previously (Section 10.3.6), Shulman *et al.* (1964) had shown that the catalytic site and the antigenic determinant of acid phosphatase were independent, by virtue of the fact that the antigen-antibody complex still showed acid phosphatase activity. Mukherjee and Ghosh (1970) had unequivocally established the independence of the two sites. Baxter (1973) took advantage of this property in his studies, and found that the anti-AP activity of the anti-semen serum cross reacted with the AP of vaginal mucus. Studies using electroimmunodiffusion (see section 2.4.3) were then conducted to quantitate acid phosphatase in these fluids. In this technique, an antiserum is incorporated into a gel (agar or agarose) support medium, and antigen-containing samples subjected to electrophoresis in the gel. As the antigen moves, the antigen-antibody reaction occurs along the path of migration, forming rocket-shaped peaks, which can be detected with acid phosphatase staining reagents. Peak height is proportional to antigen (i.e. acid phosphatase in this case) concentration in the sample (and inversely proportional to antibody concentration). SAP and VAP were found to have different mobilities in the system, and could be distinguished on this basis. SAP migrated anodally, while VAP showed an apparent cathodal and diffuse pattern, probably the result of electroendosmosis. Blind trial studies showed that false positives were not obtained for SAP in this system, but that the test could be negative in the presence of semen. The test became negative on vaginal swabs at 7–9 days postcoitus. A 6-month old stain extract could be detected. It was noted that the sensitivity of the method could be adjusted by varying the concentration of antiserum in the gel. Baxter said that the method had the advantages of speed and ease of manipulation in routine casework. Gdowski (1977) reported that she had successfully employed this procedure. It is to be noted that the technique is in some ways more closely related to the SAP/VAP separation procedures discussed in section 10.3.5 than to the other immunological techniques.

As a matter of historical interest, it should be noted that a number of workers looked into the applicability of anaphylaxis as an immunological method for the detection and species determination of seminal plasma. Any method employing immunological phenomena as a basis could theoretically be adapted to the needs and requirements of medico-legal tests. Once an antiserum has been obtained which exhibits appropriate specificity, any of a variety of immunological methods are available for detecting the antigen-antibody reaction, including precipitation, agglutination reactions, complement fixation and anaphylaxis. Applications of all these techniques have been employed at various times by various workers. More work has been carried out on immunological determination of species of origin of bloodstains than for any other purpose in forensic immunology. These studies are discussed in Unit IV. For various reasons, most workers have preferred precipitin reactions to detect antigen-antibody reactions, and still do at the present time.

Anaphylactic shock is perhaps the most dramatic indication that an antigen-antibody reaction has occurred. The guinea pig responds quickly and characteristically and has commonly been used in anaphylaxis experiments. In principle, the technique consists of injecting the test animal with a foreign protein (the sensitizing injection). After a suitable interval of time, during which there are no harmful effects, a further injection of antigen (the shocking or toxic injection) then causes the onset of anaphylactic shock. In the guinea pig, this reaction is systemic, and takes the form of restlessness, chewing, dyspnea, convulsions and usually death. The anaphylaxis reaction as a means of determining that an antigen-antibody reaction has occurred is generally as sensitive and specific as other methods.

Pfeiffer (1910) conducted anaphylaxis experiments with cattle semen, seminal proteins and blood and thought that the reaction was organ specific, i.e., shock was not induced by seminal protein if serum had been used for sensitization.

Minet and Leclercq (1911c and 1911d) first indicated that anaphylaxis could be used as a means of detecting semen-anti-semen reactions. These studies were based on their earlier experiments on the test for species determination in bloodstains (1911a and 1911b) (see Section 16.6.2). They found that the method was species-specific and specific for semen as well. Verger (1911) confirmed these results, but noted that there was some variability in the reactions of individual animals, and that a number of animals (about 10) should always be used in the test to compensate for the possibility of occasional negative reactions. Alvarez de Toledo y Valero (1915) conducted experiments on the method, but could not confirm the seminal specificity. Serum induced shock in animals sensitized with semen, and conversely. In his review in 1918, Lecha-Marzo said that the method appeared to require further evaluation and study as to its medico-legal applicability. Hektoen and McNally (1923) agreed with Lecha-Marzo. The test does not appear to have enjoyed very wide practical use, as it is obviously somewhat impractical *per se,* and probably hopelessly so for a laboratory conducting many examinations on a routine basis.

10.5 Crystal Tests

Crystal tests were the first non-morphological tests for semen to be proposed which have persisted until relatively recent times (see section 10.1). The first paper on the subject appeared in 1896, and the tests are still used in some laboratories. Any number of modifications have been proposed, and other crystal tests, based on other active constituents of the seminal plasma have been reported. The initial report of a crystal test for semen created something of a flourish of activity in the medico-legal community, everyone being anxious to have a reliable non-morphological test at their disposal.

10.5.1 The Florence Test

In 1895 and 1896, Dr. Florence in Lyon published a series of papers recounting his studies on seminal fluid and its medico-legal identification. Much of the material dealt with isolation and recognition of sperm cells in seminal stain investigation, and a good review of older methods was given as well (see in Unit IX, Translations). In the third paper, the now-familiar Florence test was introduced. Florence did not make any particularly extravagant claims for his test, and appears to have considered it primarily a useful presumptive test that would save the time required to conduct a careful search for sperm cells in every suspected seminal stain. The reagent consisted of 1.65 g KI and 2.54 g iodine in 30 mℓ water. The amount of iodine could be halved without effect. The test was found to be quite sensitive, and was always obtained with seminal stains. The characteristic crystals were not formed by nasal or vaginal mucus, urine, sweat, saliva, tears, milk, cerebral fluid or leucorrheal discharge, nor with several animal semen samples tested. The seminal component giving the crystals was called *virispermine,* and Florence said that it was not identical to Poehl's spermin (Poehl, 1891a and 1891b) which latter, he said, did not give the test. He believed that the test was specific for semen, and that a positive result provided very strong evidence of its presence, even if no spermatozoa could be found. The possibility that other materials, not yet investigated, would give identical crystals was not discounted, however. Johnston (1896) confirmed Florence's results, and said that blood, pus, nasal and vaginal secretions and urethral secretion did not give the test. He got a test on a year old seminal stain. Johnston (1897a and 1897b) noted that he had made some effort to identify the active substance in semen but had been unable to do so. The test was usually carried out on a microscope slide, the sample being put in place, a cover slip added, and reagent allowed to diffuse under the cover slip. The crystals began to form at the interface of the liquids, and were identified visually by microscopical examination.

Richter (1897a) reported that saliva and nasal mucus did not give the test, but that he could get a positive test with vaginal and uterine mucus from dead bodies, as well as with prostatic fluid and various organ extracts from decomposing tissues. He said further that Poehl's spermin did give very similar crystals, but that he did not think it was the active principle in semen. He tested a number of substances to determine whether they would give Florence's crystals. Partly on the basis of the fact that lecithin gave the test, he thought choline might be involved and perhaps other things (1897b). He did think that seminal stains always gave the test, however, and that it had value as a sorting technique. Whitney (1897) showed that seminal stains 2½ years old gave a positive test. No reaction was seen with urine, saliva, milk, fecal matter or leucorrheal or gonorrheal discharges, but morphine, strychnine and a number of alkaloids gave the test. He considered a positive result, therefore, as presumptive evidence of semen. In the following year, Whitney indicated that he thought it was the choline from the lecithin in semen that was reacting with the halogens to form the crystals (Whitney, 1898a and 1898b). That choline was in fact the reactive constituent of semen was established by Bocarius in 1902. Duquenne (1900) conducted a number of experiments on the procedure. The test could be negative in stains that had been soaked in alkali soap water, alcohol and dilute HCl, and several other solvents. Some alkaloids and pyridine and methylpyridine gave the test, but nasal, vaginal and uterine mucus did not. Duquenne's results prompted the Editor of the *New York Medical Journal* to warn, in 1901, that the Florence test should be regarded as presumptive only, until more information about its actual specificity was available. In 1909 and 1910, Dervieux said that Florence's test had no medico-legal value, whether the results were positive or negative.

De Domincis (1912) proposed a modification of the test in which $AuBr_3$ was used in the reagent. He thought that this method was specific, and had medico-legal value. Welsch and Lecha-Marzo (1912) reviewed the subject, but did not accord the test the same proof value as had De Domincis. In 1907, Lecha-Marzo had said that the test was not specific for

human semen. Hektoen and McNally (1923) considered a positive Florence test to be presumptive, but a negative test to indicate the absence of semen unequivocally. Villamil (1934) proposed the use of AuI_3 as the crystallizing agent. This reagent was sensitive to up to a 1:500 dilution of whole semen, but he did not claim any more specificity for the test than would be attributed to Florence's original test. In 1939, Bagchi noted that he had observed many examples of negative Florence tests in the undoubted presence of semen, and that negative inferences could therefore not be drawn. He believed that the test was semen-specific, however, and that positive results were certain proof of semen. Forbes (1940) did not agree. A positive result was presumptive evidence in his view, and negative results did not necessarily establish the absence of semen. Depending on the individual sample, the test failed in Forbes' hands with dilutions of whole semen of from 1:3 to 1:40. Seminal vesicle contents gave crystals in the absence of prostatic secretion, and prostatic fluid alone gave negative results. These results were in disagreement with the contention of Kahane and Levy (1939) who had said in their paper on seminal choline that the choline was contained in semen in a phospholipid, and that a prostatic enzyme was needed to hydrolyze this material and liberate the free choline. Fletcher *et al.* (1935) showed that in rats spermatic fluid was the tissue with the richest choline content. Kaye (1947) reviewed the test and some of its modifications and noted that a positive result was presumptive, but that a negative result indicated that semen was absent. Palmieri (1955) made something of the same point about the semen crystal tests that Dalla Volta (1932) had made earlier with respect to the blood crystal tests (Section 4.2.1), namely that more careful, accurate, physicochemical characterization of the crystals should be carried out before using them as a basis for an identification. His contention applied to the Florence test, and to the other crystal tests which are discussed below.

Takemoto (1970) suggested a curious kind of acid phosphatase test using the Florence method. The stain extract was incubated with choline phosphate in pH 5 acetate buffer. In the presence of AP, choline was liberated upon the hydrolysis of the choline phosphate, and could be detected by means of Florence's reagent.

Kerek (1972) reported no difficulty in getting a positive Florence test in stains which had been kept up to 14 months. Kind (1964) noted that he very rarely got a positive Florence test with vaginal swabs. Davies and Wilson (1974) said that vaginal swabs were often negative. The swabs which did give positive results were all taken within a day of intercourse, and the majority within 14 hours.

It may be noted that Kahane and collaborators conducted a number of studies on the biochemistry, metabolism and tissue distribution of choline. Seminal choline was discussed by Kahane and Levy in 1937. Human semen is said to contain from 11.2 to 14.4 mg choline/100 mℓ semen (*Blood and Other Body Fluids,* 1961). Any tissue or biological material containing sufficiently high choline concentrations would give the Florence test.

10.5.2 The Barberio Test

In 1905, Barberio in Naples reported a different crystal test for seminal fluid. The original paper (1905a) in the Italian language was translated by Dr. A. W. Herzog, and published in the American literature with Dr. Barberio's concurrence (1905b). It was noted that the original claims of species and seminal fluid specificity made by Dr. Florence for his crystal test had not withstood the experimental scrutiny to which it had been subjected. The Barberio test employed a saturated aqueous solution of picric acid. Saturated solutions of picric acid in absolute ethanol served as well. The test was carried out on a microscope slide, and the resulting refractive, yellow crystals examined microscopically. The reaction was said to be very sensitive. Positive results could be obtained with semen, seminal stains and partially putrefied seminal material. Seminal stains which had been heated to 150° for an hour still gave crystals, as did a 3 year old stain. Exposure of the stain to temperatures of 200°, however, abolished the reaction. Vaginal mucus, nasal mucus and saliva did not give the test. Barberio thought that the substance in semen responsible for the reaction was organic, was contained in seminal plasma even in azoospermic specimens, and was different from the reactive substance in Florence's test.

Cevidalli (1906) proposed that the test be carried out with glycerin containing saturated solutions of picric acid in alcohol. He did not get the crystals with dog, horse or pig semen, and thought that the active principle in human semen which was reacting with the reagent might be protamine. Bokarius (1907) used picric acid in nitric acid solutions containing glacial acetic acid or cadmium iodide for the test. Posner (1907) said that the test was specific for human semen. Lecha-Marzo in his 1907 review discussed Barberio's procedure in some detail. He regarded it as more specific than Florence's test, and much preferred it. Littlejohn and Pirie (1908) said that in their experience the test had proven to be specific for semen. The test could, however, be negative in the presence of spermatozoa, but these results had been observed in urine specimens rather than in seminal ones. They noted that they obtained better results with the original reagent than with Cevidalli's modified solution. When positive, the result was considered to be a better indication of the presence of semen than was a positive Florence test. Dervieux (1909 and 1910) said that he put no confidence in the test whatever, regardless of whether positive or negative results were obtained. Lecha-Marzo covered the subject again in his 1918 review, noting that many other body fluids, including vaginal mucus and a number of vegetable extracts, gave negative results.

Baecchi (1913) suggested that the crystals might be spermine picrate. Rosenheim (1924) mentioned that Barberio's crystals were in fact spermine picrate. He was studying spermine phosphate crystals, which form spontaneously in semen if it is left sitting for a time. He noted that the spermine phosphate crystals were first observed and described by van Leeuwenhoek in 1678, and subsequently rediscovered twice,

by Vauquelin in 1791 and by Böttcher in 1865. Poehl (1891a and 1891b) had isolated these crystals, and so-called Poehl's spermin was actually spermine. Harrison (1932) established beyond doubt that Barberio's crystals were spermine picrate. He proposed a modification of the test as well. He said that the search for the crystals was made difficult by all the amorphous protein precipitated by the picric acid. If the stain was extracted with 2.5% trichloroacetic acid, and centrifuged to bring down the protein, the test could be more easily performed on the supernatant fluid, which contained the spermine. Blood, pus, plasma, serum, urine, feces, saliva, and cow's milk all gave negative results with the test. The structure of spermine is shown in Fig. 10.5, and that of choline in Fig. 10.6. Spermine occurs in human seminal

$$H_2N-CH_2CH_2CH_2-NH-CH_2CH_2CH_2CH_2-NH-CH_2CH_2CH_2-NH_2$$

Figure 10.5 Spermine

plasma in concentrations ranging from 20 to 250 mg/100 mℓ semen. (*Blood and Other Body Fluids,* 1961). Fair *et al.* (1972) said that the spermine content of human semen positively correlated with sperm motility.

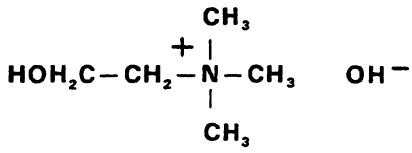


Figure 10.6 Choline

10.5.3 Puranen's Test

In 1936, Puranen proposed a microchemical test for semen using dinitronaphtholsulfonic acid, or Naphthol Yellow S, as reagent. This compound, like picric acid, reacts with spermine to form characteristic orange crystals. He thought that this method was specific for human semen. The structures of Naphthol Yellow S (Naphtholgelb S), which is the salt, and of the corresponding acid, flavianic acid, are shown in Figure 10.7.

Berg (1949) studied the reaction quite extensively. Blood, urine, feces, nasal mucus, saliva, human milk, cheese and vaginal and uterine mucus all gave negative results. Some animal semen reacted in the test, and Berg thought that the test was seminal specific but not human specific. Berg discussed the technique briefly in his 1954 paper as well.

10.5.4 Other crystal tests

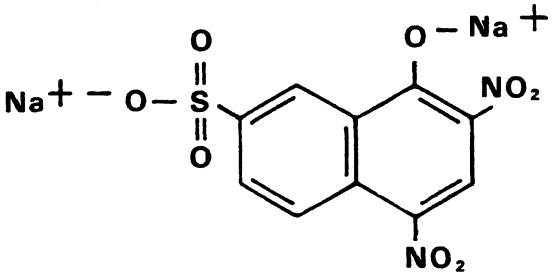
Two other tests have been proposed, but have not enjoyed very wide use. Niederland (1931) said that if seminal stain extracts are treated with dilute H_2SO_4, crystals will form. The test is not semen-specific, and is given by animal semen, vaginal secretions, serum, hen egg albumin and some other materials. It was suggested that the test had primarily negative value.

Peltzer (1931) noted that seminal stains would foam if treated with H_2O_2. If the stain material was treated with 2% eosin, long lance-like crystals, similar to Florence's, formed, and these could be converted to characteristic Florence crystals by the addition of iodine/KI solution.

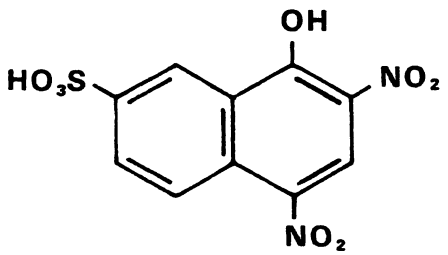
10.6 Chromatographic and Electrophoretic Methods

The chromatographic and electrophoretic methods which have been proposed as means of identifying seminal stains are based on the separation and identification of one or more lower molecular weight substances found in semen in particularly high concentrations. These are primarily choline, spermine and spermidine. Spermidine has the structure $H_2N\text{-}CH_2(CH_2)_2NH\text{-}(CH_2)_3CH_2\text{-}NH_2$, and is closely related to spermine (Fig. 10.5).

In 1957, Fiori noted that spermine and spermidine could be separated from seminal stains by descending chromatography on Whatman No. 1 filter paper in a solvent system consisting of n-butanol:acetic acid:water:40:10:50. The method was sensitive to 1 $\mu\ell$ of semen, and spermine had an R_f value of 0.03. Thoma *et al.* (1959) proposed a procedure in which spermine was extracted into chloroform



(a) Naphthol Yellow S



(b) Flavianic Acid

Figure 10.7 Naphthol Yellow S and Flavianic Acid

from seminal stain material in solutions of K_2CO_3. The chlorophorm phase was then subjected to paper chromatography. Gültingen (1961) studied this technique and concluded that it was unreliable. He also said that Thoma had told him that they had become aware of many of the problems Gültingen had encountered after the publication of their paper, and had not pursued the matter. Levonen (1960) employed ascending paper chromatography in isopropanol:acetic acid:water::50:10:40 to seminal stain extracts. The stains were extracted with 20% trichloroacetic acid, this material extracted with ether, and the aqueous phase evaporated to dryness and redissolved in dilute HCl. 10 $\mu\ell$ of semen in a stain was required for a positive result. Using Dragendorff's reagent, spermine appeared as a pink spot at R_f 0.32, while choline appeared as a deep purple spot at R_f 0.74. Satch *et al.* (1967) reported a virtually identical method, except that choline was said to have an R_f value of 0.79. They tested vaginal secretions and menstrual blood as well, with negative results, and these contaminants did not interfere with the chromatography. Stains up to 10 days old could be diagnosed. Gültingen (1961) said that he did not think the paper chromatography methods were very reliable. Djalalov (1974) reported a paper chromatographic procedure for the simultaneous separation and detection of spermine, choline, acid phosphatase and seminal amino acids.

Hessel *et al.* (1967) reported the detection of spermine and choline from as little as 1 $\mu\ell$ semen by thin layer chromatography. 1N HCl was used as an extraction medium and as developing solvent on 250 Silica Gel F plates. Choline was detected with Dragendorff's reagent at R_f 0.5, and spermine was detected at R_f 0.85 by overspraying with potassium iodoplatinate reagent. It was found that purified spermine and choline ran slightly faster than when contained in seminal plasma.

Yano (1970) reported a method for the detection of spermine and choline by thin layer chromatography. 10 $\mu\ell$ seminal plasma was required for the test. Stains up to 5 years old gave positive results, and boiling the stain in water for 1½ hrs did not destroy the spermine or the choline. A number of other body fluids, including vaginal secretions, and the juices of a number of fruits were found to contain no detectable choline or spermine. Hallcock (1974) reported a method similar to that of Hessel *et al.* (1967). Saline was used to extract the stain and 30 $\mu\ell$ of the extract was applied to the plate. She developed the spermine spot with potassium iodoplatinate (R_f = 0.85) and the choline spot by over-spraying with concentrated H_2SO_4 (R_f = 0.58).

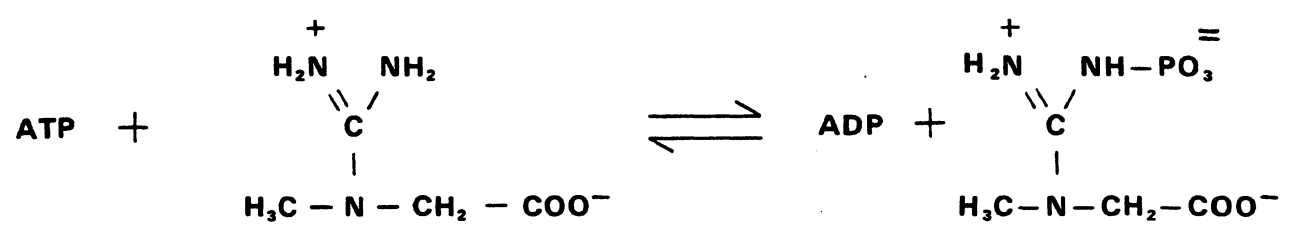
Bureš (1968) used paper electrophoresis in pH 4.6 citrate buffers for the separation of spermine and choline. Samples were extracted with 0.5N HCl, and the extracts spotted anodically. After electrophoresis for 15 min at 15V/cm, the paper was dried and sprayed with Dragendorff's reagent. Vaginal secretions, saliva, perspiration, urine and blood stains gave negative results, and did not interfere.

Kosatík *et al.* (1966) used ascending paper chromatography in butanol:formic acid:water::10:1:5 to detect citric acid for the identification of semen. Semen, it was said, contains from 80 to 410 mg % citric acid, while urine may contain 20 to 120, human milk 127 and other body fluids tested, including vaginal secretions, less than 10. Zipkin and McClure (1949) reported that human saliva contains only 0.2 to 2.0 mg % citric acid. Stains from 3–5 weeks old gave positive results, and the R_f of citric acid in the system was 0.42-0.47. The presence of citric acid in human semen was first noted by Schersten (1930). The physiological role of citric acid in semen was discussed by Humphrey and Mann (1948). Seminal citric acid originates in the seminal vesicles. Kirk had suggested the possible use of citric acid as a seminal marker in 1953.

Nickolls (1956) noted that the detection of fructose might prove valuable as a basis for seminal stain identification, but at that time no one had yet explored the possibility. Fructose is present in human semen, and is the principal reducing sugar (Mann, 1946a). Spermatozoa metabolize the fructose along the principal fermentative carbohydrate pathway, a process called fructolysis. Most cells metabolize glucose principally along this pathway (glycolysis). Once fructose-6-phosphate is formed, of course, the pathways are identical. Seminal fructose originates in the seminal vesicles, and its levels are responsive to testosterone concentration. The metabolism and physiological role of fructose in semen have been extensively studied (Cf Mann, 1945, 1946a, 1946b; Mann and Parsons, 1947). Erben (1971) showed that seminal fructose could be separated from seminal stains by paper or thin layer chromatography, and readily distinguished from glucose or sucrose. Kirk (1953) noted that a kind of paper chromatographic method very similar to the one which had been described for separating blood stains from debris (Cf section 7.2) was equally applicable to the separation of seminal stain materials from debris.

10.7 Creatine Phosphokinase

In 1964, Griffiths and Lehman suggested using the high levels of creatine phosphokinase in semen as a basis for the medico-legal identification of seminal stains. Creatine phosphokinase (creatine kinase; CPK; EC 2.7.3.2) is systematically known as ATP:creatine phosphotransferase, and catalyzes the reaction:



According to these investigators, semen contains from 385 to 14,000 units CPK per mℓ, a concentration higher than in any other body fluid tested, including normal and pathological serum, feces, gastric juice, urine and vaginal secretions. In stains the activity declined as a function of stain age. At 24 hours of age, stains showed a mean activity of 142 mols creatine formed/mℓ extract/hr. The reaction was measured using creatine phosphate and ADP as substrates. By 1 month of age, the mean activity had dropped to 132, and by 6 months of age, to 78. They said the test should be regarded as presumptive evidence for the presence of semen.

In 1968, Suyama *et al.* showed that seminal acid phosphatase hydrolyzes creatine phosphate in the absence of ADP. If a specific SAP inhibitor were added, the CPK activity of semen was found to be very low. They believed that Griffiths and Lehman had actually been measuring the AP activity of semen with creatine phosphate as substrate, and that the method was, therefore, not only not new, but in no way superior to any other acid phosphatase test.

10.8 The Lactic Dehydrogenase-X-Isoenzyme

A brief discussion of lactic dehydrogenase (LDH) was given in section 8.1.5. LDH activity in human semen was first reported by MacLeod and Wroblewski in 1958. They measured total activity in a number of samples, and found that LDH activity was lower in the seminal plasma than in whole (sperm-containing) semen. In 1963, Blanco and Zinkham reported that they had observed an LDH isoenzyme unique to human sperm. It had an electrophoretic mobility on starch in pH 8.6 buffers in between that of LDH-3 and LDH-4. In addition, its heat stability, kinetic properties with pyridine nucleotide analogs and chromatographic behavior on DEAE-Cellulose were likewise intermediate between those of LDH-3 and LDH-4. The enzyme did not occur in seminal plasma, nor in prepubertal testicular tissues. Goldberg, in the same year, independently confirmed the observation using polyacrylamide disc gel electrophoresis. He found that the enzyme could be solubilized by exposure of the sperm cells to sonic oscillations. In 1967, Farriaux *et al.* suggested the application of LDH-X, as the new isoenzyme had been named by its discoverers, to the diagnosis of seminal stains. The enzyme could be found in 35 day old stains, and showed a greater resistance to inhibition by 2M urea than some of the other LDH isoenzymes, and incorporation of the inhibitor into the procedure simplified the detection of LDH-X. The isoenzyme can be detected in men with low sperm counts (10^6 cells/mℓ), as well as in those with necrospermia and altered sperm morphology (Szeinberg *et al.*, 1967). The LDH-X activity as a fraction of total seminal LDH activity varies from one individual to another, and the isoenzyme was not detected in one person with a sperm count of 22 x 10^6/mℓ (Eliasson, 1967). Studies with split ejaculates indicated that the prostate was the primary source for the LDH isoenzymes other than X. Zinkham reviewed the subject in 1968, and again in 1972, noting that the enzyme is unique to sperm cells. The enzymatic and biochemical properties, in comparison with some of the other seminal LDH isoenzymes, were given as well. It was clear, at least in birds, that another structural gene, controlling the synthesis of an additional polypeptide chain, is involved in LDH-X production (Zinkham, 1968). Ressler *et al.* (1967) obtained data in their immunological experiments with anti-LDH-1, anti-LDH-5 and anti-LDH-X which suggested that LDH-X synthesis in humans was controlled by a third allelic locus. Evrev *et al.* (1970) noted that the isoenzyme could be found in tissue cultures of mature human testes and of seminiferous tubule cells, as well as in sperm. Dixon and Gonsowski (1974) mentioned LDH-X in their studies on the use of the LDH isoenzyme determination for menstrual blood identification (see section 8.1.5). Schmechta (1975) reported that the LDH-X activity averaged about 29% of total LDH activity in 12 samples, and that the enzyme could be detected on cellulose acetate membranes from stains up to 36 days old. Hule and Hrdlička (1975) said that LDH-X is sometimes absent in normospermic specimens. Mokachi and Madiwale (1976) recommended a disc gel electrophoretic procedure for examining seminal stains for LDH-X, and said that this method might actually be better than searching for spermatozoa in older stains. The procedure would, of course, be of no value in diagnosing azoospermic samples.

10.9 Sperm and Seminal Esterases

Nonspecific esterases in sperm were described by Beckman and Kjessler (1968). They found up to six electrophoretically separable zones of esterase activity in washed spermatozoa which were not of seminal plasma origin. Two of the zones may have been artifacts of the preparation method. Some individuals showed one zone, others showed two, and still others, three. Not all the sperm specimens examined had activity. Prasad *et al.* (1976) found that the fastest moving α-esterase was absent in vasectomized and oligospermic patients.

Of greater interest for seminal stain identification are the seminal plasma esterases. Tran Van Ky and Muller (1968) carried out a fairly extensive study of some of the enzymes in human seminal plasma. Immunoelectrophoresis was employed to assess the total antigenic protein composition; in addition, some of the precipitin arcs were identified by specific enzyme staining reagents. One lipase, two alkaline phosphatase, one chymotrypsin, two leucine aminopeptidase (see Section 8.2.3) and four esterase antigen-antibody complexes were identified in the immunoelectrophoretogram. All four esterases hydrolyzed α- or β-naphthyl acetate, but only certain ones were active with indoxylacetate, butyrylthiocholine and carbonaphthoxycholine. Only one of the esterases was common to serum. It was also found in the course of the studies that the antibodies to the various enzymes do not all appear in the immunized rabbits at the same time. Weekly injections were given, and the time of appearance of the various anti-enzyme antibodies determined. It required

70 days to obtain an anti-seminal plasma antiserum containing all the anti-enzyme antibodies reported. The authors noted the potential medico-legal application of the technique, and said that it should be superior in many ways to other methods, combining, as it does, the resolving power of immunoelectrophoresis with the specificity afforded by the specific antibodies and the specific enzyme stains. In 1970, Darwiche *et al.*, using a kind of crossed over electrophoretic method, tested the esterase identification method with seminal stains. The enzymes were found to be relatively thermostable. Several extracting solutions were tried with stains of varying age. The best results were obtained with a pH 7.4 phosphate buffer containing 10% Triton-X-100 and 10% "sputolysine" (the latter apparently a commercially available nonionic detergent). Stains from 6 to 12 months old gave positive results with this extraction medium, but stains 14 years old did not. They believed this method to be specific and relatively simple, and recommended it for seminal stain identification. Evrev (1971) tried the technique and recommended it as well. Evrev (1970) had described two esterases which hydrolyzed α-naphthylacetate, and which were detectable in immunoelectrophoretic precipitin bands. These could be detected in stains up to 30 days old, and were absent from serum, vaginal and cervical mucus, urine, saliva, nasal mucus and from the semen of bulls and rams. Roberts *et al.* (1972) characterized these seminal esterases, with β- and $\beta\gamma$- electrophoretic mobilities. The α-mobility enzyme was not always present and formed a complex with lactoferrin. The $\beta\gamma$-mobility enzyme was heterogeneous on starch gel electrophoresis, but gave a single peak on Sephadex G200 which indicated a MW of about 69,000–77,000. Hermann (1972a) described a cholinesterase and a nonspecific aliesterase in seminal plasma, both of which were detectable in antigen-antibody complexes following immunoelectrophoresis. The latter was said to be of prostatic origin.

10.10 γ-Seminoprotein

In 1969, Hara *et al.* carried out immunoelectrophoretic studies on seminal plasma with antisera to human seminal plasma. A number of precipitin lines were observed. One of these could be shown to be a protein common to semen, colostrum and saliva, but not serum. They called this protein β-seminoglobulin (β-Sm). Lactoferrin was also observed, and was called seminoferrin. One protein was found to be present in all seminal samples, and could be detected with anti-human semen serum which had been absorbed with serum, saliva and colostrum. This component was believed to be semen specific, and was named γ-seminoprotein (γ-Sm). Yamasaki (1971) in a similar set of experiments identified an α_1-glycoprotein which was common to semen, saliva and colostrum, and this protein was called α-seminoglycoprotein.

Hara *et al.* (1971) partially purified γ-Sm, and found it to be trichloroacetic acid precipitable and thermolabile. Its antigenic activity was abolished by exposure to papain or trypsin. The material gave a single precipitin line upon immunoelectrophoresis, but could be separated into several components, all with activity, upon polyacrylamide disc gel electrophoresis. Three γ-Sm components with isoelectric points between pH 5.8 and 7.1 could be observed in a purified preparation by isoelectric focusing (Koyanagi *et al.*, 1972). The purified preparation contained 74.7% protein, 0.8% sialic acid, 1.6% fucose, 3.6% mannose, 1.5% glucose, 2% galactose, 2.4% glucosamine and 1.3% galactosamine (Hara *et al.*, 1972a). The MW was estimated on Sephadex thin layers to be 28,000–30,000 (Hara *et al.*, 1972b). By sedimentation equilibrium methods, the protein was found to have a sedimentation coefficient of 2.6S, corresponding to a MW of about 19,900 (Koyanagi *et al.*, 1973a). Examination of 18 samples of seminal plasma from healthy donors indicated that the γ-Sm concentration was 1 to 6.25 mg/mℓ if a ring test was used, and 2–10 mg/mℓ if a radial immunodiffusion test was used (Hara *et al.*, 1973a). It could be shown (Hara *et al.*, 1973b) that γ-Sm was not identical with IgG or any of its components, IgA, IgM, IgD, IgE, GOT, GPT, LAP, LDH, a hyalouronidase or its inhibitor, hyaluronic acid, lysozyme, plasminogen, plasmin, thrombin, fibrinogen, carboxylesterase, PGM, 6PGD, prostaglandins or the 3.72S component of Herrmann and Hermann (1969a, 1969b) (see section 10.13.2). In 1974, Koyanagi showed that γ-Sm had four -SH groups which reacted with p-CMB, and that a very pure preparation had a sedimentation coefficient of 2.55S, corresponding to a MW of 22,500–24,000. Estimation of the MW of this pure preparation on Sephadex and Sepharose yielded a value of 23,000 (Koyanagi *et al.*, 1975a). The molecular heterogeneity of the purified preparation on polyacrylamide disc gel electrophoresis was believed to be the result of variability in the number of sialic acid residues attached to different molecules (Koyanagi *et al.*, 1975b). Hara and Inoue (1975) recommended the use of the immunological test for γ-seminoprotein as a sensitive and specific method for identification of semen in stains.

10.11 Other Methods

Two additional enzyme markers have been suggested as bases for seminal stain identification tests. In 1948, Berg found that semen and retroplacental blood contain much higher levels of diamineoxidase than do other body fluids. The diamineoxidase is a histaminase, and acts upon other substrates containing amino groups as well. He showed that the enzyme was present in azoospermic samples, and thought that this would be a good method for diagnosing seminal stains, provided blood shed at birth or abortion could be excluded. Laves (1948) assayed seminal hyaluronidase, which is a component of the sperm cell, but may occur in seminal plasma to some extent as well. He thought that this enzyme could be used as a marker for semen in stains in medico-legal cases. Neither of these techniques have been widely used. Berg (1954) mentioned them, and noted that the assays are particularly difficult and involved, and that acid phosphatase determination is probably preferred by most workers for that reason.

In 1926, Marcusson-Begun described a non-specific agglutinin in potato juice. Human and a number of animal red cells were readily agglutinated by this lectin. The agglutinin has been purified and found to be a glycoprotein containing arabinose (Marinkovitch, 1964). In 1965, Barsegyants observed that human semen inhibits red cell agglutination by the potato phytagglutinin, and on this basis, a hemagglutinin inhibition test for semen and seminal stains was devised. In 1970, he said that even seminal stains which had been washed could be detected in this way (Barsegyants, 1970a). Jakliński *et al.* (1968) carried out studies on the technique and found it to be satisfactory for seminal stain identification. It is not specific, however, since human serum and human milk both inhibit the agglutination reaction as well.

In 1954, Fiori conducted studies of a color test for spermine in terms of its applicability to the medicolegal identification of seminal stains. This test, he referred to as the Fuchs-Tokuoka reaction. Fuchs (1939) had observed that spermine reacts with copper carbonate powder to give a deep blue product upon heating. Tokuoka (1950) said that he had found spermine in neoplastic tissue and in the serum of cancer patients, and was interested in assaying the spermine as a means of diagnosing the disease. He used the copper-carbonate powder reagent to test for spermine in serum, and said that the reaction was very sensitive. Fiori (1954) found that the test compared favorably to Puranen's test (see Section 10.4.3) Although the Fuchs-Tokuoka reagent did not detect semen in stains at the lowest concentration tested in the sensitivity studies, and Puranen's reagent did detect it, both reagents reacted with the other seminal samples tested, and gave negative reactions with a variety of other body fluids and secretions which could potentially contaminate seminal stains. Gültingen (1961) could obtain positive Fuchs-Tokuoka reactions only with fluid samples. Stains, even fresh ones (5 days old), did not give the indicated color change.

Suzuki *et al.* (1980) described an enzyme-coupled technique for the determination of spermine in stains, which employed a bovine amine oxidase. The procedure was quick, they said, and could be used as a preliminary screening test.

Cortivo *et al.* (1979) examined the amino acid composition of several body fluids and secretions including semen. They noted that the amino acids present, and their relative levels, were characteristic, and suggested that the profile might provide an identifying marker pattern for semen, urine, vaginal secretions and sweat. Stains prepared from these fluids retained the characteristic patterns.

10.12 Seminal Stain Fluorescence

In 1927, Ito reported that a number of body fluid stains, including seminal stains, fluoresce under UV light. Since that time, the examination of articles submitted for blood and/or body fluid analysis under UV light has become virtually routine in most places. Many authors have mentioned this fact, including Thomas (1937), Pollack (1943), Kirk (1953) and Nickolls (1956) and many others. Characteristic fluorescence under UV light in no way establishes the presence of semen, but is an excellent, simple, non-destructive screening technique. Garbutt and Sensabaugh (see in Sensabaugh, 1977) have looked into the mechanism of seminal stain fluorescence. The fluorescence spectrum of stains is rather different from that of liquid semen. Studies on fractionated seminal fluid components dried out on substrata indicated that the visible fluorescence that develops in stains is the result of the conversion of one or more non-proteinaceous precursors in semen to several fluorescent products. These have not as yet been characterized. Liquid semen was shown to develop a yellow color upon standing, and this could be shown to be the result of the development of fluorescent compounds in the material. Two distinct fluorescence spectra could be observed: one, having an excitation maximum of about 400 nm and an emission maximum of 460 nm, was called the 400/460 fluorescence; the other had an absorption maximum of 420 nm and an emission maximum of 500 nm and was called 420/480. Preliminary experiments indicated that the 400/460 fluorescence developed first, and sometimes converted to the 420/480 fluorescence. At least five non-proteinaceous compounds were responsible for this fluorescence behavior. While the compounds could not be characterized, it could be shown that the fluorescence developed solely as a result of contamination of the samples with a strain of the bacterium *Pseudomonas fluorescens.* It is not inconceivable that this property could be used to develop a fluorescence test for seminal fluid, although the bacterium is known to interact with other body fluids to produce fluorescent products with different fluorescence spectra, Garbutt and Sensabaugh said.

Calloway *et al.* (1973) reported a low temperature phosphorescence technique which was non-destructive and could indicate the order of deposition of mixed blood and seminal stains.

10.13 Composition of Semen

A number of constituents of semen, which have been employed as markers for the identification of seminal stains, have been discussed individually in Sections 10.1–10.9. In this section, a more general, over-all discussion of the composition of semen is given. Apart from the spermatozoa, seminal plasma may be thought of as containing three major categories of components: low molecular weight compounds; enzymes; and non-enzymatic proteins. All the proteins, whether they possess an enzymatic activity or not, are potentially antigenic, and preparations of antisera to seminal plasma may contain antibodies to any of them. In addition, there are a number of antigenic proteins on the sperm cell, which may be intrinsic cellular constituents, or seminal plasma proteins which have been absorbed onto the cells (so-called sperm-coating antigens). The soluble antigens of the ABH and Lewis blood group systems will not be considered in this section, but in a later one.

10.13.1 Sperm cell antigens

The spermatozoan has various antigenic components (Scacciati & Mancini, 1975). The corresponding antibodies may be sperm agglutinins, may arise spontaneously, i.e., autoantibodies (Samuel *et al.*, 1975), and are important in considerations of fertility and sterility. Sperm agglutinating antibodies sometimes arise in women, presumably due to an antigenic stimulus, although it is not altogether clear how this process takes place (Tyler and Bishop, 1963). Various sperm agglutinins are known which bring about head-to-head agglutination, while others cause tail-to-tail agglutination (Friberg & Tilly-Friberg, 1977). The literature on this material is quite extensive, and since the subject is not of major importance to the medicolegal identification of semen, it is not reviewed in detail. The reviews of Tyler and Bishop (1963) and of Shulman (1971 and 1974) may be consulted for details and documentation. Voisin *et al.* (1975) noted that sperm antigens may be characterized as autoantigens, in which antibodies may be induced in the same organism which produced the antigen, and alloantigens, in which antibodies may be produced in other members of the same species. They used the term isoantigens to refer to spermatozoal antigens which cause the production of antibodies in women, and which may be found in serum, cervical mucus or both.

10.13.2 Seminal plasma proteins

Most of the non-enzymatic proteins of seminal plasma have been detected and studied by immunological methods. Any of the proteins may be an antigen. Some of the components are serum proteins, while others are specific to semen (Searcy *et al.*, 1964; Leithoff and Leithoff, 1965a). All the immunological tests for semen are based on the use of antisera prepared against seminal proteins. Most often, the antisera are complex mixtures of antibodies; in a few cases, such as with anti-SAP or anti-γ-Sm, they are prepared against specific components. The antigens of seminal plasma are of great importance in forensic immunology, for the immunological tests for semen in stains are not of great value if they cannot be employed in the detection of azoospermic samples.

Any number of workers have used immunoelectrophoretic techniques to study seminal plasma antigens, and up to a dozen precipitin arcs can usually be observed. The major arc areas have sometimes been designated by letters, "A" being most anodal. Individual components within the areas are then called A_1, A_2, C_3, and so forth. But the nomenclature is by no means universal. Similar designations may be used by different investigators to denote different components, and the relationships between the different components reported by different laboratories are seldom clear. There is some evidence that the antigenic composition of prostatic fluid may be different from the prostatic antigenic composition of ejaculated semen (Barnes *et al.*, 1963 and 1965). Shulman and Li (1974) and Li and Beling (1973) purified their E_1 protein component, and found that it has a MW of about 31,000 Li *et al.* (1976) purified the B_1 component, finding it to be a sperm-coating lactoferrin of seminal vesicle origin with a MW of about 33,000. Hekman and Rümke (1969) had established that lactoferrin as a prominent sperm coating antigen in seminal plasma. Herrmann (1966) purified his B_2 component, finding it to be a ceruloplasmin derivative. Herrmann and Hermann (1969a and 1969b) have purified a component having a sedimentation coefficient of 3.72S, corresponding to a MW of about 50,000. These proteins were among the eight immunoelectrophoretically distinguishable components previously described (Herrmann and Schirren, 1963). The 3.72S protein does not change its electrophoretic mobility upon treatment and neuraminidase, indicating that N-acetyl-neuraminic acid residues have not been removed (Herrmann, 1972b). The existence of sperm-coating antigens was discovered by Weil *et al.* (1956) who observed that antibodies to seminal plasma could be obtained by immunization with washed spermatozoa.

Acharya *et al.* (1966) studied seminal antigens that migrate with γ-globulin mobility on agar gel electrophoresis. These were found to be trichloroacetic acid soluble, and could be separated into three components, called TSC-1, TSC-2 and TSC-3. TSC-2 was identified as a sialomucoprotein (Kaleker *et al.*, 1967). Amano & Behrman (1968) isolated two antigens from pronase-treated seminal plasma. Both were mucoproteins, and had MW of about 40,000 and 20,000. Scacciati de Cerezo (1974) isolated two glycopeptide antigens from the trichloroacetic acid soluble fraction of seminal plasma, and determined that both their molecular weights were in the neighborhood of 10,000.

Kojima (1966) observed seven precipitin arcs by immunoelectrophoresis, calling them ρ, A_1, and P_1 through P_5, where P_5 was the most cathodic. Three of these were said to be unique to semen. Yamasaki (1971), using an anti-human semen serum which had been absorbed with serum, colostrum and saliva, as well as an unabsorbed serum, said that γ-Sm was the only component unique to semen. Albumin, an α_1-glycoprotein, α_1-antitrypsin, α_1-lipoprotein, transferrin, ceruloplasmin, Hp, fibrinogen, IgG, IgA and IgM were found to be common to serum and semen. Lactoferrin, β-SM and α-seminoglycoprotein were common to semen, saliva and colostrum. Quinlivan and Sullivan (1972) identified albumin, transferrin, lactoferrin, AP, serum β_1-globulin, IgG, seminal vesicle β_1-globulin, seminal vesicle β_2-globulin and a prostatic β_2-globulin in semen. Tauber *et al.* (1975) studied split ejaculates to try and determine the particular accessory gland origin of some of the seminal components. IgG, IgA, albumin and transferrin seemed to originate from other than the seminal vesicles, while fructose and lactoferrin were probably of seminal vesicle origin. IgM, β_1C/β_1A-globulin (C'3 component of complement), ceruloplasmin and fibrinogen could not be detected. In 1976, Tauber *et al.* continued these studies, and indicated that lysozyme, α-amylase, neutral proteinase and plasminogen inhibitor occurred in the initial fraction of the ejaculate, indicating prostatic or Cowper's gland origin. The high MW proteinase

inhibitors were of approximately the same concentration throughout the fractions, except for the pancreatic trypsin inhibitor activity which appeared to be of seminal vesicle origin. Plasminogen, prothrombin, clotting factor VIII, and several proteinase inhibitors were not detected by immunoelectrophoresis.

Recently, Sayce and Rees (1977a) looked at seminal plasma antigen profiles of a number of men, some attending a fertility clinic, and some post-vasectomy patients. Very little or no Gc-globulin, prealbumin, haptoglobin, plasminogen, cholinesterase, fibrinogen or α_2-macroglobulin was observed in these people, and no differences between the groups could be detected. Orosomucoid (α_1 acid glycoprotein), α_1-antitrypsin, albumin, transferrin, ceruloplasmin and lysozyme were detected in addition to the proteins mentioned above. Subsequently, 400 post-vasectomy patients were studied with respect to the orosomucoid component (Sayce and Rees, 1977b), using anti-human orosomucoid and anti-human α_1 acid glycoprotein sera obtained commercially (see also in Section 45.4). About 14% of the men gave a strong precipitin arc in immunodiffusion tests, there being no difference between post-vasectomy patients and patients attending infertility clinics. The orosomucoid was of serum origin. Those men not showing the strong (S) reaction showed a weak (W) reaction with the antisera, but the precipitin band was not found in the same location. Selective absorption experiments showed that the S reaction band was different from the W reaction band, i.e., that the reactions were being given by non-identical antigens. The possibility of the strong reaction precipitin band as a marker in human semen in medico-legal inquiries was raised, but the necessity of carrying out further studies to verify the specificity was noted. It could be shown that the reactivity was consistent within the same individual over a considerable number of months.

10.13.3 Enzymes and low molecular weight components of seminal plasma

Suominen *et al.* (1971) determined the activities of proteinase, AP, and an esterase in 205 seminal samples. They determined the fructose concentration as well. Semen was found to have a high fibrinolytic activity, a fact which could have serious implications in attempting to interpret results of fibrinolytic assays for menstrual blood identification if semen were present (Cf Section 8.1.2). The proteinase activity was assayed with casein as substrate, and the esterase activity was one which hydrolyzed the synthetic ester p-toluenesulfonyl-L-arginine methyl ester. Molnar *et al.* (1971) studied the ejaculates of individuals suffering from pathological conditions which caused seminal vesicle constituents to be absent. Citric acid and acid phosphatase were clearly of prostatic origin, but they believed the seminal vesicles to be the source of LDH and GOT. Nun *et al.* (1972) reported that SAP levels were significantly higher in vasectomized and azoospermic patients. Atanasov and Gikov (1972) reported that they could localize LDH, MDH, AP, alkaline phosphatase, GPT, GOT, LAP, peroxidase and α-amylase in polyacrylamide disc gels after electrophoresis of prostatic fluid. Blake and Sensabaugh (1975) surveyed human semen for enzymes displaying polymorphism, and found that AK, antitrypsin, amylase, esterase-D and peptidase-A were all present. It had been known previously that semen contains PGM.

10.14 Seminal Protein p30

Sensabaugh (1977 and 1978) has recently reported isolation of an apparently seminal fluid-specific protein, designated p30. The protein is antigenic, and was detected as one of a number of precipitin arcs given by seminal plasma by immunoelectrophoresis against anti-human seminal plasma serum. The protein has been purified to a significant degree, and specific antisera have been raised against it. It was strongly suggested that monospecific antisera to semen-specific proteins, as p30 appears to be, would provide the soundest basis for an immunological test for the identification of semen in stains. Sensabaugh could show that p30 reacted with an antiserum prepared against the E_1 component of Li and Beling (1973) (see above), indicating that E_1 and p30 are immunologically identical if not in fact the same protein. The p30 protein is a glycoprotein with MW about 30,000, probably prostatic in origin. Upon subjection to isoelectric focusing, the protein splits into several bands with isoelectric points between pH 6.5 and 8.0. The concentration of the protein in 17 men ranged from 0.24 to 5.5 mg/mℓ. These biochemical characteristics are quite similar to those which have been reported for γ-seminoprotein (section 10.10), and although direct experimental comparisons would be required to test the two proteins for identity, it may be the case that p30 and γ-seminoprotein are identical. Specific antisera to p30 have been tested with a variety of materials, including stomach contents, bile, cow milk, egg yolk, egg white, coffee, coke syrup, karo syrup, detergents, suntan lotions, Conceptrol birth control foam, several commercially available lotions, and several animal semens, and been found to be unreactive. The antiserum has been employed on casework materials as well with completely satisfactory results. Seminal stains up to a year old reacted with anti-p30. The protein was not always detected in vaginal washings from sexual assault victims, probably because the protein was too dilute.

The concentrations of a number of substances in human seminal plasma are shown in Table 10.3.

Table 10.3 Concentrations of Some Components of Human Seminal Plasma

Substance	Average Content (in mg/100 mℓ semen unless otherwise noted)	Range (same units as average)
Water (%)	91.8	89.1-94.4
Ca^{++}	18	7-30
Cl^{-}	43	28-57
Mg^{++} in meq/ℓ	12	—
Na^{+}	117	100-133
Zn^{++}	14	5-23
Total Phosphorus	112	90-120
Inorganic Phosphorus	11	—
Total protein	4.1	3.4-5.5
Acid Phosphatase★	370,000	50,000-800,000
Spermine	—	20-350
Choline	—	11.2-14.4
Citric Acid	480	0-2,340
Fructose	300	50-600
Urea	72	—
Uric Acid	6	—
Creatine	20	—
Sialic Acid	—	64.5-219

Data from Blood and Other Body Fluids (1961), White and Macleod (1963) and Santoianni (1967)

★ in King-Armstrong units (pH not given)

SECTION 11. IDENTIFICATION OF SALIVA

Saliva is encountered much less frequently than are blood and semen in evidentiary materials submitted for examination. Accordingly, it has received comparatively less attention in the literature over the years. Many authorities have not brought up the subject of saliva stain identification at all (e.g. Vibert, 1980; Hamilton and Godkin, 1894; Tidy, 1884; Lucas, 1935 and 1945; Simpson, 1965; Glaister, 1931; Gradwohl, 1954). The methods for identifying saliva are based primarily on the identification of the inorganic anions thiocyanate and nitrate, or of the enzymes alkaline phosphatase or amylase. Microscopical and immunological methods have been employed as well.

11.1 Identification of Inorganic Ions

11.1.1 Thiocyanate

According to Krüger (1898), the presence of thiocyanate in human saliva was first noted by Treviranus in 1814, but the substance was only identified as being thiocyanate twelve years later by Tiedemann and Gmelin in 1826. The old literature on the subject was reviewed by Krüger (1898) and again by Lickint (1924). In 1948, Fischman and Fischman determined that normal saliva contains from 3.1 to 27.5 mg SCN^-/100 mℓ saliva. The average content for nonsmokers was 11.7, while for smokers it was 17.5. These values are quoted in *Blood and Other Body Fluids* (1961) as well. Ruddell *et al.* (1977) determined that the concentration of thiocyanate in saliva was about 1.6 mM, or about 93 μg/mℓ (or 9.3 mg/100 mℓ). The average content was about 2.5 times higher in smokers than in nonsmokers.

Thiocyanate may be detected by addition of a small amount of dilute $FeCl_3$ to the slightly acidified sample. A pink to red color indicates a positive test. This technique is mentioned in the forensic literature by Mueller(1953), Kirk (1953) and by Dérobert (1974). The most extensive experiments on the method were done by Nelson and Kirk (1963). Fresh saliva was found to contain 150 μg SCN^-/mℓ. Freshly dried stains gave comparable values, and aqueous extracts of 7½ month old stains showed about 67% of the original concentration. After 28 months, stains on linen retained about 20% of the original concentration, while stains on cotton had only about 5%. The test is sensitive to about 3μg SCN^-, and with older stains, more and more stained cloth had to be eluted to achieve a positive result. It is believed that thiocyanate does not occur in these concentrations in other body fluids, and the test, when positive, was considered specific. Thiocyanate may be absent, or in undetectably low concentrations, however, and a negative test does not exclude the presence of saliva in the stain (Mueller, 1975). Berg (1957) said that a positive test should not be regarded as a specific test, and Dérobert (1974) does not appear to regard it as conclusive either.

11.1.2 Nitrite

Detection of nitrite anion for the identification of saliva stains was studied by Nelson and Kirk (1963). NO_2^- was detected by the Griess test (Cf Feigl and Anzer, 1972), in which α-sulfanilic acid is allowed to react with the nitrite to form a diazonium compound, which reacts with α-naphthylamine in the reagent to form the pink to red product, p-benzenesulfonic acid-azo-α-naphthylamine. A reagent consisting of 0.5% α-naphthylamine in 5N acetic acid and 0.8% sulfanilic acid in 5N acetic acid was used in the salivary stain studies. Saliva contained about 10 μg NO_2^-/mℓ, but even in stains which had been dried a few weeks previously, the ion was undetectable. This fact, coupled with the presence of nitrite in decaying nitrogenous matter, severely limits the applicability of this test. It was noted that a positive result would, however, give an indication that the stain had been deposited relatively recently. Ruddell *et al.* (1977) determined that saliva contained an average of about 100.4 μM NO_2^-, which is of the order of 4.6 μg/mℓ, in good agreement with the findings of Nelson and Kirk (1963). Gastric juice contained only about 5% as much nitrite as saliva.

That nitrite occurs in human saliva has been known at least since the work of Savostianov in 1937, who said that the concentration varied from 0.01 to 0.1 mg %. Ville and Nestrezet were said by Varady and Szanto (1940) to have suggested that the presence of nitrite in saliva could be accounted for by oral microbial reduction of dietary nitrates which are secreted in saliva. Savostianov had an identical view in 1937. Studies on the effect of dietary nitrate intake on salivary nitrite have indicated that ingested nitrates are indeed the source of salivary nitrite (Spiegelhalder *et al.*, 1976; Tannenbaum *et al.*, 1976).

11.2 Alkaline phosphatase

Nelson and Kirk (1963) assayed saliva samples for alkaline phosphatase in pH 9 barbital buffers. It was found that 24 hr incubation periods were required, and the activity of whole saliva was about 540 μg phenol liberated from phenyl phosphate/24 hrs at 37° per mℓ. Freshly prepared salivary stains had similar activity, but stains stored for 7½ months retained only about 20% of the original activity. Since alkaline phosphatase is found in many other physiological fluids, this test cannot be considered more than presumptive, but it may have some value in corroborating the presence of saliva in stains. Moon and Bunge (1968a) reported, for example, that 55 samples of seminal plasma had alkaline phophatase

activity ranging from 2.3 to 106.6 Sigma units/mℓ. The presence of alkaline phosphatase in human saliva was first noted by Chauncey *et al.* in 1954. It is not clear whether the enzyme originates in the salivary glands or in the oral epithelium (Levitskii *et al.*, 1973). There may be some contribution from the oral flora as well. With p-nitrophenylphosphate as substrate, saliva had an alkaline phosphatase activity of about 0.04 μmol p-nitrophenol liberated/min/mℓ (Lindqvist *et al.*, 1974). Pini Prato (1970) said that two alkaline phosphatase activity bands could be isolated from human saliva by polyacrylamide disc gel electrophoresis.

11.3 Amylase

11.3.1 Applications of amylase detection to saliva stain identification

Detection of amylase, and in some cases quantitative determination of its activity, is almost unquestionably the most extensively used test for the identification of salivary stains. Amylase is one of the oldest enzymes known. Chittenden (1881) who studied the enzyme quite extensively, said that the activity in human saliva was first noted by Leuchs in 1831. Dixon and Webb (1964) noted that Payen and Perzoz had described an amylase activity in alcoholic precipitates of malt extract in 1833, which they had called "diastase". This term persisted in the literature for many years. Later, salivary amylase came to be known as "ptyalin". Roberts (1881) said that Kühne had proposed the general name "enzyms" for the soluble "ferments", including amylases, and Roberts suggested that the term be Anglicized as "enzymes". Roberts was conducting studies of pancreatic amylase and his paper is one of the oldest references to the now familiar starch-iodine test. Iodine solutions cause starch to turn a deep blue color. In the presence of starch-hydrolyzing enzymes, the disappearance of the blue color can be used as an indicator of the progress of the reaction. Schoch (1961) noted that iodine reacts with the linear molecule amylase rather than with amylopectin (see section 11.3.2) to give the blue color. Amylose in solution forms helices, each turn of the helix containing six glucose units. The blue color is attributed to the binding of one diatomic iodine per turn of the amylose helix. The color intensity and shade will depend on the number of helical turns and, therefore, on the length of the chain. Amylose chains of less than 12 glucose units and two helical turns give no color. As chain length and number of helical turns increase, the color is brown, red, purple, and finally the characteristic blue at chain lengths of more than 45 residues and 9 helical turns. Because amylopectin has branches of about 20–30 residues, Schoch said that it gives a red color with iodine.

Mueller (1928) was the first medico-legal investigator to suggest the identification of salivary amylase as a basis for diagnosing salivary stains. Using 8 hr incubations, he detected substrate disappearance using Lugol's iodine, and product (glucose) formed by means of Fehling's solution. He carried out a number of studies on the assay of the enzyme in saliva, and was able to get positive results from salivary stains. Kirk (1953) noted that the amylase test for saliva stain identification had not yet been standardized. Berg (1957) mentioned the amylase test, and said that he carried it out using iodine to detect the starch. Nickolls (1956) discussed the test in his book, and recommended it as a method for saliva stain identification.

Schaidt (1956) proposed a modification of the amylase procedure, taking advantage of an improved method for detecting the reducing sugar products on a micro scale. He employed the Folin-Wu technique for the colorimetric detection of glucose. Folin and Wu published their basic method in 1919, and a number of refinements in the technique were offered as time went along (Folin and Wu, 1920; Folin, 1929). In effect, the method employs a slightly alkaline solution of Cu^{2+} tartrate for oxidation of the sugar, the Cu^{+} then being detected colorimetrically using a phosphomolybdic acid-sodium tungstate reagent. Schaidt read the color at 440 nm. Good results were obtained, he said, even with a saliva "stain" from a piece of a postage stamp. Incubation times with starch were for 10 min at 40°, much shorter than had been used by Mueller in the original studies in 1928.

In 1960, Yoshida reported that salivary traces on postage stamps and envelope sealing flaps could be detected using a modified amylase test. Stain extract was incubated with starch solution for 30 min at 37.5°, and the reducing sugar detected with triphenyltetrazolium chloride in base. The tetrazolium salt was thereby reduced to a red insoluble formazan. Thoma (1961a) who had apparently arranged for a translation of Yoshida's paper, and discussed some of the experimental details contained in it, conducted some experiments on this method. Reliable results could not always be obtained using Yoshida's procedure, but Thoma presented a modified technique which was said to be reproducible. Pieces of postage stamps or envelope sealing flaps 0.5 cm^2 were said to give positive results, provided the incubation period was long enough. It has been known for a long time that triphenyltetrazolium chloride reacts with ascorbic acid as well as with reducing sugars (Feigl, 1966). Thoma (1961c and 1964) said that this fact should provide a basis for using the tetrazolium salt to determine saliva traces based upon the ascorbic acid content of saliva. Glavind *et al.* (1948) determined that there was 2.4 μg/mℓ (0.24 mg/100 mℓ) levels of Vitamin C in unstimulated saliva, and 1.7 μg/mℓ (0.17 mg/100 mℓ) in paraffin-stimulated saliva. Hafkesbring and Freeman (1952) noted that the level ranged from zero to 0.372 mg/100 mℓ, this figure being quoted in *Blood and Other Body Fluids* (1961) as well. Thoma said that the characteristic red formazan precipitate formed with saliva stain extract in the presence of the tetrazolium salt, while with water controls either no color was obtained or a red color formed but without any precipitate. Radam (1965) critically evaluated this method. He did not think that the test was measuring the ascorbic acid content of saliva at all. Taking the ascorbic acid concentration determined by

Glavind *et al.* (1948) for saliva, 2.4 μg/mℓ, and assuming that the amount of saliva on a postage stamp is 10μℓ, he calculated that the amount of ascorbic acid Thoma was actually testing with the tetrazolium salt, after dilutions and removal of the aliquot for the test, was 1.2 ng. Feigl (1966) said that the limit of detection of ascorbic acid with triphenyltetrazolium chloride was 0.2 μg. Saliva does contain some endogenous glucose, from 11.28 to 28.08 mg/100 mℓ according to *Blood and Other Body Fluids* (1961). This amount alone would not be sufficient to account for the positive tetrazolium test using Radam's assumptions and method of calculation, but he said that saliva contained some dextrins (partially hydrolyzed starch polymer) as well, and that the action of amylase on these compounds would increase the endogenous concentration of reducing sugars. He thought, therefore, that Thoma was in reality measuring reducing sugars. Radam preferred the starch-iodine test in the amylase determination in salivary stains. Mueller (1975) said that von Haas, in his 1968 dissertation at the University of Marburg, had been unable to confirm Radam's explanation for the tetrazolium test being positive in the absence of added starch substrate with saliva extracts.

Thomas (1961b) suggested an alternative method for detecting reducing sugar products of the salivary amylase reaction with starch. It was based on a spray reagent developed by Partridge (1948) for the detection of reducing sugars following paper chromatography. The reagent consisted of 0.1N $AgNO_3$ in excess (5N) NH_4OH, and the reducing sugars brought about deposition of elemental silver on the paper. Thoma applied the amylase reaction products to filter papers which had been previously impregnated with $AgNO_3$, and then exposed them to ammonia. The method, he said, was very sensitive.

In 1974, Willott applied dyed starch substrates for the amylase assay to the medico-legal identification of saliva for the first time. A commercially available blue starch polymer was used as amylase substrate (Phadebas test, Pharmacia). The blue dye is covalently attached to the starch, and upon hydrolysis, a product is obtained which is readily determinable colorimetrically. Saliva, blood, semen, fecal material on anal swabs, vaginal swabs without semen, urine, sweat, human milk and a number of vegetable and plant materials were tested for activity. The test can be carried out quantitatively by determination of the OD at 620 nm following incubation, or qualitatively by incorporation of the substrate into agar gel, and noting the color change upon hydrolysis visually. Saliva stains showed considerable variation in activity (0.013 to 0.183 I.U./ℓ; these values represent the actual activity measured in $3mm^2$ segments of stained cloth in a final volume of 5 mℓ, converted to I.U./ℓ from the OD-activity chart supplied by the manufacturer). Fecal stains showed wide variation as well, two samples having high amylase values (0.4–0.5 I.U./ℓ). One of the vaginal swabs showed 0.021 I.U./ℓ. Since some of the materials tested did show amylase activity levels which fell into the range of activities shown by saliva stains, albeit rarely, the amylase test, even when carried out quantitatively, could not be regarded as being entirely specific for saliva. Blood and semen levels were found to be lower than those of saliva, and identification of saliva in the presence of either of these should not present a problem.

The use of dyed starch substrates for assaying amylase was introduced in 1967 by Rinderknecht *et al.*, who could covalently link a blue dye, Remazol Blue R (Farbwerke Hoechst AG) (see Table 5.3) to starch. Upon hydrolysis, soluble blue product was released, and could be determined by its absorbancy at 590 nm. Ceska *et al.* (1969b) found that a blue dye called Cibachron blau F3G-A (see Table 5.3) could be covalently linked to starch polymer. This material in turn could be cross-linked to form a three dimensional polymer network which swelled in water. The formation of soluble blue product was proportional to substrate concentration over a wide range, and the reaction was linear with time for about 10 min. This substrate was shown to be completely suitable for amylase determinations in serum and urine (Ceska *et al.*, 1969a). In 1971, Sax *et al.* employed a different substrate called Procion Brilliant Red M-2BS Amylopectin (available at the time from Reliable Reagents, Maryland Heights, MO, USA) (see in Table 5.3). This substrate was shown to be suitable for amylase determination in serum and urine, the OD of liberated product being read at 517 nm. In 1973 Rosalki and Tarlow evaluated several commercially available dyed starch substrates in amylase assays. The activity data correlated well with the Remazol Brilliant Blue R Amylopectin (Amylopectin-RBB, Calbiochem), the Cibachron Blue Starch (CB Starch, Pharmacia) and the Procion Brilliant Red Amylopectin (Amylopectin PBR, Dade), but the results varied from those obtained with a quantitative starch-iodine assay. These authors recommended the dyed substrates. Lehane *et al.* (1977) compared nephelometric, dyed starch and starch-iodine techniques for the determination of amylase in serum and urine. The nephelometric and dyed-starch results correlated with one another, but not with the starch-iodine result. Furthermore, the nephelometric and dyed-starch techniques gave above-normal activities in every case of clinical pancreatitis, but the starch-iodine method gave normal values in two of eight cases. Nephelometry was considered more convenient than dyed-starch, and starch-iodine technique was said to be less reliable, based on the results. Rosalki (1970) reported that the Remazol Brilliant Blue Starch (Amylose Azure) could be used to localize amylase on cellulose acetate membranes following electrophoresis. The cellulose acetate foil was placed onto an agar gel containing the substrate, and incubated at 37°. Salivary and pancreatic amylases which had been separated could be localized as blue spots. Hall *et al.* (1970) noted that salivary and pancreatic α-amylases showed differential substrate specificity with soluble starch and Amylose Azure.

Baxter and Rees (1975) carefully compared the starch-iodine assay with a method employing Remazol Brilliant Blue R Amylopectin (Amylose Azure) for the identification of salivary stains. The starch-iodine assay was carried out on

a series of doubling dilutions of buffer extracts of approximately 0.5 in^2 stain, after incubation with soluble starch for an hr at 37°. A boiled extract was included as a control, as were identically treated cloth control extracts. The iodine test for starch was negative to about 1:128 with saliva stain extract, whereas boiled extract and cloth controls were negative to about 1:4 to 1:8. The Amylose Azure assay was found to be somewhat more sensitive, and to give good results after 40 min incubations at 56°, even with extract dilutions of 1:1000. The test was usually done with extracts from ⅛ in^2 of stained cloth. Saliva stains were tested, along with semen, blood, serum, urine, milk, tea, coffee, mixtures of saliva with urine, blood and semen, extracts of malt, potato and yeast, and stains made from dilutions of saliva up to 1:32. The test was specific for saliva, but did not detect salivary stains made from saliva more dilute than 1:4. It was successful in diagnosing saliva on cigarette ends, postage stamps and envelope sealing flaps.

Kipps and Whitehead have done extensive studies on the use of Procion Red MX2B Amylopectin as an amylase substrate and its applicability to the diagnosis of amylase in body fluids. A quantitative assay method for amylase was devised using agar gel plates. The substrate, dissolved in phosphate buffer at pH 6.9 and containing 10 g NaCl/ℓ, was incorporated into 1% gels. Small wells, into which test solution could be placed, were punched into the gel. After 24 hrs incubation at 37°, a red concentric ring indicating amylase activity formed around the well, its diameter being proportional to enzyme activity (Kipps and Whitehead, 1974). Substrate concentration was inversely proportional to diameter. The diameter of the ring could be related to absolute units of enzyme activity by using purified amylase of known specific activity in one of the wells. This technique was employed to assess the amylase concentration in saliva and a number of other body fluids (Kipps and Whitehead, 1974 and 1975). These results, along with the results obtained by several other workers, are given in Table 11.1.

In examining Table 11.1, note that Somogyi units were originally defined for serum amylase, the activity being detected by the use of copper reagents to determine reducing sugar product colorimetrically. The number of Somogyi units/100 mℓ serum is equivalent to that amount of enzyme which yields copper reducing power from starch equal to that number of mg of glucose. For example, 120 U/100mℓ would mean that 100 mℓ serum had the same copper reducing power as if it had contained 120 mg glucose (Somogyi, 1938, 1940, 1941, and 1960). Street-Close Units were defined for an assay using amylose as substrate, and iodine

Table 11.1 Amylase Concentrations in Various Body Fluids

Body Fluid	Mean or Average	Range	Units	Reference
Saliva	0.42	—	mg amylase/mℓ	Schneyer, 1956
Submaxillary	0.25	—		
Sublingual	0.26	—		
Parotid	1.03	—		
Saliva				
Caucasians	616	—	Somogyi/mℓ (auto)	Boettcher & De La Lande, 1972
	37.15	—	I.U./mℓ (manual)	
Aborigines	1071	—	Somogyi/mℓ (auto)	
	64.57	—	I.U./mℓ (manual)	
Saliva	350,000	72,000-1,300,000	I.U./ℓ	Kipps & Whitehead, 1975
Serum	160	84-300		
Urine	850	130-3500		
Sweat	575	45-895		
Tears	—	870-2150		
Lip Mucous	903	170-2900		
Semen	95	28-200		
Azoospermic	22	3-150		
	—	680-13,000		
Semen			Street-Close/100 mℓ	Moon & Bunge, 1968b
Normal	9	3-75		
Abnormal	8	2-22		

to detect residual reactant. A non-enzyme-containing "standard" was run next to the "test" solution, and activity was defined as:

$$\text{Units} = \frac{100}{S}(S - T), \text{ per 100 m}\ell \text{ serum, where}$$

S and T were OD readings at 620 nm. If all the starch were hydrolyzed, the OD would go to zero in "T", and Units would equal 100 per 100 mℓ serum, i.e.

$$\text{Units} = \frac{100}{S}(S - O) = 100 \text{ per 100 m}\ell \text{ serum}$$

(Street and Close, 1956). Amylase was first reported in semen by Goldblatt (1935) and Povda (1962) located amylase in paper electropherograms of seminal plasma. Brantzaeg (1971) examined amylase secretion rates in saliva in terms of μg/min/gland, and noted that the amylase values rose 16-fold upon extended gustatory stimulation.

Kipps and Whitehead (1974) noted that Schneyer's value of about 0.4 mg amylase/mℓ corresponded to about 100,000 I.U./ℓ, and that Moon and Bunge's values could be converted to a range of 17 to 1434 I.U./ℓ (see Table 11.1).

The data of Kipps and Whitehead (1975) indicated that the lower limits of amylase concentration in saliva could be close to the upper limits shown by some of the other body fluids. In one experiment, the amylase level in the saliva of the same person varied from 14,500 to 832,000 I.U./ℓ at 11 different times over the course of about 3 months time. Care must therefore be exercised in the interpretation of amylase assay results from salivary stains. Moreover, depending upon the lower limit of sensitivity of the assay being used, a negative test might not necessarily indicate the absence of saliva.

Whitehead and Kipps (1975) devised a test paper for saliva stains based on the Procion Red dyed starch. Moistened test paper and cloth were placed in contact for 15 to 30 min. Test paper areas which had been in contact with saliva stained areas showed pale regions on the paper in contrast to the red background. The resolution could be improved by giving the paper a water rinse, since the soluble products were then washed away. Negative or very weak tests were given by other body fluid stains, and this technique is perhaps the best available for localizing salivary stains on garments. It is analogous to the procedure devised by Kind (1964) with acid phosphatase test papers for localizing seminal stains (Section 10.2.2). Kipps *et al.* (1978) carried out studies on the detection of saliva stains located underneath bloodstains on several different substrata, using the amylase test paper method. They were successful in being able to identify such stains. Willott and Griffiths (1980) have recently described amylase test papers, prepared from blue dyed-starch substrate.

Kipps *et al.* (1975) noted that saliva stains could be detected on the fronts, cuffs and pockets of worn clothing sampled randomly and not related to any case. These findings show that the finding of a stain on garment and the assessment of its evidentiary value are different matters, just as has been previously discussed in connection with bloodstains (Owen and Smalldon, 1975; Hunt *et al.*, 1960) (section 6.8) and seminal stains (Owen and Smalldon, 1975) (section 10.2.2).

Dawes (1972) showed that the secretion of a number of substances in saliva exhibited circadian rhythm. The rhythms were not the same in stimulated and unstimulated saliva. Barsegyants (1970b) said that amylase in salivary stains could be detected after washing in detergents. In 1971, he indicated that a number of pathological conditions, treatment with various drugs and alcohol ingestion did not affect the ability to detect amylase in stains from the saliva of individuals so affected.

11.3.2 Some properties of amylase and starch

Amylases are found in a wide variety of plants and animals. They hydrolyze the C_1–O_4' bond of $\alpha 1 \rightarrow 4$ linked glucose polymers, with transfer of the glucosyl residue to water. Amylases are found in animals and some plants, and yield hydrolytic products of the α-configuration. They are endoenzymes, acting at most internal bonds, can bypass branch points ($\alpha 1 \rightarrow 6$ linkages) in branched polymers, and yield a mixture of oligosaccharide products. β-Amylases appear to be restricted to plants. They are exoenzymes, hydrolyzing every other bond from the non-reducing end of the chain, and yielding maltose as product. They cannot bypass branch points. α-Amylase is systematically called α-1,4-glucan-4-glucanohydrolase (EC 3.2.1.1) and β-amylase is called α-1,4-glucan maltohydrolase (EC 3.2.1.2). Salivary amylase in an α-amylase. Amylases is general were reviewed by Thoma *et al.* (1971).

Salivary amylase, like other α-amylases, has an absolute requirement for Cl^-, and all assay mixtures contain chloride salts, usually NaCl. The amino acid composition of the purified enzyme is known, and Metzbaum and Schulz (1965) reported that the MW of the pure enzyme, calculated from the sedimentation coefficient, is 55,200.

It may be noted that starch, the storage polysaccharide of higher plants, consists of two types of glucose polymers, amylose and amylopectin. Amylose is a linear polymer of glucose units held in the chain by $\alpha 1 \rightarrow 4$ glucosidic linkages (Fig. 11.1). Amylopectin is a branched polymer, containing $\alpha 1 \rightarrow 6$ linkages at the branch points (Fig. 11.2) and is a much larger molecule than amylose. The amylases do not hydrolyze the $\alpha 1 \rightarrow 6$ linkages, and cannot therefore degrade amylopectin completely. Another enzyme, an $\alpha 1 \rightarrow 6$ glucosidase, or debranching enzyme, is required to hydrolyze the branch point bonds.

11.4 Immunological Methods

The first medico-legal application of an immunological method to saliva identification appears to be that of Diniz (1925). He was concerned with the determination of whether dead infants had been born dead or alive, sometimes called docimasia. There was a connection, he believed, between inflation of the lungs with the first breath of air taken by a newborn, salivary gland secretion and the swallowing reflex.

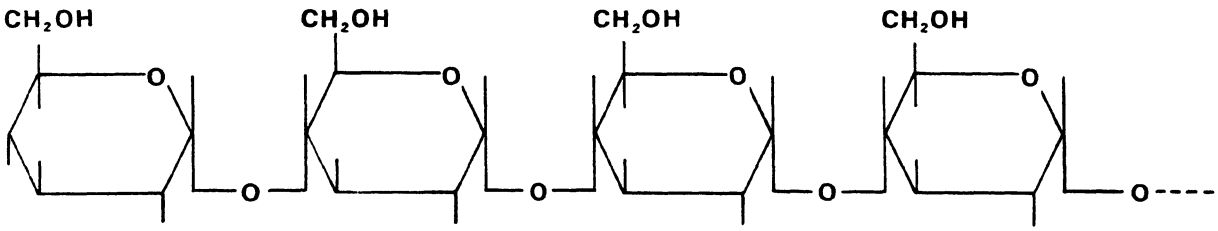


Figure 11.1 Amylose

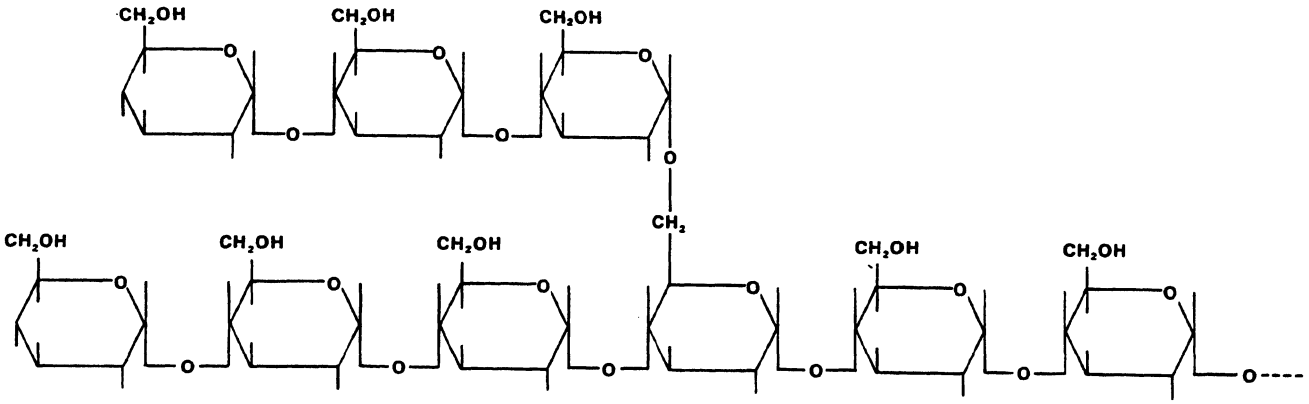


Figure 11.2 Amylopectin

Identification of saliva in the infant stomach indicated that it had been born alive. Few experimental details were given, but he appears to have prepared an anti-saliva serum, and said that the antigen-antibody reaction could be detected, with good results, by precipitation, complement fixation and anaphylaxis. Seitz (1913) could get anaphylactic reactions in guinea pigs using human saliva as both sensitizing and shocking injection material (see section 10.3.2). Mueller (1928) noted that the absence of experimental detail in Diniz's paper made it impossible to repeat the experiments. He said that he was not set up to carry out complement fixation tests, but he did conduct experiments on the precipitin and anaphylaxis methods. The anaphylactic shock studies yielded equivocal results, and Mueller did not think that this approach was particularly promising. Anti-human serum serum gave a precipitin test with fresh saliva and salivary stain extracts, but large areas of stained material were required, and the antiserum naturally cross reacted with a number of other body fluids. Mueller noted that the solution might lie in preparing a specific anti-saliva serum. Witebsky and Henle (1933) prepared rabbit immune precipitin antisera to human saliva. It cross reacted with serum, but reacted more strongly with saliva. Similarly, anti-serum serum cross reacted with saliva, but reacted much more strongly with serum. Rex-Kiss (1942) confirmed these observations, using saliva from group 0 people for immunization to avoid the problem of antibodies being formed to the soluble blood group substances. Some animal anti-saliva sera which he prepared showed salivary specificity, but the human one cross reacted with serum. Saliva and serum share a number of antigens in common, but saliva does contain some specific proteins.

Stoffer *et al.* (1962) found by immunoelectrophoresis that saliva contained the serum proteins albumin, ceruloplasmin, β-lipoprotein, transferrin, β_2-macroglobulin and gamma globulin. There was no fibrinogen. Leach *et al.* (1963) confirmed these findings, and reported serum α_2-glycoprotein, α_1-lipoprotein and β_{2A}-globulin in saliva as well. Ellison and Mashimo (1958) and Ellison *et al.* (1960) found a number of saliva specific proteins using rabbit anti-saliva serum. Simons *et al.* (1964) found nine antigenic components in saliva using an anti-saliva serum, and the antiserum contained an anti-amylase antibody. Furuya (1968) reported that an anti-human saliva serum prepared in rabbits gave 10–13 precipitin arcs upon immunoelectrophoresis, but that after absorption with serum and semen, only one precipitin arc was obtained against saliva, and this could be identified as amylase-anti-amylase complex. Masson *et al.* (1965) identified several salivary globulins by immunoelectrophoresis. Culliford (1964 and 1967) noted the desirability of a specific antiserum to salivary proteins for use in the immunological tests. He also observed that anti-human semen serum reacted more strongly with saliva than did antihuman serum serum. Baxter and Rees (1975) tested an antiserum to

human group A saliva, and found that it cross reacted with seminal plasma. Absorption with pooled semen failed to render the antiserum saliva-specific, and amylase activity could not be detected in antigen-antibody complexes.

11.5 Microscopical Methods

Microscopy may be used to identify the characteristic exfoliated buccal epithelial cells associated with saliva. Most authorities extract the stain, centrifuge the extract, and examine the particulate material thus obtained. A wide variety of histological stains may be employed for examining smears. Radam (1965), for example, used a hematoxylin stain following alcohol fixation. If enough cells are available, cytological sexing methods may be attempted with these cells. Cytological sexing techniques are discussed in a subsequent section. Microscopical methods were discussed briefly by Camps (1968), by Mueller (1975) who did not think the method was as useful as some of the others, and by Dérobert (1974) who indicated that the microscopical confirmation of buccal epithelial cells represented the only certain method of saliva identification.

11.6 Fluorescence Under Ultraviolet Light

Saliva stains exhibit a bluish-white fluorescence under ultraviolet light, which is very useful for carrying out preliminary examinations of garments. The fluorescence helps merely to localize areas for more detailed examination; it does not have any value by itself in the identification of any specific body fluid stain (Mueller, 1953 and 1975; Kirk, 1953).

SECTION 12. IDENTIFICATION OF URINE

Methods for identifying urine have been based on the detection of inorganic anions, especially phosphate and sulfate, and of a number of organic compounds which concentrate in urine, including creatine, creatinine, urea, urinary indican, urochrome, free purine and pyrimidine bases and steroid derivatives. Even though a number of these occur in urine in higher concentrations than in other body fluids, some authors have advised the detection of two or more constituents in an effort to make the identification more probable. Methods have been proposed based on the identification of a single constituent as well.

12.1 Microscopical Methods, Ultraviolet Fluorescence and Odor

Human beings excrete about 9 to 29 mℓ urine/kg body weight/day, containing about 55–70 g solids (*Blood and Other Body Fluids,* 1961). Microscopical methods take advantage of the fact that the "solids" in urine contain various crystalline substances, and epithelial cells characteristic of the urinary tract linings. This approach may provide satisfactory presumptive evidence of the presence of urine in liquid specimens, but has not been very successful in the examination of stains. In the presence of infections, bacteria can be identified as well (Balthazard and Rojas, 1922).

Urine stains fluoresce under ultraviolet light, a fact which helps in localizing stains on garments for subsequent analysis (Kirk, 1953). The color of the fluorescent light varies if abnormal substances are present, e.g., in glycosuria (Hansen, 1945).

One of the more characteristic properties of urine is its odor. Some authors have recommended gentle heating and detection of this odor. Camps (1968) mentioned this method, and Kirk (1953) said that the characteristic odor detection might be among the most specific tests which can be done for urine in stains.

12.2 Inorganic Ions

Balthazard and Rojas (1922) mentioned that urine contained relatively large amounts of Cl^- and PO_4^{3-}, the former being determined with $AgNO_3$, the latter with ammonium molybdate reagent. Kirk (1953) mentioned both phosphate and sulfate as being present in urine in appreciably higher concentrations than in other biological fluids. Sulfate may be detected as its barium salt, and phosphate may be detected as crystals of magnesium ammonium phosphate. According to *Blood and Other Body Fluids* (1961), "inorganic" phosphate is present in serum, saliva, semen and sweat at concentrations of 2.4–3.6, 7.4–21.1, 11 and 0.009–0.043 mg/100mℓ, respectively. Urine contains 10–15 mg phosphate/kg body weight/day. Assuming a 70 kg person, excreting 1ℓ urine in a day, the concentration of phosphate would be 70–105 mg/100 mℓ, assuming constant concentration over time. Similarly, the sulfate concentration is higher than that of serum or perspiration. Chloride in urine is excreted at the rate of 40–180 mg/kg/day. Using the same assumptions as above, the concentration would be 280–1260 mg/100 mℓ, as compared with 295 for serum, 29.8–62.8 for saliva, 99.4–202.4 for semen and 36–468 for sweat. The assumption that the concentration of a substance in urine is constant with time is almost surely not true, but the values give at least some idea of what general ranges of concentrations might be expected. The ions are not unique to urine, although the phosphate and sulfate concentrations are normally considerably higher than in other common body fluids. Swaroop (1973) presented a simple method for detecting sulfate in urine, in which a known amount of barium is complexed with sodium rhodizonate forming a chromophore which absorbs at 520 nm. In the presence of sulfate, the barium is precipitated and the absorbancy is decreased. The sulfate concentration was determined by the absorbancy difference, which was shown to be linear over the range of 0–32 μg sulfate/mℓ.

12.3 Urea

In 1914, Policard suggested the use of xanthydrol, which forms a crystalline compound with urea, for the identification of urine in stains. The test could be carried out on a few threads of stained material, and the characteristic crystals identified microscopically. The reaction had been described by Fosse (1907 and 1912). Maiocchi (1915) carried out studies on the xanthydrol test, noting that some other body fluids including serum, saliva and tears, gave a positive reaction. Balthazard and Rojas (1922) recommended the xanthydrol test, and said that they had gotten negative results with blood, egg white, semen, milk and fecal material. The xanthydrol was dissolved in 95% alcohol, and the test reagent prepared by mixing this solution with glacial acetic acid just prior to use. Ordinarily crystal formation occurred within 30 min, even with older stains. In 1945, Manson took up studies on the procedure. He refined the technique for the actual identification of the crystals by applying a microsublimation procedure described by Kofler and Kofler (1945). Material containing the suspected stain was teased apart into fibers, and treated with alcoholic xanthydrol and acetic acid, and the crystals allowed to form. The crystals were then subjected to the microsublimation procedure and their melting point determined. He said the test was specific for urea and regarded it as virtually specific for urine. The

reaction of xanthydrol with urea is indicated in Fig. 12.1. Ishler *et al.* (1947) said that they had obtained false positive reactions with the xanthydrol test.

In 1940, Gee reported that several of the commonly used tests for urine including color, odor, behavior under ultraviolet light, biuret test, and tests based on ammonia evolution, were not very satisfactory for urine stains on woolen carpets because of interference by substances within the wool. A method was devised in which the urea was extracted into alcohol-acetone, dried down, and the residue again extracted with acetone. The last extract was slowly evaporated, one drop at a time, on a microscope slide, keeping the area occupied by the "spot" as small as possible. Characteristic, needle-shaped crystals soon formed, which could be identified microscopically. The urea crystals could then be converted to urea nitrate crystals by treatment with concentrated HNO_3 as well. Gee said that the extraction was necessary because substances in the wool itself could give false positive results with the test otherwise. Nickolls (1956) recommended Gee's test. Ishler *et al.* (1947) noted that they had encountered problems in trying to convert the urea crystals to urea nitrate.

Ishler *et al.* (1947) utilized a urease enzymatic test for urea in order to identify urine stains on fabrics. Urease (EC 3.5.1.5) is systematically known as urea aminohydrolase, and catalyzes the reaction:

$$\text{urea} + H_2O \rightleftharpoons CO_2 + 2\,NH_3$$

The ammonia may be detected in a number of ways. Ishler *et al.* moistened a small portion of the stained cloth with urease solution, and placed it over a steam bath in contact with a test paper impregnated with Mn and Ag nitrates. A black spot appeared on the test paper within 30 sec if urine was present in the original sample. The test paper reagent was based on the reaction (Feigl and Anger, 1972):

$$Mn^{2+} + 2Ag^{+} + 4\,OH \rightleftharpoons MnO_2 + 2\,Ag^{\circ} + 2\,H_2O$$

Sulfides would give false positive tests if in direct contact with the test paper. A control was recommended in which test paper was placed into contact with fabric material that had been moistened with a non-urease solution. With thick fabrics, a fabric wetting solution could be employed. A positive test could be obtained with a stain made from 0.01% urea solutions.

Cook (1948) suggested a urease test for urea (not specifically for urine) in which the ammonia was detected by its basicity using the acid-base indicator bromthymol blue. The test could be conducted with test paper impregnated with urease and BTB or in 2% agar gels into which the reagents had been incorporated. A bright blue color was a positive test. Thrasher (1970) noted that the agar technique was greatly superior to the impregnated filter paper method in cases where it was difficult to achieve good contact between the filter paper and the contaminated material. He was concerned with the detection of mammalian urine contamination of wheat grains, and said that the agar gel method would detect 2.5 μg urea distributed over the surface of a single grain. Thoma and Kuchinke (1953) reported a urease method for stain examination in which the ammonia was detected using Nessler's reagent. Test papers treated with reagent were employed in the test.

Kirk (1953) did not think that the detection of urea was of very much value in identifying urine in stains.

Rhodes and Thornton (1976) recently described a test for urine in stains based on the reaction of urea with p-dimethylaminocinnamaldehyde (DMAC). It had been observed by other workers that 0.1% solutions of DMAC could be used to detect latent fingerprints. This concentration was too high for a urine test, since all urea-containing physiological fluids (Table 12.1) reacted. At concentrations of DMAC of 0.005%, however, the reagent (prepared in 180 mℓ acetone and acidified with 2 drops concentrated HCl) reacted with the higher urea concentrations found in urine, but not the lower ones found in semen, saliva, perspiration or serum. Stains suspected of containing urine were tested by thoroughly extracting a 1 cm^2 piece of material with 1 mℓ distilled H_2O. A piece of filter paper, dipped into the extract and allowed to dry, is then sprayed with or dipped into a solution of DMAC reagent and heated. Positive results were indicated by a dark pink to red reaction product. In much the same way, the procedure could be used to search for urine stains on fabrics, bedding, etc., by placing the suspected material in between two wet pieces of filter paper, sandwiching them between polyethylene sheets, and allowing the assembly to sit under a weight for an hour. The filter paper could then be dried, and tested. Background color develops in 15 min to 1 hr, so the results had to be read right after the test. DMAC does exhibit reactions with other com-

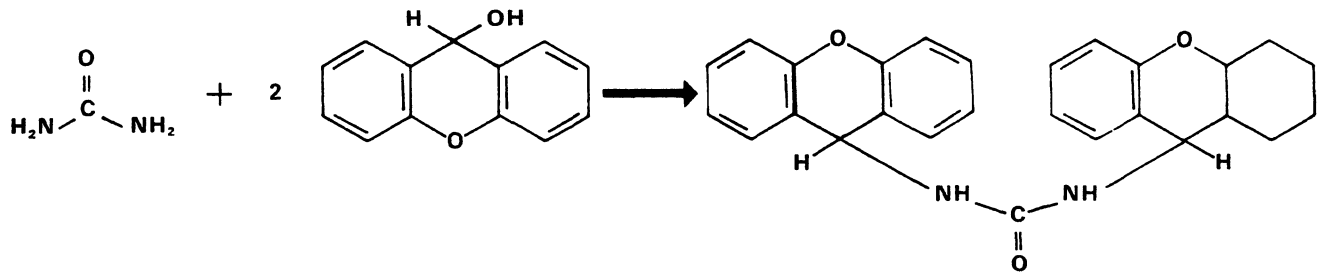


Figure 12.1 Reaction of Xanthydrol (Xanthanol) with Urea

pounds, and it was said that this was not a specific test, but it was recommended as a presumptive test and as a searching aid in locating urine stains.

Human urine contains 200–500 mg urea/kg body weight/day (*Blood and Other Body Fluids,* 1961), corresponding to a concentration of 1400–3500 mg urea/100 mℓ in a 70 kg person who produces 1ℓ urine in a day, assuming no fluctuations in concentration over time. By comparison, serum contains 16–35 mg urea/100 mℓ, semen 72 mg/100 mℓ and sweat 12–57 mg/100 mℓ. Saliva contains 0–18.1 mg/100 mℓ, according to *Blood and Other Body Fluids* (1961). Updegraff and Lewis (1924) determined that saliva contained an average of 4.2 mg/100 mℓ, while Wu and Wu (1951) said that the value ranged from 7.4 to 18.6 mg/100 mℓ.

12.4 Creatinine

The determination of creatinine in urine samples has been of interest to clinical chemists for at least 75 years. One of the oldest tests for the detection of creatinine is that of Jaffe (1886). He found that picric acid reacts with creatinine, in the presence of base, to form a bright red product. In 1904, Folin applied this test to the clinical determination of creatinine in urine. The Jaffe test, as it is usually called, has undergone scores of modifications over the years, primarily by the clinical chemists. The test is applicable to the detection of creatinine in urine stains. Heredia del Portal *et al.* (1971) showed that urine spots dried on filter paper for 15 days could be tested for creatinine using a modified Jaffe test without significant reduction in activity. Many structures have been proposed for the red Jaffe reaction product. These were reviewed in 1974 by Blass *et al.*, who isolated and carried out structural studies on the product. They proposed the structure indicated in Fig. 12.2.

Varma *et al.* (1968) reported a quantitative test for creatinine based on the so-called Salkowski test, in which sodium nitroprusside gives a prussian blue when heated with creatinine. Potassium ferricyanide was employed as oxidizing agent, and a soluble prussian blue complex could be obtained, the absorbancy of which at 680 nm showed a linear relationship with creatinine content over the range 0–10 μg.

Narayan and Appleton (1972) used a countercurrent distribution method to test the specificity of the Jaffe test and the Sakaguchi test for creatinine. The latter, first described by Sakaguchi in 1925 and studied in detail by van Pilsum *et al.* (1956) is based on the reaction of creatinine with o-nitrobenzaldehyde. Narayan and Appleton found that the Jaffee test was more specific for creatinine.

Creatinine occurs in urine to the extent of 15–30 mg/kg body weight/day. A 70 kg person excreting 1ℓ urine per day would have a urinary creatinine concentration of 105–210 mg/100 mℓ, assuming no concentration variation with time. Values of creatinine concentration in other body fluids in the same units (mg/100 mℓ) are serum 0.6, saliva 0.275–0.455, and sweat 0.1–1.3 (*Blood and Other Body Fluids,* 1961). It is known that creatinine excretion varies as a function of time, however, as well as from one individual to another. Greenblatt *et al.* (1976) found that the mean excretory rate in 8 individuals was 21.4 to 25.4 mg/kg body weight/day. There was considerable variation in the mean mass of excreted creatinine by the same individual on different days.

Ladell (1947) studied creatinine losses in sweat during work in hot, humid environments. The rate of excretion decreased at first, but in some cases increased again to values in excess of the zero time value. The salivary creatinine was unaffected (mean value 0.455 mg/100 mℓ), and ingested creatinine which increased serum levels, did not affect the excretory rate in sweat.

12.5 Indican

Indican is excreted in urine at the rate of 0.5–2.0 mg/kg body weight/day, corresponding to 3.5–14 mg/100 mℓ in a 70 kg person excreting 1ℓ urine per day, with no concentration change over time. Serum contains about 0.095–0.105 mg/100 mℓ of this substance (*Blood and Other Body Fluids,* 1961).

Indican is 3-indoxylsulfuric acid. In 1920, Lattes discussed the determination of indican as a method for the diagnosis of urine in stains. He did not think the test was specific for urine stains.

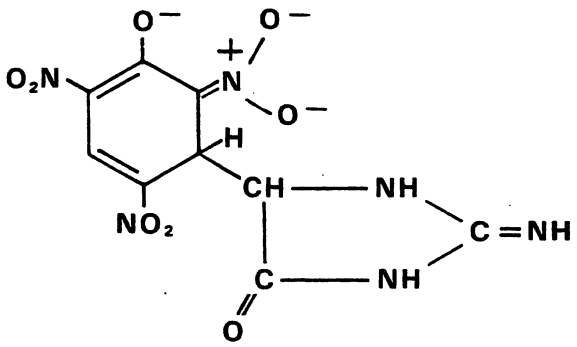


Figure 12.2 Jaffe Reaction Product (after Blass et al., 1974)

12.6 Chromatographic Methods

In 1956, Thoma devised a paper chromatographic method with butanol:acetic acid:water::120:70:10 as the developing solvent (Thoma, 1956a). Urea was detected at R_f 0.4 using p-dimethylaminobenzaldehyde reagent. Rhodes and Thornton (1976), in their paper on the DMAC test (section 12.3), noted that urea reacts more strongly with DMAC than with p-dimethylaminobenzaldehyde. Urine stains could be distinguished in the system from blood, saliva, semen and bile. Thoma (1956b) also noted that allantoin occurs in animal urine but not in human urine. Detection of allantoin using a phenylhyrazine reagent could thus be used to distinguish human from animal urine. In 1966, Weinke *et al.* reported a thin-layer chromatographic method using n-butanol:acetic acid:water::4:1:1 as developing solvent. The solvent front advanced about 10 cm in 70–80 min. Urea was detected as a yellow spot at R_f 0.5 with HCl-p-dimethylaminobenzaldehyde, and creatinine was detected as an orange spot at R_f 0.19 using 5% alcoholic picric acid containing 10% NaOH. Urine stains on various substrates were tested after aging at room temperature. Both compounds could be detected in most cases after 10 days, and in some cases after as much as 40 days.

Giusti and Panari (1972) proposed that several other components of urine be detected by thin layer chromatography, in addition to creatinine and urea, in order to make the identification more specific. Urochrome, indican, purine and pyrimidine bases and steroid derivatives were detected on Silica Gel G plates with isopropanol:concentrated ammonia:water::10:1:1 as developing solvent. They recommended that urea and creatinine be detected first using the method of Weinke *et al.* (1966). Urine stains could be differentiated from perspiration stains, in that the latter showed only urea, creatinine and the purine-pyrimidine base spot at R_f 0.00.

Concentrations of some of the substances in urine and in other body fluids which have been quoted in Section 12 are collected in Table 12.1.

12.7 Immunological Methods

Lee *et al.* (1977) reported that they had prepared an anti-human urinary protein serum, and tested its applicability to the detection of urine in stains. The antiserum was not entirely satisfactory, but the approach was regarded as a promising avenue for future experiments.

Table 12.1 Concentrations of some components of urine and other body fluids

Substance	Urine (mg/kg body weight/day)	Urine (mg/100ml) ★	Serum ●	Saliva ●	Semen ●	Sweat ●
phosphate	10-15	70-105	2.4-3.76	7.4-21.1	11	0.009-0.043
sulfate	3.5-17.5	14.5-122.5	0.45	—	—	0.7-7.4
chloride	40-180	280-1260	295	8.4-17.7 ✓	28-57 ✓	36-468 385 profuse [4] 469 profuse- [5] intermittant [4] 1091 intermittant [4]
creatinine	15-30 21.4-25.4 [1]	105-210	0.6	0.275-0.455	—	0.1-1.3
creatine	0-2	0-14	2.7	—	20	—
urea	200-500	1400-3500	16-35	0-18.1 4.2 [2] 7.4-18.6 [3]	72	12-57 68 profuse [5] 275 intermittant [5]
uric acid	0.8-3.0	5.6-21	1.6-3.9	0.5-8.7	6	0.07-0.25
indican	0.5-2.0	3.5-14	0.095-0.105	—	—	—

Values in Table from Blood and Other Body Fluids (1961) unless otherwise noted

● Values in mg/100 mℓ unless otherwise noted

✓ Values in meq/ℓ

★ Calculated from the mg/kg body weight/day value assuming a 70 kg person excreting 1 ℓ urine per day with no variation in concentration as a function of time

[1] Greenblatt et al., 1974

[2] Updegraff and Lewis, 1924

[3] Wu and Wu, 1951

[4] Lobitz and Osterberg, 1947c

[5] Lobitz and Osterberg, 1947a

SECTION 13. IDENTIFICATION OF FECAL MATERIAL

Microscopy is the oldest method for identifying fecal material and still probably the best technique for achieving a reasonably satisfactory result. The undigested residues of food material ingested 12–24 hrs previously will be found in fecal material. Characteristic fibrous material from animal meats, fish and from vegetables and fruits, seeds, pits and other plant material which is undigestible can all be identified, if present, by an experienced observer. One of the oldest papers devoted to the medico-legal identification of fecal material is that of Moeller (1897) although Robin and Tardieu had identified fecal stains in a case on which they reported in 1860. Vibert (1908) discussed the microscopical method in his text. In 1899, van Ledden Hulsebosch published a complete monograph on fecal examination, with complete descriptions of the various materials that might be found and over 250 plates showing photomicrographs. This book was a standard reference in forensic medicine for several decades. Van Ledden Hulsebosch's son published a paper in 1922, describing an apparatus he had devised for preparing fecal material for microscopical examination. Reuter (1934) reviewed the subject briefly, and Hepner (1952) described in detail his technique for photomicrography in connections with examination of fecal material.

There was always interest in finding methods for comparing fecal samples, which might indicate common origin. Microscopical examination can be of help in establishing that samples were deposited by the same person within a relatively short period of time. Kraft (1929) took advantage of what he thought should be a fairly unique property of fecal material at any one time, its chlorophyll content. Ethereal extracts of fecal material contain chlorophyll if green plant foodstuffs have been ingested, and these solutions show the characteristic chlorophyll fluorescence. The amount of chlorophyll is a function of the amount of green plant food materials which have been eaten. Kraft said that, in one case, he could differentiate two fecal samples on this basis.

Hoen (1929) took an interesting, if somewhat complicated, approach to the problem of individualizing fecal samples. There was evidence that the different strains of coliforms inhabiting the G-I tract, and appearing in feces, showed a certain degree of individual specificity. Hoen thought that, by preparing antisera to the bacteria and differentiating them serologically, different samples should be able to be compared as to common origin. In the particular case at issue, he found the two specimens to be identical by microscopical examination, and felt that the serological identity of the bacteria, which he demonstrated, gave a very strong indication that the specimens had a common origin. The serological profile of the coliforms was relatively constant in the same person over the course of time, and the approach was regarded as very promising. The technique is quite involved, of course, it being necessary to culture and harvest the bacteria, prepare antisera to them, and then carry out the serological tests.

Another method for the identification of fecal material is the detection of bilirubin, a precursor of the normal fecal pigment. The compound may be oxidized to a pink-to-red product with $HgCl_2$, and this test may be used for relatively small fecal stains (Asada and Kominami, 1924). Nickolls (1956) referred to a variation of this test in which 10% $HgCl_2$ in amyl alcohol was used as reagent, and $ZnCl_2$ added to the supernatant fluid following centrifugation. This, he referred to as the Edelman test. Mueller (1975) said that the test was not always reliable.

Vanni (1949) reported on the potential importance of parasitology in examination of fecal material. Any number of harmless, nonpathogenic parasites may inhabit the human gut, and the parasites themselves or their eggs may be found in fecal samples. Vanni elaborated a procedure for carrying out the examination for parasites. It is noteworthy that Robin and Tardieu, in 1860, identified the eggs of *Ascarides lumbricoides* in fecal stains on a garment, and used the finding as a criterion for judging the stains to be fecal in nature. It is necessary to have a sample from the suspected person in order to perform a parasitological comparison for identification purposes. Jarosch and Marek (1959) reviewed briefly the medico-legal examination of fecal material, and included a discussion of bacteriological, parasitological and morphological techniques.

Johnson (1948) reported on a case in which a defendant was convicted of burglary, largely on the strength of expert testimony linking fecal matter at the scene with that found on his overshoes at the time of arrest. The expert compared the samples as to color, odor, and consistency, and carried out a detailed microscopical examination in which a number of animal and plant products could be identified. On this basis, the expert testified that there was only a slim possibility that the samples were from different sources. The New Hampshire Supreme Court agreed that the evidence supported the guilty verdict [State v Burley, 57A (2d) 618, N.H., 1948].

Giersten (1961) gave a very good review of the entire subject. He thought the detection of urobilin was a much more specific test for fecal material than the detection of bilirubin from which it is derived. The latter may occur in vomit as well. Camps (1968) recommended the detection of urobilin in identifying urine stains. The nomenclature of the fecal and urinary prophyrin derivatives is confusing because the early investigators based many of their conclusions on work with impure materials. Both urine and feces contain

two urobilinogens, mesobilane ("urobilinogen"), and tetrahydromesobilane ("stercobilinogen"), and two urobilins, mesobilene ("urobilin IX") and tetrahydromesobilene ("stercobilin") (Lemberg and Legge, 1949). Giersten also noted that identification of enterococci is strong presumptive evidence for the presence of fecal material.

Konzak (1980) suggested coprostanol as a marker for fecal material. The compound could be detected by TLC of an extract of the test substance. Petersen Inman (1980) said that detectable amounts of IgA immunoglobulin, alkaline phosphatase and amylase could be detected in fecal material extracts. These were proposed as potential identification markers for fecal material. It may be noted, too, that the amylase detected in fecal material is pancreatic in origin (AMY_2 locus) and is known to show some genetic variation (see in section 37.3).

SECTION 14. IDENTIFICATION OF OTHER BODY FLUIDS AND SECRETIONS

It may be necessary on occasion to attempt to identify uncommon physiological fluids or secretions. These include amnionic fluid, meconium, colostrum, milk, pus, nasal mucus, sweat and tears. Textbooks in forensic medicine have often discussed one or more of these substances. Most authorities recommend microscopical examination as the best method for differentiating these materials, except for perspiration and tears which are seldom mentioned. In many cases, histologically identifiable cells, globules or other characteristic structures are present which serve to indicate the origin of the material. Proteinaceous fluids may be distinguished form urine, sweat and tears by means of one of the general protein tests, such as the biuret test.

Sweat and tears are similar in composition to urine except that they contain less urea and creatinine. Kirk (1953) noted that urine can best be distinguished from perspiration on the basis of its odor upon heating. Giusti and Penari (1972) noted that their TLC method distinguished urine from perspiration, as discussed in Section 12.6. Lobitz and Osterberg (1945, 1947a, 1947b and 1947c) carried out extensive studies on palmar sweat. The concentrations of various substances varied, depending upon whether sweating was profuse, profuse-intermittent, or intermittent. These data are collected in Table 12.1 There was considerable individual variability in the rates of excretion of various substances.

Josephson and Lockwood (1964) prepared an antiserum to human tears, and examined the antigenic composition of lachrymal fluid by immunoelectrophoresis. Several serum proteins including ceruloplasmin and β_2A-globulin were found, and mild trauma, such as rubbing the eyes, caused transferrin, serum albumin and γ-globulin to appear. The data indicated, however, that tears do contain specific, non-serum proteins. In 1956, Erickson described an albumin in tears which had an electrophoretic mobility faster than that of serum albumin, and was said to constitute about half the total tear albumin. Josephson and Weiner (1968) studied this protein further, saying that it did not occur in serum, saliva, cerebrospinal fluid or urine. It had a MW between that of lysozyme and serum albumin by gel filtration, and was referred to as the anodal tear protein. Electrophoretically, it behaved as a prealbumin. Bonavida *et al.* (1969) named the protein "specific tear prealbumin", and found that it had a MW by gel filtration of about 15,000. It could be further shown that the protein is synthesized in the lacrimal gland. Some animals have this protein in their lacrimal gland secretions as well (Erickson, 1956; Bonavida *et al,* 1969).

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Bibliographic Notes to References for Unit III

§ 1 *Japanese Journal of Legal Medicine* (*Jpn. J. Leg. Med.*) has Japanese title: *Nippon Hoigaku Zasshi*

§ 2 *Reports of the Scientific Police Research Institute* has Japanese title: *Kagaku Keisatsu Kenkyujo*

§ 3 *Rendiconti dell'Accademia Scienze Fisiche Mathematiche* (Napoli) has alternate title: *Reale Accademia Scienze Fisiche Mathematiche* (Napoli). *Rendiconti.*

§ 4 *Acta Criminologiae et Medicinae Legalis Japonica* has Japanese title: *Hanzaigaku Zasshi* and see note §10 to References for Unit II

§ 5 *Acta Medica* (Fukuoka) has Japanese title: *Igaku Kenkyu*

§ 6 *German Medical Monthly* is a cover-to-cover translation of *Deutsche Medizinische Wochenschrift*

§ 7 *Journal of the Kumamoto Medical Association* has Japanese title: *Kumamoto Igakkai Zasshi*

¶ Symbol indicates that a translation of the paper appears in Unit IX

UNIT IV. DETERMINATION OF SPECIES OF ORIGIN

SECTION 15. OLDER METHODS

15.1 Introduction

If the results of the examination of unknown bloodstains are to have very much probative value, the species of origin must be established. It may be necessary on occasion to carry out a determination of species of origin on a body fluid or secretion other than blood, but since the question arises much less frequently with body fluids than with blood, less attention has been paid to the problem. If immunological tests are to be used, the principle remains the same, of course, regardless of which fluid is under examination. Most current methods in common use for determining species of origin are immunological ones. The basis for these methods was established in the last few years of the 19th Century, and the techniques actually devised and put into practice in 1901. Prior to that time, medicolegal investigators were very concerned about the problem, but the techniques were not very satisfactory.

15.2 Chemical Methods

In 1829, J.-P. Barruel introduced a method for determining the species of origin of bloodstains. Blood or bloodstains would emit a completely characteristic odor, he said, when treated with an excess of concentrated sulfuric acid. He claimed, in addition, that human blood could be differentiated with regard to sex of origin based upon the pungency of the odor. Orfila was apparently convinced of the usefulness of this approach, for in 1835 the technique was used in a homicide case in which he served as the medico-legal expert along with Barruel and Chevallier (see section 3), although no results could be obtained with the exhibits submitted. In 1842, Mandl noted that the method was unreliable, and not suited for use in legal medicine. Schmidt (1848) evaluated Barruel's technique, and said that it was not reliable. In 1853, Tardieu *et al.* re-examined the technique in connection with a case they were working on, and concluded, after a series of blind trial tests on stains of known origin, that the test was not reliable.

In 1848, Casanti reported that the blood of different mammals, including human beings, formed a characteristic residue when dried and treated with concentrated phosphoric acid. He said, too, that menstrual blood could be differentiated from circulating blood in this fashion. The method does not appear to have enjoyed any popularity, doubtless because of its unreliability.

15.3 Micrometric Methods

Micrometry, the measurement of red cells as precisely as possible in smears made from fresh blood as well as in reconstituted stains, came into use as a method for species determination of the 19th Century. There was considerable disagreement among various authorities over the certainty which could be attached to the results of these determinations. The controversy was never actually resolved. It simply became irrelevant shortly after 1900, when the immunological methods were discovered and quickly put into use in medico-legal practice.

For the most part, the technique involved the reconstitution of the erythrocytes from bloodstains, and examination of a fairly large number of cells microscopically. Careful measurements were performed on the size of the cells, and these compared with values for the known sizes of red cells from many different species. Most vertebrate red cells are enucleate, and it was usually not difficult to diagnose invertebrate blood. But, particularly among the mammals, a number of species have red cells which are quite similar in size. The efforts which were made, and the disagreements which existed, must likewise be considered in the context of the accuracy of the methods which were then available. There was never complete agreement that species could be correctly diagnosed using microscopical measurements, or micrometry. A good many authorities came to believe that a proper examination could establish only that blood was of mammalian origin.

The notion that red cell size might be exploited for the determination of species of origin can be traced to some of the earliest discussions of medico-legal blood examinations in France. In 1821, Prevost and Dumas had established that the red blood cells of various species differed in size. In 1827, Dulong is said to have remarked at a meeting of the Royal Academy of Medicine that the size of red cells is a very characteristic feature, even when the blood is old and dried, and that this property could be used to help decide the species of origin of the blood. Orfila (1827), however, was not convinced, having carried out a number of experiments to test the possibility. Orfila's reputation being what it was, the matter does not appear to have come up again for several years.

In 1842, Mandl published an extensive paper on the use of microscopy in medico-legal investigations, and strongly advocated the measurement of the red cells as a means of determining species origin. There was no difficulty in distinguishing the nucleate red cells of birds, reptiles, and so forth, from the enucleate ones of mammals. Mandl said, however, that one could not discriminate among the red cells of mammals by this method. Schmidt (1848) briefly discussed the micrometric method in his book, and indicated that he thought it was more promising than the methods which had been in use up to that time. Robin and Salmon readily distinguished human from duck bloodstains in a case in 1857. The bloodstains at issue, they reported, had all the

microscopic characteristics of human blood and could not have come from a duck as the defendant was claiming. Fleming (1861) discussed the technique, but did not think it was very reliable. Roussin wrote a paper on the subject in 1865, advocating the method strongly. He introduced his solution for the preservation of red cells from bloodstains (see section 5.3) in this paper, but he did not think that the blood of mammals could be differentiated by the measurements. The successful use of the method in two cases was reported. In 1869, Richardson in Philadelphia, who became one of the foremost advocates of the method, published a paper saying that one could, by careful measurement of the red cells, distinguish readily between human, sheep and pig blood. He extended his studies to other species, noting several years later the importance of using high power objectives in carrying out the measurements, so as to obtain sufficiently high magnification to be able to distinguish relatively small differences (Richardson, 1874a, 1874b). He thought that there was no difficulty in distinguishing human blood from the blood of pig, ox, deer, cat, horse, sheep and goat, even in stains that were five years old. Later, he examined blood from a number of people of different ethnic backgrounds, and said that there were no significant differences in the size of the red cells among the different races of humankind (Richardson, 1877). Woodward (1875) took exception to Richardson's claims, noting that the cells of the dog were in fact very similar in size to those of humans. He said that he did not believe there was any method available for the unequivocal diagnosis of human blood. Tidy (1882), while noting with interest Richardson's work, said that in giving testimony to a Court about the species of origin of a bloodstain, ". . . it is better, in the present state of science, at once to confess our inability to give a definite reply." Hemphill (1875) noted that he thought it possible to diagnose mammalian blood in stains, but not to establish which particular species was involved.

In 1875, Gulliver published one of the most extensive single investigations of the sizes and shapes of the red cells of vertebrates. Dozens of species of animals were included in the studies, and extensive tables prepared to summarize the findings. These data became known as "Gulliver's Tables", and were widely quoted and sometimes reproduced in the medico-legal literature for 25 years. The relationships he found in the sizes of red cells of various species were summarized in a figure in his paper, which is reproduced as Fig. 15.1.

In 1873, a committee appointed by the Societé de Médecine Légale in France reported that it thought the organization should adopt the stance that it was possible for a qualified expert to decide by micrometric methods whether or not a bloodstain was of human origin (Mialhe *et al.*, 1873). "Il [referring to the expert] mesurera les globules et pourra ainsi affirmer s'il s'agit ou non de sang humain," they wrote.

The major objections voiced by opponents of the accuracy of the methods were that the differences in size between many mammalian species cells were very small, and fell within the experimental error of the measurements, that the interspecific variations were sometimes exceeded by intraspecific variation, and that the cells underwent irregular changes in size and shape upon drying and reconstitution. Richardson and other adherents of the method did not fully accept these arguments. He thought that red cell size varied only very little within a species, and that the reconstituted cells were exactly the same size as those which had originally dried out.

In 1882, Vibert carried out a number of studies on the technique, proposing two solutions for the conservation of red cell size and shape as well. He did not think that human blood could be differentiated from mammalian blood, however. Masson reviewed the material on this matter extensively in 1885 in a series of papers. He conductd a number of experiments as well. A number of the solutions which had been proposed were tested both with fresh blood and with bloodstains, Vibert's solution being recommended for the former, Virchow's for the latter. A number of experiments were done to try and find out what factors cause the changes in red cells upon drying. Masson thought that the faster the blood dried out, the better the cells were conserved in terms of shape and size. He thought that unknown stains could be assigned to one of several categories of animals, arranged according to the size of the red cells, but he did not think a specific diagnosis was possible with a bloodstain. Formad (1888) in his book reviewed the entire subject extensively and well. Most of the opinions which had been expressed on the accuracy of the method were covered, and the references given. Formad himself was persuaded that Richardson's conclusions were valid in many respects. He thought that there was no difficulty in distinguishing human blood from that of domestic animals in stains, assuming that a large enough number of cells could be examined and measured (500 was recommended), and omitting the guinea pig from consideration. Human blood could not be distinguished from every animal blood, and he noted that the probative value of testimony presented by an expert who had conducted such examinations relied in great part on the way in which the questions were asked of him. He thought it would be misleading for the Court to gain the impression that human blood could not be distinguished from *any* animal bloods by the micrometric method. Ewell (1892a) measured 4000 red cells, and found variations within the same species. He also said that different observers would get different values, and that the variation was large enough to make one doubt the published figures. He did not think red cells in stains could be restored to their *in vivo* proportions. He thought that the micrometric method could be useful in diagnosing blood as being of mammalian origin, but not more.

In 1892, Jones reported on a murder case in Ascension Parish, Louisiana, in which he had examined bloodstain evidence. The body of a man named Simeon Cascio had been discovered September 22, 1892. Suspicion fell on the victim's brother-in-law, one Joseph Polito who had been seen with the victim shortly before the murder, and who had some of the victim's possessions with him at the time of his arrest.

Dr. Jones examined stains on the blade and handle of a knife, on a pocket book, on the collar of the victim's shirt and several stains on paper money bills. The stains on the knife and pocket book, he said in his report, "presented the characteristics of human blood." The stains on the currency were identified as being blood, and he said that the stains on the shirt collar were "due to human blood." Ewell (1892b) took very great exception to Jones' conclusion that he had determined the stains on the collar to be human blood. "In view of the consensus of opinion among microscopists who have examined this question, that in the present state of science it is impossible to identify human blood, as such, it would be interesting to know how Dr. Jones reached a conclusion at variance with the almost unbroken current of authority," he wrote. Jenne (1896) agreed with the view that human and animal blood in stains could not be distinguished by micrometric methods.

Axtell (1895) reported in detail on a murder case in Denver, Colorado, in which he had been able to determine by micrometry that the stains on the suspect's clothing were mammalian in origin, and consistent with human blood. The defendant had claimed that the stains were from the blood of a rabbit, but Axtell was able to exclude several different species of rabbits as being the source of the bloodstains on the basis of differences in red cell sizes. The defendant in the case entered a plea of guilty before Axtell had the opportunity to present his findings before a Court. In 1892, Bell reviewed the micrometric method in some detail, and presented the veiws of most authorities. To illustrate the kinds of tables that were employed, Bell's table of comparative measurements is reproduced as Table 15.1. Corin (1901) gave a brief review of micrometric methods. Several papers by 19th Century French authors, which go into the subject in considerable detail, have been included in the Translations (Unit IX).

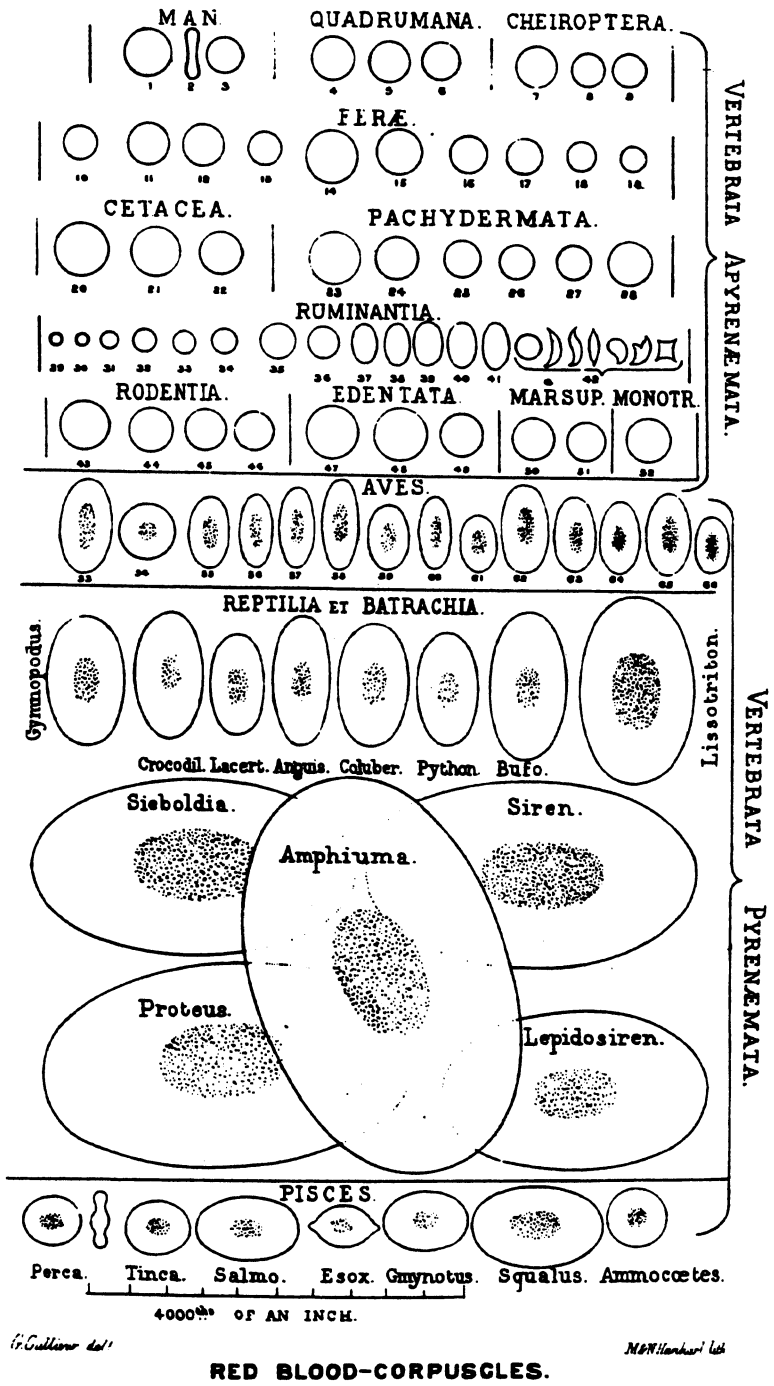

Figure 15.1 Relationships of red cell sizes of many species (after Gulliver, 1875)

All the objects are red blood-corpuscles done to one and the same scale, which is at the foot of the drawing. The whole length of the scale represents $\frac{1}{100}$ of an English inch, and each one of its ten divisions $\frac{1}{1000}$ of an inch, as described at page 475. Only corpuscles of the average sizes and quite regular shapes are given; and they are all magnified to the same, or nearly the same, degree—to wit, about 800 diameters.

VERTEBRATA APYRENÆMATA.

Homo.

1. Corpuscle lying flat.
2. The same on edge.
3. Membranous base of the same, after removal by water of the colouring-matter.

Quadrumana.

4. Simia troglodytes.
5. Ateles ater.
6. Lemur anguanensis.

Cheiroptera.

7. Cynonycteris collaris.
8. Vespertilio noctula.
9. Vespertilio pipistrellus.

Feræ.

10. Sorex tetragonurus.
11. Ursus labiatus.
12. Bassaris astuta.
13. Cercoleptes caudivolvulus.
14. Trichechus rosmarus.
15. Canis dingo.
16. Mustela zorilla.
17. Felis tigris.
18. Paradoxurus pallasii.
19. Paradoxurus bondar.

Cetacea.

20. Balæna boops.
21. Delphinus globiceps.
22. Delphinus phocæna.

Pachydermata.

23. Elephas indicus.
24. Rhinoceros indicus.
25. Tapirus indicus.
26. Equus caballus.
27. Dicotyles torquatus.
28. Hyrax capensis.

Ruminantia.

29. Tragulus javanicus.
30. Tragulus meminna.
31. Tragulus stanleyanus.
32. Cervus nemorivagus.
33. Capra caucasica.
34. Capra hircus.
35. Bos urus.
36. Camelopardalis giraffa.
37. Auchenia vicugna.
38. Auchenia paco.
39. Auchenia glama.
40. Camelus dromedarius.
41. Camelus bactrianus.
42. Cervus mexicanus (see page 483)

Rodentia.

43. Hydrochœrus capybara.
44. Castor fiber.
45. Sciurus cinereus.
46. Mus messorius.

Edentata.

47. Myrmecophaga jubata.
48. Bradypus didactylus.
49. Dasypus villosus.

Marsupialia.

50. Phascolomys wombat.
51. Hypsiprymnus setosus.

Monotremata.

52. Echidna hystrix.

VERTEBRATA PYRENÆMATA.

Aves.

53. Struthio camelus.
54. The same, made round and deprived of colour by water.
55. Vanga destructor.
56. Lanius excubitor.
57. Bubo virginianus.
58. Syrnea nyctea.
59. Columba rufina.
60. Columba migratoria.
61. Dolichonyx oryzivorus.
62. Buceros rhinoceros.
63. Psittacus augustus.
64. Phasianus superbus.
65. Pelecanus onocrotalus.
66. Trochilus, sp.

Reptilia et Batrachia.

Gymnotus ægyptiacus.
Crocodilus acutus.
Lacerta viridis.
Anguis fragilis.
Coluber berus.
Python tigris.
Bufo vulgaris.
Lissotriton vulgaris.
Siebolidia maxima.
Siren lacertina.
Proteus anguinus.
Amphiuma tridactylum.

Pisces.

Perca cernua, one corpuscle lying flat, the other on edge.
Tinca vulgaris.
Salmo fontinalis.
Esox lucius.
Gymnotus electricus.
Squalus acanthias.
Ammocœtes branchialis.
Lepidosiren annectens.

Figure 15.1 Explanation of the red cell size diagram on the facing page from the original article.

COMPARATIVE TABLE OF THE AVERAGE RESULTS OF MEASUREMENTS OF RED BLOOD CORPUSCLES OF MAMMALS.

Each column giving the average size (diameter) of the Corpuscles as obtained by various observers, expressed in fractions of the English inch, side by side with the common expression (roughly) in millimeters.

	Gulliver, 1845 and 1875.		Wormley, 1885.		C. Schmidt, 1848. Mallinin, 1875.		French Medico Legal Society, 1873, and Wilker.		Masson, 1885.		Hans Schmid, 1878.		Woodward, 1875.		Personal Observations.	
	In.	M. M.	In.	M. M	In.	M. M.	In.	M. M.	In.	M. M	In.	M. M	In.	M. M.	In.	M. M.
Elephant	1.2745	0.0092	1.2788	0 0093												
Great Anteater	1.2769	0.0092														
Walrus	1.2769	0.0092														
Sloth	1.2965	0.0095														
Ornithorynchus	1.3000	0.0061														
Whale	1.3099	0.0080														
Opossum	1.3557	0.0071	1 3145	0 0080												
Capybara	1.3190	0.0080	1.3164	0.0080												
Man	1.3300	0.0079	1.3250	0 0078	1.3300	0.0077	1.3257	0 0078	1.3257	0.0078	1 3412	0.0074	1 3092	0 0082	1.3200	0.0079
Seal	1.3281	0.0078														
Beaver	1.3325	0.0076														
Muskrat	1.3550	0.0073														
Porcupine	1.3769	0.0075														
Monkey	1.3412	0.0074	1 3382	0.0078												
Kangaroo	1.3440	0.0074	1 3410	0 0074												
Guinea Pig	1.3558	0.0071	1.3323	0.0079					1.3300	0 0077			1 3213	0.0079	1.3400	0 0076
Wolf	1.3600	0.0070	1 3422	0 0074											1.3450	0.0074
Dog	1.3539	0.0071	1.3561	0 0071	1.3636	0.0070	1.3486	0 0073	1 3577	0.0071	1 3846	0.0066	1 3246	0.0078	1.3580	0.0071
Rabbit	1.3607	0.0070	1.3658	0.0070	1.3968	0.0064	1 3953	0 0069	1 3636	0.0070					1 3602	0.0069
Ass	1.4000	0.0063	1.3630	0.0070												
Rat	1.3754	0.0065	1 3658	0 0070	1 3968	0.0064					1 5000	0 0051				
Bear	1.3698	0.0070	1.3656	0.0069												
Mouse	1.3814	0.0067	1 3742	0.0067	1 5000	0 0051										
Mule			1.3760	0 0067												
Squirrel	1.4000	0.0064	1.4140	0.0061												
Ox	1.4267	0.0060	1.4219	0.0060	1.4364	0 0056	1.4545	0.0056	1.4237	0.0060	1.4695	0.0054			1 4200	0 0060
Pig	1.4230	0.0060	1 4098	0.0062	1.4098	0 0062	1.4098	0 0062	1.4098	0.0062	1.4098	0.0062			1.4250	0.0060
Horse	1.4600	0.0055	1 4043	0.0059	1.4464	0 0057	1.4545	0 0056							1 4310	0 0059
Cat	1 4404	0.0058	1 4373	0 0058	1.4545	0.0056	1.4682	0.0055	1.4440	0.0057						
Sheep	1 5300	0.0049	1 4912	0.0051	1 5649	0.0045	1 5076	0 0050	1.4464	0.0057	1 6060	0.0042			1.5000	0.0051
Goat	1 6366	0.0040	1 6164	0.0041	1.6360	0.0040	1.5385	0.0046							1.6100	0.0042

Table 15.1 Comparative Red Cell Measurements by Several Authorities (after Bell, 1892). References in the table may be found in the reference list, except "Gulliver (1845)" and "Welker". French medico-legal society (1873) is listed as Mialhe et al. (1873) in the reference list.

SECTION 16. IMMUNOLOGICAL TESTS WITH BLOODSTAINS

16.1 Precipitin Test

16.1.1 Development of the precipitin test and its medicolegal application

The development of a specific immunological test for determination of the species of origin of bloodstains was a major event in the evolution of legal medicine. It provided for the first time a technique for unequivocally establishing species of origin in stains. All authorities soon came to agree on the specificity and certainty provided by the new method, Wood (1902a) having said to the Massachusetts Medico-Legal Society that one could finally testify absolutely as to the species of origin of bloodstains, rather than having to say that the results were "consistent with" the stain having originated from a particular species. Precipitin reactions are discussed more generally in section 1.3.4.2. Recognition of precipitating antibodies, and their application to forensic investigations, must be regarded as natural outcomes of the rapid and ever-widening development that was occurring in immunology in the last years of the 19th century, and which continued into the present one. In the years 1901 to 1920, five Nobel prizes for Physiology and Medicine were awarded in immunology: in 1901 to Emil A. von Behring "for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths"; in 1905 to Robert Koch "for his investigations and discoveries in relation to tuberculosis"; in 1908 to Elie Metchnikoff and Paul Ehrlich "in recognition of their work on immunity"; in 1913 to Charles R. Richet "in recognition of his work on anaphylaxis"; and in 1919 to Jules Bordet "for his discoveries relating to immunity."

Immunology had gotten its start as a proper scientific field of inquiry because of concern with infectious and highly contagious diseases, epidemics of which affected large segments of the population at times. The recognition, and ultimate acceptance, of the notion that these diseases were caused by specific microorganisms provided a basis for detailed investigations into the subject of immunity and its mechanism.

Behring and Warnecke (1892) made the important discovery that animals injected with diphtheria toxin elaborated specific antitoxins. Pfeiffer (1894) discovered that if animals were treated with bacteria, specific antisubstances form in the serum of the injected animal. If this serum and the bacteria are mixed together and injected into the peritoneal cavity of a healthy animal, the bodies of the bacilli are degraded. If bacteria are mixed with the antiserum *in vitro*, the bacteria are agglutinated, and will settle out of the suspension as a mass. Uhlenhuth (1911) attributed this discovery to Gruber and Durham in 1896. In 1897, Kraus made the important observation that bacterial antibodies have the property of precipitating cell free bacterial extracts. This paper marked the first description of precipitin antibodies, which would shortly play an important role in immunological species differentiation. It was soon realized that bacterial substances were not the only ones to which animals would elaborate antibodies. Bordet (1898) found that guinea pigs produce a potent red cell agglutinating serum against rabbit red blood cells when immunized with defibrinated rabbit blood. Similarly, rabbits which had received several injections of defibrinated hen blood yielded a serum which actively agglutinated (agglutinin) and lysed (hemolysin) hen red cells. In addition, it was found that the antiserum contained a specific precipitin for hen serum. This discovery was made, Bordet said, by Tschistovitch at the Pasteur Institute. In 1899, Tschistovitch found that rabbits elaborated a precipitin antibody to eel serum with which they had been injected. The precipitates were soluble in solutions of strong base. To Tschistovitch, therefore, belongs the credit for the recognition of the fact that animals will form precipitating antibodies to the serum of an animal of another species. Bordet (1898) could show that milk proteins were antigenic in rabbits, giving rise to precipitins for the casein. The serum containing the antibodies was referred to as "lactoserum". Fisch (1900) in this country confirmed Bordet's observations on the antigenicity of casein. Wassermann and Schütze (1900) extended these studies to show that the precipitin test could be used to differentiate the milk of different species of animals immunologically. In 1900, Myers showed that crystalline hen egg albumin was antigenic in rabbits, giving rise to an antiserum which cross reacted to some extent with duck ovalbumin solutions. The antiserum was entirely specific for the ovalbumins, however, and did not react with a number of other animal protein solutions. Independently, Dr. Paul Uhlenhuth, whose name came to be more or less synonymous with the precipitin test in its forensic applications, published a paper in 1900 in which he said that he had successfully differentiated the egg albumin from several species of birds using precipitating antisera prepared in rabbits. In the course of these experiments, he found that rabbits immunized with chicken blood elaborated precipitating antisera for the chicken serum, and this antiserum did not react with the sera of horse, donkey, cow, sheep or pigeon. Further, the antisera prepared against hen blood showed only a slight cross reaction, and this after a long time, with hen ovalbumin, demonstrating that immunological specificity could extend not only to the proteins of different species, but also to proteins from different sources within the

same species. As Uhlenhuth remarked about these experiments some years later in the Harben Lecture (1911), ". . . . *the foundation was laid for the forensic method of distinguishing between different specimens of blood,* for the serum of rabbits treated with the blood of man or of any other animal gave a precipitate only with blood solutions of the corresponding species to that whose blood had previously been injected to the rabbit."

The stage having thus been set, 1901 saw the publication of a number of papers which specifically addressed the immunological differentiation of human from animal bloods using the precipitin test, and recommended that the method be adopted in medico-legal examinations of blood stains. Uhlenhuth (1901a) reported in the February 7 issue of *Deutsche Medizinische Wochenschrift* that he had been able to distinguish cattle blood from horse, donkey, pig, ram, dog, cat, deer, hare, guinea pig, rat, mouse, rabbit, chicken, goose, turkey, pigeon and human bloods using a rabbit immune anti-cow precipitin serum. Similarly, human blood could be differentiated from the others using serum from rabbits which had been immunized with human blood. Normal rabbit serum did not react with saline solutions of any of the bloods. Further, it could be shown that saline extracts of human bloodstains dried on pieces of wood could be distinguished from horse and cattle bloodstains treated similarly. The stains were a month old when tested. A summary of this work appeared in a later issue of *Muenchener Medizinische Wochenschrift* (Uhlenhuth, 1901b). Independently and almost simultaneously, Wasserman and Schütze (1901) arrived at the same conclusions regarding the species specificity of the reaction and its applicability to dried bloodstains, this paper appearing in the February 18 issue of *Berliner Klinische Wochenschrift.* They noted that serum could be used as immunizing antigen in order to obtain antisera lacking antibodies to the cells (the agglutinins and hemolysins). Uhlenhuth (1901c) reported the technique in detail in the medico-legal literature in May, noting that species differentiation was possible in 3 month old bloodstains, in blood frozen for 2 weeks in the snow, and even in putrefied blood, provided a completely clear extract could be obtained for testing. A number of badly putrefied samples reacted readily with the antiserum, and human menstrual blood in a urine specimen could be detected easily. Human urine containing albumin reacted with the antiserum as well (Uhlenhuth, 1901d). This observation was also made by Dieudonné (1901). Further studies on the precipitin test indicated that bloodstained materials from criminal cases, made available from past cases by the judicial authorities, could be reliably diagnosed in every case. Results could even be obtained with bloodstains that had been washed (Uhlenhuth, 1901e). It was noted that cross-reactions occurred between more closely related species, e.g. anti-ram reacted to some extent with goat and cattle blood. Uhlenhuth recognized that the relationship between immunological cross reactivity in the precipitin test and species relatedness was a matter of fundamental biological importance. Anti-human serum had been shown to react with albumin-containing urine, and it also reacted with human semen and with pulmonary mucus from tuberculosis patients to some extent. The antiserum, therefore, detected human albumin, and was not necessarily specific for blood (Uhlenhuth, 1901e; Wassermann and Schütze, 1903). Uhlenhuth noted that antiserum preparation required considerable experience, and that there was variability in the rabbits used to produce it. He said that the use of high quality, carefully tested antisera was of paramount importance in forensic investigations, and suggested that antiserum production be carried out in a few specialized institutes.

Nuttall (1901a) confirmed the specificity and value of the precipitin test. He noted further that the serum used for immunization needn't be fresh in order to elicit antibodies. He showed also that the reaction worked well in dried bloodstains and in putrefied blood. The investigations on the phylogenetic relationships among various animal species, using immunological cross reactivity as a basis, were begun at this time (Nuttall and Dinkelspiel, 1901a and 1901b), and it was noted that anti-human serum cross reacted with the blood of several species of monkeys. Antisera could also be prepared against human pleural fluid, and this serum cross reacted to some extent with human tears and nasal mucus. Medico-legal application of the precipitin method was strongly recommended. Ziemke (1901a and 1901b) confirmed the value of the technique, noting that species could be diagnosed in bloodstains up to six years old as well as on a variety of substrata. Frenkel (1901) and Stern (1901) likewise showed that the precipitin test was fully applicable to bloodstains, and both noted the cross reactions with monkey bloods. The term "antiserum" appears to have been introduced at this time simultaneously and independently by Nuttall (1901a) and by Stern (1901) to apply to the serum containing the antibodies (usually, immune rabbit serum). Corin (1901) noted in his review that the precipitin antisera were more specific if the serum globulins were isolated and used for immunization.

The bulk of evidence thus indicated that the precipitin test was both specific within certain limits and fully applicable to forensic samples, and the use of the test rapidly became widespread. Many authorities confirmed its value and noted its limitations. Kratter (1902) dissented for a time from the current of opinion, saying that he had been unable to confirm a number of Uhlenhuth's observations on specificity, reliability and medico-legal applicability of the test. Uhlenhuth (1902) responded to Kratter's article, giving further detailed experimental justification for the test's reliability and specificity in the diagnosis of species in bloodstains on objects involved in criminal cases. Stonesco (1902) said that because of the known cross reactions of the antisera, it would be better to use the term "probably" in giving evidence on the species of origin of bloodstains in which a positive test had been obtained for human blood. Schulze (1903) more or less agreed with this view, saying that the Court should be made aware of the test's limitations as well as of its great value. Whitney (1902 & 1903) described successful results with the method, and Wood (1902b and 1903) gave an

account of the technique he used, and said that he had been able to identify human blood on an alleged murder weapon (a stone) in a case from New Hampshire. Ewing (1903) and Mallett (1903a and 1903b) both said they thought antihuman sera prepared in chickens were more specific, and gave fewer cross reactions than rabbit antisera. Mallett thought there might be interracial immunological differences which could be detected by means of the precipitin test, this based on a few experiments with serum from Caucasians and Negroes. Bruck (1907) was able to obtain differences in the intensity of reactivity with the sera of people from different races using the complement fixation technique (section 16.6.1), and thought that the method might be useful in this respect. Marshall and Teague (1908), in extensive experiments using both the precipitin and complement fixation techniques, also observed that there were slight differences in reactivity of the sera of persons from different races with antisera prepared against the serum of a member of a particular race. But these differences were so slight that they did not agree with Bruck that racial origin could be diagnosed by immunological techniques.

Some authorities around this time began using the term "humanized serum" to apply to the antiserum. Thus, rabbit immune anti-human serum was referred to as "humanized" rabbit serum. DeLisle (1905) prepared antihuman sera in horses, and recommended this large animal as a source because of the large quantity of antisera which could be obtained. It is now known that horse and a few other animal species often produce precipitin antisera which have somewhat different characteristics from most rabbit antisera. The horse antisera are characterized by a narrower zone of equivalence between antigen and antibody, that is, the antigen-antibody complex is soluble in either excess antigen or excess antibody, and the range over which precipitation will occur is narrower than with rabbit antisera (Maurer, 1971). This matter is discussed in more detail in section 1.3.4.2. In 1903, Hitchens reviewed the highlights in the development of immunology, emphasizing the value of precipitin antisera in medico-legal work. Ewing and Strauss (1903) gave an extensive review of precipitins and their medico-legal applications.

An extremely comprehensive series of studies on the application of the test to forensic materials was reported by Graham-Smith and Sanger in 1903. Many of the test's parameters were explored in detail, and studies were conducted on blood stains on a large number of different materials under different conditions. A few chemicals exerted a deleterious effect on the antiserum, and some stains on leather substrata were not able to be diagnosed. Very high titer antisera could be produced using relatively small quantities of immunizing sera, of the order of 3 to 5 mℓ at each of three to four injections. Many workers had employed considerably larger quantities of immunizing serum.

Robertson (1906) recounted his experiences with the test, and noted that he got positive tests in stains that were 9 years old. In 1908, Gay reported what was probably the first application of the precipitin test in this country in the enforcement of the game laws. Uhlenhuth (1901f) had shown earlier that the method was applicable to the differentiation of meats from different species of animals. Gay distinguished between precipitins against sera (seroprecipitins) and those against meat protein (musculoprecipitins), and attributed this distinction to Vallee and Nicholas. In the case in question, a man in Massachusetts was accused of illegally killing a deer, based on the finding of an animal heart in his possession by the police. The suspect claimed that the heart was from a cow. Dr. Gay prepared antisera to extracts of authentic beef and deer heart muscle as well as to extracts of the suspected heart. It could be shown that the heart found in the man's possession was in fact that of a deer. Clarke (1914) reported a similar case in California, in which he identified deer meat in a poaching case, which went to a jury trial. In 1917, Hunt and Mills reported on a case in which the precipitin test failed to react with several bloodstained objects in a criminal case. Two anti-human sera were used, one prepared by them and the other prepared abroad. These bloodstains were later shown to be of human origin by the complement fixation technique. They cautioned, therefore, that a negative precipitin test does not necessarily exclude the presence of homologous antigen (blood) in a stain.

In 1909, Uhlenhuth and Weidanz published a monograph which reviewed the subject of forensic immunology to the time, and provided very detailed experimental procedures and methods for every aspect of the precipitin technique. This book was a standard reference for a number of years. In 1911, Uhlenhuth delivered the Harben lecture in England, recounting many of the experiments that had led to the development of the precipitin test, and giving his own recollections. This paper was the only one of his many contributions which appeared in English.

In 1914, Hektoen discussed the production of antisera to human and animal sera, finding that a single injection of 30 mℓ immunizing serum was about as effective as three successive ones of 5, 10 and 15 mℓ, respectively. In 1917, Hektoen carried out experiments to determine whether antibody production in rabbits was enhanced if animals were used which had previously been employed for antibody production (not necessarily with the same antigen). There were no differences between such animals and immunologically virgin rabbits, and Hektoen suggested that antisera were therefore best prepared in the latter. Ewing (1903) and Mallott (1903a and 1903b) had recommended the production of antisera in fowl. Sutherland (1914) preferred to prepare his antisera in domestic fowl, but these antisera exhibited some peculiar characteristics. Sutherland and Mitra (1914) noted that too rapid thawing of frozen antisera caused it to exhibit interspecific cross reactions. These could sometimes be eliminated by allowing the serum to sit in the dark for a time, or by dilution with normal nonimmune fowl serum. Hektoen (1918a) found that fowl were more reliable, liberal producers of antisera than were rabbits. Good, high titer antisera could be obtained in most cases in 10–12 days after a single intraperitoneal injection of 20 mℓ defibrinated blood or of serum. He noticed some of the same peculiarities which

Sutherland and Mitra had earlier observed, and in another paper (1918b) suggested that it would probably be best to use rabbits for making antisera. Hektoen and McNally discussed the precipitin test in detail, including the preparation and evaluation of antisera in their review of medico-legal examination of blood stains in 1923. Fujiwara (1922a) said that heat-coagulated serum elicited antibody production as well as did fresh serum. Schmidt (1921), working with egg albumin, had shown that antibodies obtained with denatured protein could react with the denatured protein as well as with the native molecules. Blumenthal (1927) reviewed in detail the methods for making antisera, and gave what he regarded as the best protocol for immunization. Proom (1943) was able to obtain potent antisera in over 80% of the rabbits used with alum-precipitated antigen. Over the years, many investigators have looked into the preparation of antisera for the precipitin test. Many of these studies are discussed in some detail in Schleyer's (1962) review.

At present, many laboratories obtain antisera from commercial sources. Schleyer (1962) said that antisera should meet certain criteria before being employed in medico-legal work. They should be sterile, free from turbidity, of high potency and species-specific. By high potency was meant that the antiserum should have a minimum titer of $1{:}10^4$ to $1{:}2 \times 10^4$ against homologous antigen, and quite obviously the antiserum must not cross react with the sera of other animals to be exlcuded in the tests at the dilutions at which the test is performed. Otto and Somogyi (1974) said that most of the cross reactions of antihuman sera with nonhuman animal sera were accounted for by antibodies to IgG and to α-globulins.

It is worthy of mention that the use of adjuvants in immunizing animals to obtain antisera has permitted better yields of higher titer antisera in many cases. Since the work of Freund (see, for example, Freund and McDermott, 1942), many immunologists have employed adjuvants in the preparation of antisera. Herbert (1973) discussed the use of water-in-oil adjuvants, giving a review of the technique as well as some of the experimental details.

16.1.2 More recent developments—gel methods

Another important development has been the evolution of methods which use gel media for detection of the antigen-antibody reaction. Before gel media were introduced, the precipitin test was carried out in tubes. Often, antiserum, which is more dense than the usual aqueous (e.g. saline) antigen solutions, was placed into a tube and antigen solution carefully layered over the top so as to form an interface at which the precipitate could then form. Alternatively, the antigen solution could be placed in the tube first, and the antiserum solution layered underneath it by means of a capillary pipette. This procedure is sometimes called a "ring test". The earlier workers all employed this sort of method, in many cases using what would be considered by present standards excessive quantities of reagents, on the order of one to several mℓ. Material can be conserved by carrying out the test in small capillaries. The use of capillary tubes was first recommended by Hauser in 1904. Schoenherr (1952) gave a technique for the precipitin test in Pasteur pipettes. The ring test is still in use in many laboratories (see, for example, Boyd, 1946 and Hunt *et al.*, 1960). The test can be carried out on a microscope slide and the precipitate observed in the microscope under dark field illumination (Marx, 1920).

The first immunodiffusion experiment was done by Bechhold in 1905, although his primary interest was not in antigen-antibody reactions. He allowed goat serum to diffuse into a gelatin medium into which had been incorporated rabbit antiserum to the goat serum. The experiment was done to obtain a precipitate different from those obtained when inorganic chemicals were allowed to diffuse together in gels and form precipitates. One can, for example, put a drop of $Na_2Cr_2O_7$ onto a gel into which has been incorporated $AgNO_3$, and a series of concentric rings of $AgCr_2O_7$ precipitate will form. These are called "Liesegang rings", and Bechhold attributed their discovery to R. E. Liesegang in 1898. No notice appears to have been taken of Bechhold's experiments by the immunologists of the time. In 1920, Nicolle *et al.* devised a single immunodiffusion technique for the identification of bacteria. Bacteria were grown in agar medium containing specific antisera for particular bacterial species. If the bacterial cell wall antigens were homologous to the antiserum, a halo formed in the gel around the colony. None of the early workers properly appreciated the meaning and potential applicability of multiple precipitin band formations, which were occasionally observed, but thought they were similar to the Liesegang phenomenon. The possibility that they might represent more than one antigen-antibody system, present in the same test system, was overlooked.

In 1946, Oudin published his first paper on immunodiffusion. This work represented the beginning of the development of the technique as a powerful immunological tool. Antiserum containing gels were overlayed with homologous antigen solutions and the behavior of the precipitin band which formed in the gel was studied. The precipitin band appears to diffuse through the gel in proportion to the initial concentration of antigen, its diffusion coefficient, and inversely to the antibody concentration. Multiple antigens cause the formation of multiple bands, which migrate independently. In succeeding years Oudin developed the method further and devised a theoretical basis for the observations (Oudin, 1947, 1948 and 1949). A review of the principles underlying Oudin's technique and immunodiffusion generally may be found in Oudin (1971), Aladjem (1971) and Oudin and Williams (1971).

Oudin's technique is called single immunodiffusion. It is discussed in connection with the principles of immunodiffusion in section 2.2.

The late 1940's witnessed the development of techniques for double diffusion analysis of antigen-antibody reactions in gels by Elek in England and Ouchterlony in Sweden. The method was developed primarily for assessing the diphtheria bacillus toxin reaction with homologous antitoxin. Elek pub-

lished his preliminary findings in 1948, a more detailed account following in 1949. Ouchterlony published his first account in 1948 as well (Ouchterlony, 1948a and 1948b). The theoretical basis was laid down in a series of papers in the following year (Ouchterlony, 1949a, 1949b and 1949c). Elek's interest in the subject did not continue, but Ouchterlony has continued to work in the field, one of the best single references being his monograph, published in 1968. This work covers not only the agar gel method, but discusses some of the other support media such as cellulose acetate membranes. Detailed descriptions of various methods are given, as well as the underlying theoretical basis for the variations which have been described. Crowle's book also gives a very complete treatment of the subject (Crowle, 1973). Oakley and Fulthorpe (1953) developed the double diffusion technique for use in tubes, with special reference to its application to the analysis of bacterial toxins. Double diffusion is discussed in section 2.2.2.

Muller *et al.* (1950) applied Oudin's technique to medicolegal practice. Gum acacia was tried as a medium but was abandoned in favor of agar and agar-gelatin mixtures (Muller & Michaux, 1952). In 1958, Muller *et al.* noted the applicability of the double diffusion methods. Species determination could be carried out using a microdiffusion technique in agar originally devised by Hartmann and Toillez in 1957 (Muller *et al.*, 1959). Mansi (1958) devised a microimmunodiffusion technique which was used and recommended by Fiori (1963). Gajos and Brzecka (1968) said that threads from bloodstained fabrics could be incorporated directly into the gel for species determination, thus avoiding not only the time required for extraction, but the concomitant loss of material. Maresch and Wehrschütz (1963) described a precipitin test for species determination carried out on microscope slides in 2 mm thick agar gels. Feinberg (1961) devised a "microspot" double diffusion test, which could be carried out on microscope slide cover slips. Katsura (1976) utilized this method to diagnose the species of origin of very small amounts of blood using anti-human Hb antisera (see section 7.1). The precipitin lines were visualized using a phase contrast microscope, and even stains 48 years old were said to react within 3 hrs at 30°. Feinberg (1962) published a modification of his spot test for use on cellulose acetate membranes. El-Guindi (1972) reported successful results with an ordinary macroimmunodiffusion procedure in agar gels. He used several arrangements of sample and antiserum wells.

In 1959, Bussard suggested taking advantage of the electroendosmotic properties of agar in carrying out immunoelectrophoretic analysis of antigen-antibody reactions. A system could be devised in which the antigen and the antibody migrated toward one another, a precipitate forming at the point of their interaction. This technique, he called electrosyneresis. It has also been called electroprecipitation and crossed over electrophoresis or crossing over electrophoresis (see section 2.4.2). Culliford (1964 and 1967) devised a crossed over electrophoretic technique for species determination in forensic casework which allowed a large number of samples and controls to be run simultaneously in a short period of time, using only a few $\mu\ell$ of material for each test. In this method, small wells about 1.5 mm in diameter are punched in an agar gel about 5 mm apart. The stain extract (antigen) is placed in the cathodic well of a neighboring pair, and the antiserum in the anodic one. The γ-globulin antibodies migrate cathodically because of electroendosmosis, while the other serum proteins migrate anodically. The net result is a precipitin reaction occurring about midway between the paired wells. The test was performed in veronal buffers, pH 8.6, containing calcium lactate. Čarny reported a similar technique in 1971. Grunbaum (1972) noted that crossed over electrophoresis could be done on cellulose acetate membranes as well. Although it is usual practice to prepare antisera against whole human or animal sera, Tran Van Ky *et al.* (1968) recommended the use of antisera prepared specifically against the γ-globulin fraction.

Lincoln (1975) reported on a very interesting series of cases involving alleged witchcraft, illustrating how the events were unraveled by determining the species of origin of the articles involved.

16.1.3 Effects of some external influences

The age of a stain alone apparently does not prevent a positive precipitin test. Haseeb (1972) got a positive test on a 12 year old stain kept at room temperature. Linoli (1971) examined the "flesh and blood" from the eucharistic miracle that is said to have occurred at Lanciano, Italy, in the 7th Century A.D. The blood, which was about 1200 years old at the time of examination, gave a positive precipitin test with anti-human serum. Smith and Glaister (1939) reported a positive precipitin test on extracts from mummified tissue many thousands of years old. It has been known for a long time, however, that old blood stains do not easily yield serum proteins to mild, aqueous extracting solvents, like water or saline. Dorrill and Whitehead (1979) said that 5% (v/v) 0.880 ammonia was a much more effective extraction medium for older stains than water for purposes of extracting serum protein for species determination in older stains.

Muller *et al.* (1966) showed that a positive precipitin test could be obtained on stained garments which had been dry cleaned. There are circumstances, however, under which the precipitin test may fail. Since antisera are almost universally prepared against serum proteins, serum protein must be present in, and extractable from, the stain if a reaction is to be expected. There is not a necessary relationship between the amount of hemoglobin and the amount of serum protein in a stain extract. One cannot judge how much serum protein may be present on the basis of the amount of pigment present, as was pointed out by Okamoto in 1902. With fairly freshly dried stains there is a strong likelihood that considerable blood pigment will be extracted, but sometimes without sufficient serum protein to give a positive precipitin test, particularly if extraction time is short (Mueller, 1934). 24 hour extraction times are recommended in such cases. Mezger *et al.* (1933) noted that with a blood stain on wood,

the dissolved superficial layers of stain did not give a precipitin test. It was necessary to extract the wood substratum, the serum protein having soaked into the wood more deeply than the red portion of the stain. Olbrycht (1950) showed that extracts of dried human blood mixed with earth can fail to give a positive precipitin test with anti-human serum, presumably because of the absorptive properties of the soil. In all cases in which the precipitin test failed, the extract did not contain detectable protein. The same results were obtained with dried blood mixed with aluminum oxide or finely pulverized iron ore. Some bloodstained earth samples did yield precipitin-positive extracts, as did bloodstained limestone and bloodstained sand. A few chemicals caused nonspecific precipitation of the antiserum, and of normal control serum as well. These included aluminum and iron chlorates, aluminum chromate, tannic acid and an extract of spruce bark. Some organic solvents, dilute acids and bases, and peroxide cause non-specific precipitation of the antiserum (Vollmer, 1949).

Exposure of bloodstains to lengthy steeping in cold water does not prevent their giving a positive precipitin test. Older stains yield less protein to the steeping fluid than do fresher ones, and older bloodstains retained more serum protein in 50° water than in cold water (Smith and Glaister, 1939). Detergents do interfere with the precipitin test. Some detergents, even in dilutions up to $1:10^4$, will give a flocculent precipitate with antiserum as well as with normal serum (Burger, 1956; Klose, 1961). Burger (1956) thought these reactions were caused by alkyl- and arylsulfonates or sulfates in the detergents. Water rinsing does not entirely remove these chemicals, but rinsing stained fabric with a solution of methanol-ethanol-amyl alcohol-trichloroethylene prior to saline extraction does rid the substratum of the interfering substances, and allows the test to be performed. Burger noted that these observations underscored the importance of running cloth controls. Schoenherr (1957) noted that detergent residue on glass can cause precipitation of a test serum, a point well worth noting if the test is done in glassware that is washed and reused.

Exposure of bloodstains to heat causes the precipitin reaction to become weaker, and reaction time longer. There is an upper limit of exposure, beyond which the reaction will be negative. The limit is a function of both the temperature and the time of exposure. Smith and Glaister (1939) could still get a positive test on stains exposed to 130° or 150° for 15 min, or to 200° for 2 min. Exposure to heat also tends to fix the stains and render the protein increasingly insoluble. This phenomenon was observed very early by Katayama (1888) and Hammerl (1892) with regard to hematin compounds. Stains which have been ironed may be extremely insoluble (Schech, 1930). Much longer extraction times are required for stains that have been exposed to heat than under ordinary circumstances (Schech, 1930; Schleyer, 1948 and 1962). It is sometimes necessary to use 0.1N NaOH for extraction in these cases since saline will not extract the proteins even after extended periods of steeping (Schleyer, 1948).

Lee and DeForest (1976) devised an interesting variation of the precipitin test, applicable to stains which contain species-specific antigens, but which do not readily yield them up to extraction media. They could show that a substantial fraction of the species-specific serum protein (saline-extractable protein) was lost upon steeping bloodstained cloth in water or detergent solutions. The loss of protein positively correlated with the temperature of the washing water from 25° to 100°. This result was not in accord with that of Smith and Glaister (1939), who observed that hotter water extracted less protein from stained fabric than room temperature water, and thought that the hotter water tended to fix the stain, and prevent protein loss. Losses were greater in Lee and DeForest's experiments if a cold water rinse step was included following the washing step. The results indicated that only a very weak precipitin test at best could be obtained from bloodstains exposed to washing water at temperatures of 75° or hotter, without agitation. It was thought that some species-specific protein might still be present in the stains, however, notwithstanding the fact that it could not be extracted with saline in quantities sufficient to give a precipitin test. An inhibition test was therefore devised in which the washed stained material was incubated with relatively low titer antihuman serum (1:16) for 24 hr at 4°. Aliquots of the antiserum were then removed and tested in an Ouchterlony double diffusion system with fresh human serum to see whether the stained material had substantially reduced the titer of the antiserum. Positive results were obtained, and it could be shown that washed control bloodstains made from cow, pig, horse, sheep, duck, chicken, rabbit, dog and cat bloods had no inhibitory effect on the antihuman serum. It was noted that this procedure could be applied equally well in situations in which the bloodstain contained a soluble substance which interfered with the ordinary precipitin test. The interfering substance could be washed out, and the stain then subjected to the inhibition procedure.

Itoh (1979) devised a somewhat similar kind of inhibition test for species determination using anti-human serum. He said that the procedure was applicable to bloodstains and to muscle tissue. Here, anti-human serum of appropriate titer was incubated with the material to be tested. The absorbed antiserum was then titrated with latex particles, coated with serum protein antigens (see also in section 16.5).

At various times, there has been interest among immunologists in the preparation and characteristics of antisera against heat-denatured serum proteins, particularly the comparison of reactions of such antisera with native and denatured antigen (see, for example, Schmidt, 1908). These experiments were of significance in advancing immunological knowledge, but not of much practical significance for medico-legal investigations (Schleyer, 1962). It is clear that moderate exposure to heating, e.g., 10 min at 55–70°, causes denaturation in serum proteins leading to changes in the shape of the molecules and giving rise to aggregation, but such proteins retain their antigenicity (Peeters *et al.*, 1970).

16.1.4 Tests with anti-human hemoglobin antisera

Preparation and use of anti-human hemoglobin sera has been discussed in section 7.1. These antisera can be used for the determination of species of origin as well as for the identification of the presence of blood. Anti-hemoglobin sera are generally not as potent as anti-serum sera, because hemoglobin is not as antigenic as most of the serum proteins. The same considerations apply to the use of anti-human hemoglobin as do to the use of anti-human serum reagents. Assuming species specificity has been established for an anti-human Hb serum, however, its reaction with a bloodstain extract may be regarded both as an indication of the presence of hemoglobin (presence of blood) and a diagnosis of human origin.

16.1.5 The antigen-antibody reaction. Optimization of reactant concentrations

No complete review of the theory of antigen-antibody reactions would be appropriate here. A good deal of work has been done on the subject and some discussion is given in section 1.3.4.2. Munoz and Becker (1950) and Becker *et al.* (1951) gave a detailed treatment of the reaction as it takes place in Oudin's single diffusion system. The precipitin reaction in solution was discussed by Kendall (1942). The effect of salt concentration was studied in detail (Aladjem and Lieberman (1952) and Lieberman and Aladjem (1952). A very good review by many specialists may be found in Chapters 13 and 14 of Williams and Chase (1971).

With some variations, depending upon the system under study, antigen-antibody reactions have certain characteristics in common. It has been known for a long time that, if one titrates a fixed quantity of antigen with decreasing amounts of antibody, the antigen-antibody reaction does not occur at extreme excesses of either component. In many precipitin systems, for example, no precipitation is observed at the excess antigen or the excess antibody extremes of the titration series. It is important, therefore, to obtain some idea of where the optimum precipitating concentrations lie in order to insure that the conditions chosen will not be characterized by an excess of either component. In carrying out the precipitin test for determination of species, it is common practice to make dilutions of the serum, or of the stain extract. The antiserum is often employed undiluted or at a particular constant dilution. Since there is considerable variation in the concentration of proteins in bloodstains, as well as variation in the efficiency of extracting them, some attention has been paid to determining the protein concentration of stain extracts. The extract can then be diluted if necessary to optimize the protein concentration prior to performing the test. Allison and Morton (1953) devised a simple test for approximating the amount of protein in a stain extract. Drops of progressively doubly diluted blood solutions were placed onto a filter paper and dried. A dried drop of stain extract was treated similarly, and the concentration of protein estimated by eye on the basis of color. If a more accurate estimate is wanted, the dried spots could be stained with a protein stain, the paper background destained, and the color of the extract spot compared with the colors of the "standard" spots. Lynch (1928) mentioned three other methods for estimating the protein concentration in stain extracts. The so-called "foam test" is based on the fact that 1:1000 dilutions of serum in saline, which have air bubbled through them, will form bubbles at the surface which persist for about 10 min while in greater dilutions of serum, the bubbles disappear almost at once. Nitric acid precipitates protein, and extract can be layered over a nitric acid solution to see whether an opalescent precipitate forms at the interface. In 1:1000 dilutions of serum, a faint precipitate will be observed, while in greater dilutions, no precipitate will form. A similar test is based on the protein precipitating power of sulfosalicylic acid. The opalescence caused by addition of $\frac{1}{20}$ of a volume of 50% sulfosalicylic acid to bloodstain extract may be compared to that in a series of standards prepared from dilutions of whole serum. Schleyer (1962) discussed the sulfosalicylic acid test in his review. It is usually said that a 1:1000 dilution of whole serum or its equivalent in a stain extract is optimal for carrying out the precipitin test.

It may also be mentioned that the pH of stain extracts should be in the neighborhood of neutrality for best results with precipitin reactions, and to diminish the possibility of pseudoreactions. Normally, there will be no problem because saline is usually used for extraction, but if 0.1N NaOH or ammonia were required as an extraction medium, some neutralization would be necessary.

16.2 Antihuman Globulin Serum Inhibition

Antihuman globulin serum (AHG serum, Coombs' serum) is an antiserum prepared against human serum globulin. Since the antibodies of serum are in the globulin fraction, AHG serum contains antibodies to the other antibodies, a fact whose significance will become clear in the discussion below, and see section 1.3.4.1.

In 1949, Wiener *et al.* suggested a new serological test for the determination of human serum globulin. The serum globulin is human specific, and the application of the method to species determination is based on this property. There are certain kinds of antibodies to the Rh_o (D) receptor of human red cells which do not agglutinate Rh+ cells in saline. These so-called "incomplete" or "blocking" antibodies do, however, combine with the antigenic receptor and sensitize the Rh+ cells (see sections 1.3.4.1 and 22.4). The presence of such antibodies on red cells can be detected by using anti-human globulin serum. The AHG antibodies are capable of combining with the anti-Rh antibody which is, in turn, bound to the red cell. If AHG serum is added to red cells with incomplete antibody bound to their Rh_o (D) receptor, agglutination will occur. Cells with incomplete antibodies attached to their receptors are said to be "sensitized". This principle is illustrated in Fig. 16.1. A bloodstain contains the human serum globulin proteins, and, if incubated with AHG serum, will bind the antibodies and reduce the titer of the AHG serum. An inhibition test can therefore be devised, in

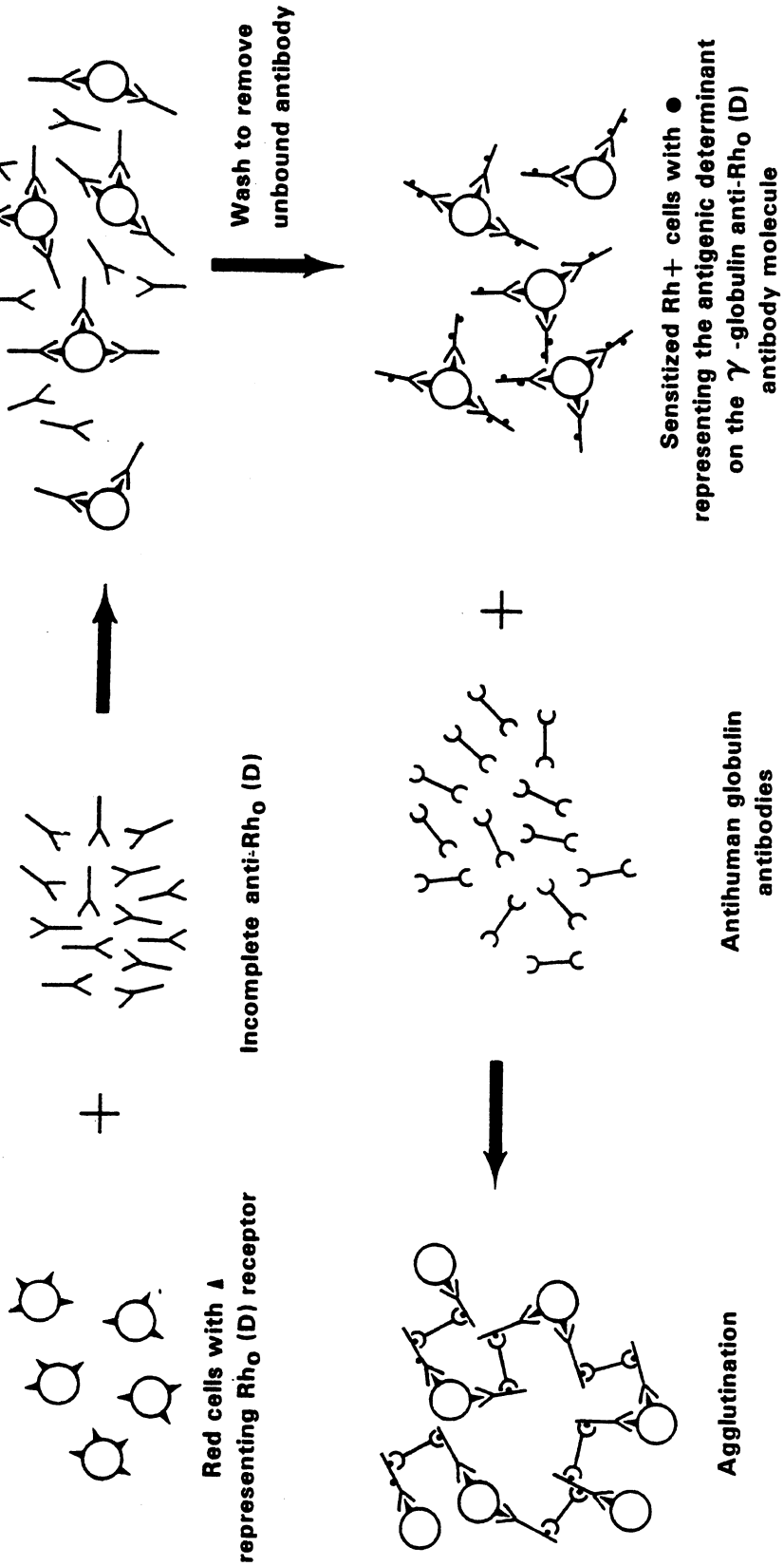

Figure 16.1 Scheme of Agglutination of Sensitized Rh+ Cells by Antihuman Globulin Serum

which bloodstained material is incubated with AHG serum, and the serum then tested to see whether it will agglutinate sensitized Rh+ test cells. If no agglutination occurs, the bloodstained material has removed the antibodies from the AHG serum, and it is, therefore, of human origin. If agglutination does occur, the stain did not reduce the combining power of the AHG serum and it is, therefore, not of human origin. The test for human origin is illustrated schematically in Fig. 16.2. In practice, the test is slightly more involved, in that appropriate dilutions of the AGH serum must be chosen so that the reduction in titer brought about by the serum globulin in a human bloodstain can be detected by the test system. Wiener *et al.* utilized a doubling dilution titration technique, and could quantitate the serum globulin in the test sample. Urine, semen, cerebrospinal fluid, saliva, and the sera of ox, horse, rabbit and Rhesus monkey did not inhibit the AHG reaction, and it was suggested that the method might find a medico-legal application.

Anderson (1952) reported that the method was more specific than the precipitin test, and could be applied to the detection of any animal serum, provided specific anti-globulin serum was available. The work was extended in 1954, and he could show that the technique was applicable to bloodstains. Human blood could not be distinguished from chimpanzee blood, but could be differentiated from all other animal bloods tested. Allison and Morton (1953) confirmed Anderson's (1952) findings. They showed that the test worked well with bloodstains on different substrata. It also gave positive results with alkali-denatured serum protein, although the inhibitory effect was reduced, and a positive test for human blood was obtained on cadaveric material from a mummy 5000 years old.

The value of the test was soon confirmed by a number of other laboratories. Several different techniques were proposed, some calling for titration, while others arranged the dilutions of the reagents in such a way that a one-step "all-or-none" procedure could be followed. Vacher *et al.* (1955) gave their method and said that semen, saliva, urine, meconium and fecal matter did not give inhibition. A variety of natural and synthetic textile materials were found not to interfere. Ruffié and Ducos (1956) published their method for carrying out the test, and Dérobert *et al.* (1957) enlarged upon their earlier (Vacher *et al.*, 1955) studies.

Jungwirth (1956) in Germany, Dell'Erba (1957) in Italy and Liberska and Smigielska (1958) in Poland all confirmed the specificity and sensitivity of the method. Ducos (1958a) described the techniques for the AHG serum inhibition test, as well as for the precipitin test and the passive hemagglutination test (see Section 16.3) in detail. He noted that if all three tests were performed on a sample, interpretation is simple if all results agree, but that it becomes very difficult if different tests give conflicting results. Cramp (1959) described a relatively simple version of the test which he said had been found to be quite satisfactory in 170 cases in New South Wales. Mosinger *et al.* (1960) described a two dimensional titration protocol for the AHG serum and the anti-Rh_o serum which, it was said, should be carried out with the reagents in order to select the optimal concentrations for actually performing the test. Schleyer (1962) discussed this technique in his review. Grobellar *et al.* (1970a) said that they selected their antisera for the test based on the criteria established by Proom (1943) for precipitin sera, namely that human serum should react at dilutions of 1:8000 while other animal sera should not react at dilutions of 1:50 or greater. It is important to include proper controls in this test, including the usual cloth control, and controls on the various reagents used in the test. Cramp (1959) said that he routinely included a positive human bloodstain control and two negative animal bloodstain controls as well. Hunt *et al.* (1960) showed that the antihuman globulin inhibition test was possible with a 1 cm^2 piece of cloth containing no more than 0.1 $\mu\ell$ of blood. Saliva and tears caused reduction as well if they were not too dilute. They recommended an AHG serum which, when diluted 1:400, gave a titer of 1:8 against Rh_o+ (D+) cells strongly sensitized with anti-D (anti-Rh_o).

Klose (1962) indicated that the presence of detergents in test samples will interfere with the test, unless a separation procedure is employed to get the globulin away from the surfactant material. The procedure using paper chromatography, described in section 7.2, was found to be satisfactory, and the detergent material could be located by its fluorescence under UV light.

Patzelt *et al.* (1977) said that the test could be done using human γ-globulin-coated latex particles. These are available commercially for use in testing for Rheumatoid Arthritis Factor in serum. Rheumatoid Arthritis Factor behaves as an antibody to human γ-globulin. Patzelt *et al.* said that false positive reactions could be gotten with the blood of higher mammals, and false negative results with sera from people with high concentrations of Rheumatoid Arthritis Factor. The false negatives were not seen, however, in bloodstain tests.

16.3 Passive Hemagglutination Techniques

In 1951, Boyden observed that certain preparations of inulin could render red cells able to absorb proteins onto their surfaces from solution. Inulin is a storage polysaccharide in certain plants (e.g. the Jerusalem artichoke) and is a polymer analogous to starch except that it is made up of fructose units instead of glucose units. Boyden found that preparations of inulin which rendered the cells able to absorb protein could, at high dilution, agglutinate the cells. Inulin preparations not having the property did not agglutinate the cells. It had been known since the work of Reiner and Fischer (1929) that dilute tannic acid solution could agglutinate red cells, and it was decided to test tannic acid solution for their ability to render red cells protein-absorbing. It was found that sheep red blood cells treated with 1:20,000 dilutions of tannic acid readily absorbed proteins from saline solutions. The cells could then be washed, and an antiserum homologous to the absorbed proteins would readily cause agglutination of the cells. Red cells which have been treated

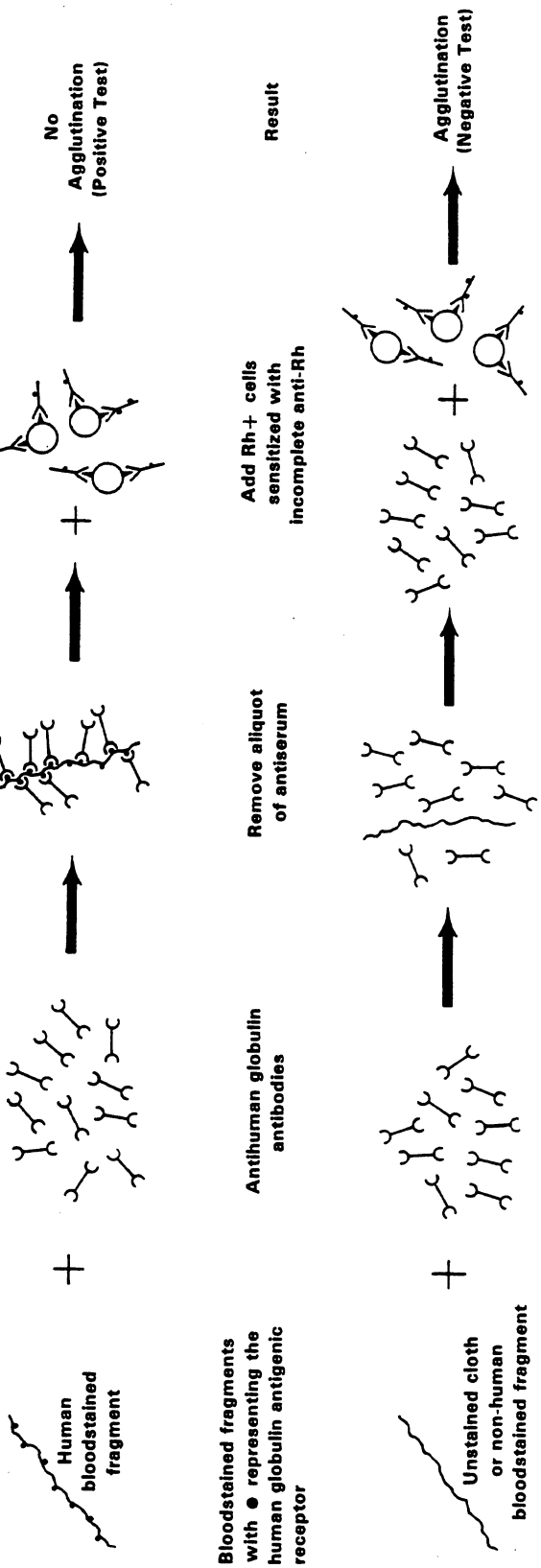

Figure 16.2 Scheme of Testing Bloodstain for Human Origin by the Antihuman Globulin Inhibition Method

with dilute solutions of tannic acid to render them protein-absorbing are referred to as "tanned cells".

Coombs *et al.* (1952) used a somewhat different technique to achieve essentially the same result, namely to devise an agglutination method for the assay of soluble antigens. Using benzidine tetrazonium chloride as a conjugating agent, egg albumin and chicken globulin proteins could be conjugated to incomplete Rh antibody molecules without affecting the latters' ability to combine with the Rh receptor on the red cell. Red cells could then be sensitized with anti-Rh antibodies to which had been conjugated either ovalbumin or chicken globulin protein. Antisera to either of these proteins would then bring about agglutination of the cells having the homologous antigen present.

Ducos (1956) put the tanned red cell technique to use in medico-legal species determination. Using a modified tanned red cell preparation method described by Dausset *et al.* (1955), he incubated bloodstain extracts with the tanned red cells. The cells were then washed, and tested for agglutination with AB serum, a series of blood group antibodies, and AHG serum. Of greatest interest was the result obtained with AHG serum. Only cells which had been exposed, after tanning, to human bloodstain extract, and which had therefore absorbed human globulin, were agglutinated by the AHG serum. The reaction was very specific and Ducos noted that it had the advantage over the AHG-inhibition method (section 16.2) that agglutination in this case represented a positive test, while a positive test in the former case was represented by the absence of agglutination. Ducos extended these studies (1958a, 1958b) showing that, while this technique was somewhat less sensitive than the precipitin and AHG-inhibition tests, it was more specific. In cases of exposing tanned red cells to human bloodstain extracts in doubling dilutions, and testing with AHG serum, agglutination could be observed at dilutions of 1:5000. In 1960, Ducos again described the method, suggesting that in medico-legal cases, several of the techniques should be used in order to be more certain about the species of origin. Hara *et al.* (1969) incubated stain extract with anti-human serum, washed the samples, then eluted the bound antibody, which was detected using tanned red cells to which had been absorbed human serum protein.

16.4 Mixed Antiglobulin Technique

In 1963, Styles *et al.* described a mixed agglutination technique for species determination based on the species-specific serum globulin present in bloodstains. The principle underlying mixed agglutination is as follows: a complete antibody to an antigen contained in the bloodstained fibril is allowed to react with the receptor in the stain, and the excess antibody is then washed away. Red cells containing the same antigen are then incubated with the "sensitized" stain fiber, and will be bound by the remaining combining site of the antibody, resulting in the cells arranging themselves in an orderly fashion along the fiber. The test cells are in effect "agglutinated" to the fiber rather than being agglutinated to one another.

A test for human species origin of a bloodstain was devised using this principle, and the technique called the "mixed antiglobulin reaction" by its originators. A bloodstained thread, which, if human blood is present, contains human globulin, is incubated with AHG serum. The test material is then washed to remove excess, unbound antibodies. Test cells are Rh positive and have been sensitized with an incomplete anti-Rh antibody. The mixed agglutination (mixed antiglobulin) reaction occurs if any AHG has been bound to the fiber, i.e., if the fiber contined a human bloodstain. The principle is illustrated in Fig. 16.3. Hara *et al.* (1969) employed the method, although they did not use AHG serum. An anti-human serum serum, which had been absorbed with monkey serum, was used. The test cells were tanned erythrocytes, to which had been absorbed human serum proteins. They said the technique was more sensitive than the precipitin test either in tubes or in agar gels. They referred to the technique as the "double combination method", a term which the Japanese workers have usually preferred to "mixed agglutination" (see section 1.3.4.1).

16.5 Sensitized Particle Techniques

16.5.1 Sensitized colloidon particles

In 1925, Freund established the groundwork for particle agglutination techniques in his physico-chemical studies on the agglutination of tubercle bacilli, and of protein-lipid extracts from these cells. The cells and the lipoprotein particles derived from them are maintained in a dispersion by a surface potential difference. When the potential is reduced below a threshold value, the dispersion is destabilized and the particles aggregate. All sera have the effect of reducing the potential, but if the particles are coated with an antigen, specific antiserum reduces it more effectively to a subthreshold value, and aggregation occurs. Jones (1927) applied this principle to the agglutination of colloidon particles, to which had been absorbed ovalbumin, by a specific anti-albumin serum. In 1940, Cannon and Marshall described a technique for the accurate determination of the titer of precipitin antisera using sensitized colloidon particles. Antigen was absorbed onto the particles, which were then washed to rid the suspension of unabsorbed antigen. The antisera could then be titrated with accuracy using these "indicator" particles.

Giaccone (1958) applied this principle to the determination of species of origin of bloodstains. Bloodstain extracts were incubated with washed colloidon suspension for 12–24 hrs at 4°. The particles were then thoroughly washed and resuspended in saline. A small aliquot of the particles was incubated with an equal volume of antiserum, the mixture centrifuged, shaken lightly, and read for agglutination either macroscopically or microscopically. Positive, specific reactions were obtained with undiluted and with 1:10 diluted bloodstain extracts. Concentrated control sera incubated

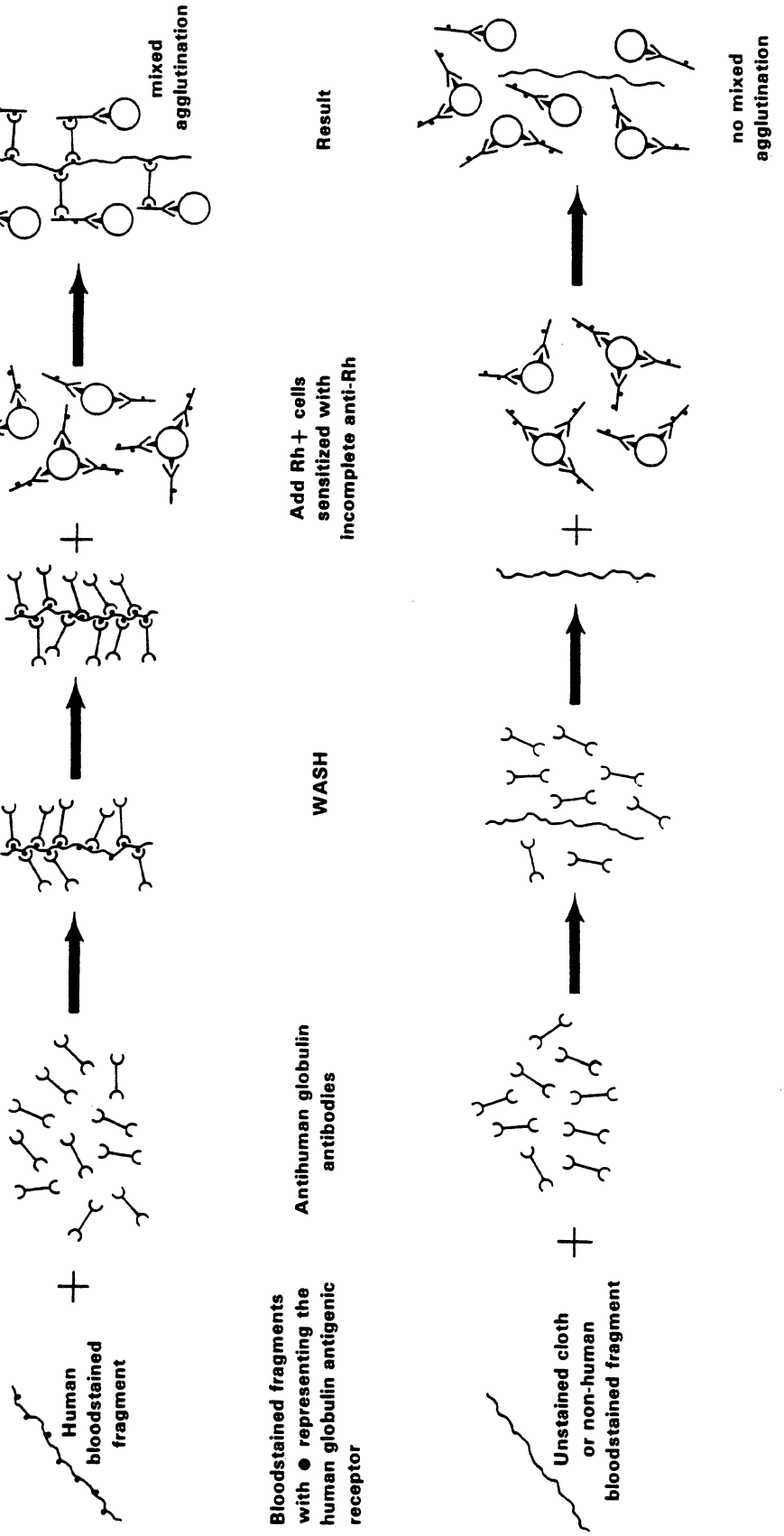

Figure 16.3 Scheme for Testing Bloodstains for Human Origin by Mixed Antiglobulin Technique

with the colloidon mixture instead of bloodstain extract sometimes caused spontaneous agglutination. Spontaneous agglutination also sometimes occurred when stain extract was added, but did not survive the washing steps.

Schleyer (1962) reported that he had conducted experiments on this method in collaboration with Hilgermann in 1960. The method was found to be specific and useful, and no spontaneous agglutination was observed in the course of these studies. Human serum in dilutions of 1:8000 gave positive, though weak reactions, whereas the precipitin test could not be obtained at dilutions of human serum greater than 1:4000.

16.5.2 Sensitized latex particles

In 1956, Singer and Plotz applied the particle agglutination principle to the serological diagnosis of rheumatoid arthritis, using sensitized latex particles. Robbins *et al.* (1962) showed that latex particles coated with human chorionic gonadotropin (see section 8.2.1) were agglutinated by a specific anti-HCG serum, and suggested that the technique might well lend itself to the development of a pregnancy test. In 1973, Cayzer and Whitehead applied the sensitized latex particle technique to medico-legal species diagnosis. The latex particles were sensitized with immunoglobulins, isolated from a sheep immune anti-human serum, in glycine buffered saline at pH 8.2. Protein concentrations of between 1 and 2 mg/mℓ with equal volumes of 2% latex suspensions were routinely employed for sensitization, but these conditions varied depending upon the particular globulin preparation. Dilutions of serum or of stain extract were mixed with the sensitized latex particles on a glass plate, the mixture rocked gently for 2 min, and agglutination read macroscopically against a dark background. The test was positive with human serum dilutions as high as 1:10,000. Cross reactions with the sera of closely related primates were positive at similar dilutions. All other animal sera tested gave negative results at serum dilutions greater than 1:100, and in some cases at dilutions greater than 1:40. Extracts of 2 year old human and primate bloodstains gave strongly positive reactions while extracts from stains of turkey, rabbit, hamster, rat, cat, mouse, dog, sheep, horse, cow, pig and donkey bloods gave negative reactions. Neat human saliva gave a strongly positive reaction but the reaction diminished rapidly at saliva dilutions greater than 1:100. Neat human semen gave a weak positive reaction. These studies were extended to animal bloodstains (Whitehead *et al.*, 1974) and latex particles were sensitized with the immunoglobulins of antisera against deer, cow, sheep, dog, cat, horse, mouse, hen and guinea pig as well as human sera. Some cross reactions were seen in the experiments with sera, but with the bloodstain extracts, the only cross reaction was a weak reaction of cow bloodstain extract with anti-deer particles. A few of the anti-animal particles reacted with human rheumatoid sera, but not at dilutions greater than 1:500. A number of other substances were tested for non-specific reactions with the anti-human particles. Soap solutions and fabric softener gave weak reactions, the only strong reaction (to a dilution of 1:1600) being given by milk. It was found, however, that milk agglutinated the unsensitized latex particles as well. Whitehead and Brech (1974) indicated that the latex particle technique could be applied to an extract from a single thread, which could then be employed for blood grouping by the elution technique since the species-specific antigens are water soluble whereas the blood group antigens are not.

16.6 Other Immunological Tests

16.6.1 Complement fixation

Complement (or alexin) is a multi-component system of serum proteins, and participates in the action of lytic antibodies (see section 1.3.5). Antibody must be present on a cell, bound to its receptor, before complement can combine with that cell causing lysis. In most antigen-antibody reactions, the resulting complex will combine with complement if it is present. Such combination is known as complement fixation. For purposes of the present discussion, complement fixation can serve as an indicator that an antigen-antibody reaction has occurred (see section 1.3.5.3). Since complement participates in the lysis of red cells by specific hemolysins, and is in fact required for hemolysis, hemolytic systems are convenient tools for the detection of the presence or absence of complement. All sera contain complement, but the amount varies even among members of the same species. Guinea pig serum is a common source of complement in complement fixation assays. If an antigen and its antibody are allowed to react in the presence of complement, some complement will be taken up, or fixed. The amount of complement added to the system can be arranged so that all of it is fixed. If the solution is now tested with a hemolytic system, its hemolytic power will be found to have been lessened or abolished. A common hemolytic system used in these assays consists of sheep red blood cells which have been sensitized with an anti-sheep red cell hemolysin prepared in rabbits. If such test cells are incubated with a test solution, and hemolysis occurs, complement was still present in the test solution indicating that it has not been fixed, i.e. that the antigen-antibody reaction which was being tested has not taken place. Conversely, if no hemolysis occurs, complement has been fixed and the antigen-antibody reaction may be inferred to have occurred. The complement in the serum containing the hemolysin is inactivated by heating prior to sensitizing the cells.

It may be noted that a number of the early studies on complement, and its ability to be "fixed" in antigen-antibody reactions, were carried out by the Nobel laureate immunologist Jules Bordet and his collaborators. Bordet showed in 1898 that an anti-rabbit erythrocyte hemolysin, prepared in guinea pigs, would first clump and then dissolve (hemolyze) rabbit red cells. He could show further that both antibody and complement were required for the lysis to take place, and that complement, but not antibody, was inactivated by heating for about 30 min at 60°. Since it was not clear at the time that one and the same antibody molecule

could bring about a variety of different effects when the antigen-antibody reaction occurred (such as agglutination, lysis, fixation of complement, etc.), antibodies were given descriptive names, based upon their action in a particular system (e.g. agglutinin, precipitin, lysin, etc.). Antibodies which "sensitized" cells so that they could subsequently be lysed in the presence of complement were called "sensibilisatrices" by the French workers and "amboceptors" by the German workers. Gengou (1902) established that complement could be fixed in precipitin reactions as well as in lytic ones. This fact, he demonstrated using rabbit antiserum to cow milk proteins and to ovalbumin, as well as in several other precipitating antigen-antibody systems. These studies were enlarged upon by the work of Gay (1905a and 1905b) and of Bordet and Gay (1906).

The complement fixation test for the medico-legal diagnosis of species of orgin was introduced by Neisser and Sachs (1905 and 1906a). They were prompted to carry out their experiments by the studies of Moreschi (1905) on the anti-complementary properties of certain sera. Neisser and Sachs used a hemolytic system consisting of rabbit immune anti-ox erythrocyte serum, known to be hemolytic for sheep red cells, and the sheep red cells were sensitized with this antiserum. Fresh guinea pig serum served as the source of complement. The antiserum had had its complement inactivated. 1 mℓ of a 5% suspension of sheep cells was readily hemolyzed in the presence of 5 $\mu\ell$ antiserum and 50 $\mu\ell$ guinea pig serum. Anti-human serum serum, prepared in rabbits, did not interfere with the hemolytic reaction. However, if 0.1 $\mu\ell$ human serum or 1 $\mu\ell$ monkey serum and 0.1 $\mu\ell$ anti-human serum was incubated with the complement, and the indicator system then added, no hemolysis occurred. Rat, pig, goat, rabbit, horse and ox sera had no such inhibiting effect on the hemolytic reaction. Saline extracts of 3 month old human bloodstains, diluted in some cases to the extent that a precipitin test could not be obtained, inhibited hemolysis in the system just as had human serum. Saline extracts of similarly aged bloodstains from sheep, fowl, rabbit, guinea pig, ox and horse did not inhibit hemolysis. They recommended that the test be employed as a control on the basis of the fact that a hemolytic reaction is easier to read than a weak precipitin reaction, that hemolytic sera are easier to obtain than precipitin sera, and that opalescence of the antiserum, which is a problem in a precipitin tube test, presented no difficulties in this test.

In another series of experiments Neisser and Sachs showed that with normal rabbit serum as the source of complement, hemolysis was inhibited by as little as 10 nℓ fresh human serum incubated with 10 $\mu\ell$ anti-human serum. They said that an antiserum which would inhibit hemolysis with 0.1 $\mu\ell$ fresh human serum, i.e. a very high titer antiserum, should be used in carrying out forensic tests. Friedberger (1906) conducted a number of experiments on the technique, and thought that its sensitivity might be a drawback. He obtained a "human" reaction with a saline extract of a stain of human perspiration and chicken blood. He was using a very high titer antiserum, however, and appears to have overlooked the fact that sweat may contain serum proteins which can react with an anti-human serum serum (Sutherland, 1907). Ehrnrooth (1906) got inhibition of hemolysis with 1:80,000 dilute human serum in a system which used rabbit serum as the source of complement and a goat red cell-rabbit immune anti-goat erythrocyte antiserum as the detection device. Muir and Martin (1906) showed that complement fixation occurred with 10 nℓ human serum incubated with 50 $\mu\ell$ of a potent rabbit anti-human serum. 1 $\mu\ell$ of human serum was required to obtain a definite precipitin test, indicating that the complement fixation test was about 100 times more sensitive than the precipitin test. They suggested, as had Friedberger (1906), that only very potent antisera, which could bring about complement fixation with 10 nℓ human serum, should be used in medico-legal investigations.

Uhlenhuth (1906a) tested a number of articles in his collection by complement fixation and obtained a number of false positive reactions. Some materials contained substances which could bring about the non-specific fixation of complement. A number of other materials, such as cotton-wool, pasteboard and gauze, which could be substrata for bloodstains, were negative. He got a complement fixation test in a case (Uhlenhuth, 1906a) from a bloodstain on a sack, when the precipitin test was negative and where examination of the bloodstain showed quite unequivocally that it was of avian origin. He was inclined not to put too much confidence in the test, therefore, and did not think it should be trusted as a check on the precipitin test (Uhlenhuth, 1906b). Neisser and Sachs replied (1906b) that adventitious substances could sometimes bring about non-specific complement fixation, and that a boiled bloodstain extract control should be run, since boiling would destroy the specific reaction but not the non-specific one. Another means of detecting such non-specific reactions is the running of a control in which stain extract is incubated with saline or non-immune serum (Sutherland, 1907). Schütze (1906) found the complement fixation test to be specific and more sensitive than the precipitin test. Graetz (1910 and 1912) carried out a number of studies on the method. Sutherland (1907) discussed the test, and his own experiments on its applicability. Stockis (1910) reviewed this method along with the other major tests for species determination. Complement fixation was reviewed in detail by Pfeiffer in 1938. In 1914, Sutherland mentioned that the test was not done routinely in India because sufficiently potent antisera could not be obtained. Olbrycht (1950) noted that the test is employed only infrequently in medico-legal practice, because of the experience required to carry it out properly, the controls that are necessary, and the fact that the precipitin test is usually more convenient and simpler. In the older literature, complement fixation is sometimes referred to as "complement deviation".

16.6.2 Anaphylaxis (Hypersensitivity)

The principle of anaphylaxis as a means of detecting antigen-antibody reactions was discussed briefly in sec-

tion 10.4.2, and more generally in section 1.3.6. Anaphylaxis as an immunological device for the medico-legal diagnosis of species of origin was explored by the earlier workers, but the technique is too time-consuming and cumbersome to be of any real value in practice, when simpler, equally effective alternatives are available. The anaphylaxis test is also sometimes called the hypersensitivity test (Überempfindlichkeitsprobe).

A number of workers appear to have suggested that the anaphylactic test be applied to medico-legal problems around the same time, and independently of one another. Thomsen wrote a full paper on the subject in 1909, which appeared in the March 25 issue of the *Zeitschrift für Immunitätsforschung und Experimentelle Therapie.* Shortly thereafter, Sleeswijk (1909) published a paper on serum hypersensitivity. On the page preceding the title page of this article, he made a point of noting that he had discussed the forensic applicability of the technique at an Academy proceeding in Amsterdam on March 27, 1909, and that his work had been done independently. Pfeiffer's paper appeared in 1910, and had been delivered at a September, 1909, meeting of the German medico-legal organization. Uhlenhuth took credit for having been the first to note the medico-legal applicability of the anaphylaxis method (Uhlenhuth and Weidanz, 1909). This claim is in fact true. Although he did not publish a full paper on the subject at the time, he did make the point clearly in a discussion which took place at the Berliner Militärärztliche Gesellschaft on December 14, 1908 (see in *Dtsch. Militaeraerztl. Z.* **38,** Vereinsbeilage, pp. 3–4, 1909).

In principle, the test is simple, but time consuming. An animal, usually a guinea pig since this animal tends to exhibit relatively consistent and characteristic symptoms of anaphylactic shock, is injected with sensitizing antigen. Extremely small doses are required to bring about the subsequent hypersensitivity, which becomes evident after about 10 to 14 days. A second, usually much larger dose of homologous antigen will then bring about anaphylactic shock in most of the animals. They normally go into convulsions, while displaying a characteristic set of symptoms, and die fairly soon. Some sensitized animals do not show the characteristic shock reaction, however. Sera to be used for sensitization must be heated to 55–60° for 30 min so that it does not cause a reaction in the animals. And the animals must be given injections which are as close to their body temperature as possible, or the symptoms very similar to anaphylaxis may result and mislead the observer (Sutherland, 1910). Rosenau and Anderson (1907, 1908 and 1909) conducted extensive experiments on the anaphylaxis, hypersusceptibility phenomenon. Among other things, they could show that a variety of proteinaceous substances would cause anaphylaxis in guinea pigs, that maternal hypersensitivity was transferred to the fetus whether sensitization had occurred prior to or after conception, that the active principle in horse serum causing sensitization was inactivated by heating to 100° for 1 hr, that exceedingly small doses (0.1 $\mu\ell$) of sensitizing antigen were required if administered intracranially, and that dessicated proteinaceous matter worked equally as well in inducing hypersensitivity as the fresh substances.

Thomsen (1909) could show that bloodstain extracts were completely suitable as sensitizing antigens. The reaction was not completely specific, however, in that some monkey sera could cause the reaction in animals sensitized with human serum. Pfeiffer (1909) showed that guinea pigs which have been hypersensitized to an antigen show a marked decrease in body temperature upon administration of the shocking injection, even if the other symptoms of anaphylaxis are not present. This temperature change, he believed, should be the sole criterion for judging the presence of the reaction in an animal in medico-legal work (Pfeiffer, 1910). Uhlenhuth and Haendel (in Uhlenhuth and Weidanz, 1909) showed that even old material could cause hypersensitivity in test animals. A fourteen year old, decomposed bloodstain, which would not give a precipitin reaction, gave a positive anaphylaxis test. Sutherland (1910) pointed out that, were it possible to hypersensitize the test animals in advance and have them sitting around at the ready, and then simply test bloodstain extracts as they were received in the laboratory, some of the objections to the test's inconvenience would be answered. Unfortunately, the shocking dose must be quite large, and there is seldom enough bloodstain to produce a sufficiently large amount of extract for the purpose. Since the sensitizing dose can be very minute, the bloodstain extract must normally be used in this way, necessitating the 10 to 14 day waiting period. Minet and Leclercq (1911) conducted extensive experiments on the technique with bloodstained materials. Stains on a variety of substrates were tested, and a number of chemicals, including ammonia, potassium permanganate and phenol, were mixed with bloodstains before testing. In all cases, the reaction took place. Stains 12 and 20 years old gave results that were indistinguishable from fresh blood, while a 28 year old stain gave a somewhat less intense reaction. They recommended that Pfeiffer's temperature decrease criterion be used in determining the presence or absence of the reaction. The technique was recommended for medico-legal investigations, and particularly in situations where the precipitin and complement fixation tests would not give a result. Pfeiffer (1938) discussed the method in some detail in his review, and concluded that the technique should be used where other, simpler ones would not suffice. Olbrycht (1950) noted in his review that the method was prohibitively cumbersome, and also said that the judgment of the presence of the anaphylactic reaction was too subjective, even if the body temperature decrease is used as the criterion.

16.6.3 Hemolysins

Hemolysins are antibodies which bring about the destructive lysis of the red cells which contain the homologous antigen. In 1869, Creite observed that if an animal received a transfusion of foreign blood, it developed hemoglobinuria. He also noted that the animal's red cells were dissolved (lysed) by foreign serum, but he apparently did not appreciate the relationship between the two observations. Landois

(1875) carried out extensive experiments on the transfusion of blood from one animal to another. Transfusions between unrelated species were always accompanied by hemoglobinuria in the recipient animal, and Landois recognized that this was the result of hemolysis brought about by the donor serum. In transfusions between closely related species, these results were not seen. In 1898, Belfanti and Carbone showed that artificial hemolysins could be produced by immunization of an animal with the blood of another animal, and that they were specific for the red cells of the animal whose blood had been used for immunization. Artificially produced hemolysins are used in the complement fixation assays described in sections 16.6.1 and 1.3.5.3. In 1901, Deutsch proposed the direct use of hemolytic antisera for the diagnosis of species of origin of bloodstains. The method relied on the lysis of the red cells in the bloodstain by the antiserum. Hemolysins could be prepared against any desired animal or human red cell, and complete hemolysis was obtained within 24 hrs of incubation of homologous bloodstain with antiserum. Normal serum, used as a control did not bring about hemolysis. The difficulty with such a method is in judging the degree of hemolysis which has occurred in a dried bloodstain. In a red cell suspension, it is relatively easy to assess hemolysis, but in bloodstains, which may already be partially hemolyzed, there are many problems in interpretation. Nuttall (1904) noted that he thought there were many potential sources of error in this method, and that it would be better to use the more reliable precipitin test. The subject was briefly reviewed by Sutherland (1907) and does not appear to have been pursued.

16.6.4 Agglutinins

When the serum of an animal is mixed with the red cells of another, not too closely related animal, the cells are agglutinated. Sutherland (1907) attributed the initial observation of this phenomenon to Landois in 1890. In 1904, Marx and Ehrnrooth attempted to devise a species test for bloodstains based upon this principle. A saline extract of a nonhuman, mammalian bloodstain would cause fresh human cells to be agglutinated, while with an extract of a human bloodstain, there was either no effect or else rouleaux formation occurred. They thought that the isoagglutinins of human serum did not persist in an active state in bloodstains longer than 1 month. In cases of bloodstains less than a month old, they said that a drop of saline diluted human serum, obtained by allowing a saline diluted drop of human blood to settle for 24 hrs, should be added to the text mixture. This addition would cause the clumping by homologous isoagglutinins to become more marked, while it would tend to break up the aggregates formed by heterologous agglutinins. They said that the test would be a useful preliminary one, to be carried out prior to doing the precipitin test. Pfeiffer (1904) obtained positive results with the test on bloodstains on wood and linen that were 37 and 24 years old, respectively, and felt that it was a good preliminary test. De Domincis (1904) found that heating bloodstains to 150° prevented the reaction, and he obtained false positive reactions with a number of body fluid substances other than blood. Carrara (1904) looked into the technique. He found that the agglutinins were inactivated by a few minutes exposure to 70°. He also said that saturated borax solutions or Paccini's solution (see Section 5.3) could be used to extract agglutinins from bloodstains that had become insoluble. Martin (1905) carried out extensive experiments on the test with a number of different animal bloods, and concluded that it was not reliable and should not be used in forensic practice. Sutherland (1907), having gotten agglutination of fresh human cells with an extract of human menstrual blood, and having failed to get it with a number of animal bloodstain extracts, agreed with Martin.

Leers, in his 1910 monograph, described his own procedure for carrying out the test, but emphasized that it was not to be regarded as a substitute for the precipitin test, and that it was necessary to use proper controls. Most authorities agreed that a negative test was noninterpretable, because it could not be known whether agglutination had failed to occur because the blood was human, or because the agglutinins had become inactive in a stain that was in fact of animal origin. Baecchi (1910a and 1910b) took a look at the issue of the presence of human isoagglutinins in the stains. Marx and Ehrnrooth had originally suggested that the isoagglutinins of human blood deteriorate faster in bloodstains than do the heteroagglutinins in animal bloodstains. Further, they suggested that the two could be distinguished on the basis of differences in agglutination behavior. Baecchi recognized the problem, and said that one should carry out the test with a number of examples of human red cells. If these were all agglutinated uniformly, he said, the probability of the stain having contained heteroagglutinins, and therefore, of being of animal origin, would be very much increased. Lattes (1913) took up the problem of distinguishing between the isoagglutinins and the heteroagglutinins in this procedure, and appears to have been the first investigator to have suggested a simple method for it based on the principles governing isoagglutination in human blood (see section 19 for full discussion). His objection to Baecchi's approach was that it was "blind", in that no account was taken of the blood group of the test cells used. False positive results in the test, caused by the presence of human isoagglutinins in human bloodstains, could be avoided, Lattes said, by the very simple expedient of using group O test cells. Under these conditions, he noted, the test could be of value provided it gave positive results. Negative results could not be interpreted. He felt that the test could be used to screen samples for a subsequent precipitin test, and in addition, could serve to indicate possible human-animal blood mixtures in the stain in cases where the agglutination test was positive and the precipitin test with anti-human serum was also positive.

16.6.5 Serum-hemoglobin precipitation

In 1967, Kimura published a series of papers on the precipitin reaction of human hemoglobin with human serum. The reaction was first observed in Ouchterlony gels, and was noted to be independent of the ABO group (1967a). A series

of experiments was conducted with a number of animal sera and hemoglobins as well as with human serum and Hb. The only homologous reaction besides the human one which occurred was that of monkey hemoglobin with monkey serum, and it was weaker than the human reaction. Human serum reacted weakly with monkey and rabbit hemoglobins (1967b). It could be shown by immunoelectrophoresis that hemoglobin was reacting with the albumin of the serum (1967c). A medico-legal application of the phenomenon was proposed (1967d) in which human serum was used as test reagent, the bloodstain serving as the source of hemoglobin. Extracts of 6 months old human bloodstains gave the test with serum dilutions of up to 1:16.

In 1971, Hartmann and Oepen said that they had tested many combinations of sera and hemoglobin, and could not confirm Kimura's findings. They said that the precipitin reactions between serum or albumin and hemoglobin were nonspecific and could be avoided in immunodiffusion tests by keeping the distance between wells less than 6 mm and the temperature lower than 20°. Human serum would react with a number of animal hemoglobins in their experiments, and they said that the method was of no value in diagnosing species of origin.

16.6.6 Phytoprecipitin and phytagglutination methods

That the seeds of certain plants contain agglutinins for red cells has been known for a long time. In 1888, Stillmark found that aqueous extracts of *Ricinus communis* were hemagglutinating (Gold and Balding, 1975). Plant extracts which agglutinate red cells are usually called lectins, or phytagglutinins. More recently, the term "receptor specific proteins" has been introduced, and includes agglutinins from animal sources (protectins) as well. This subject is now quite complicated and its literature immense. More is said about lectins in section 19 (ABO system). Some plant extracts can also cause precipitation of animal sera. These are called phytoprecipitins.

In 1963 Haferland reported that aqueous extracts of *Bryophyllum diagremontienum* contained a phytoprecipitin specific for human serum. There was a weak reaction with some monkey sera. In 1964, he showed that the precipitin detected human serum in bloodstains up to 5 years old on a number of substrata by the agar gel diffusion test. In one instance, the phytagglutinin reacted when anti-human serum did not. Haferland recommended the phytoprecipitin for medico-legal species diagnosis.

In 1963, Raszeja reported that the phytagglutinin from the mushroom *Laccharia laccata Berk* was specifically inhibited by human serum, as well as by human saliva, semen and milk. The sera of 35 animals showed no inhibition. This principle was proposed as the basis of a forensic species diagnosis test, and an extract of a 36 year old human bloodstain showed the inhibition reaction. The lectin has an anti-H specificity, and it was found in subsequent studies that "anthropomorphic" monkey (e.g. chimpanzee) sera also cause inhibition, but that the sera of "zoomorphic" monkeys (e.g. Rhesus monkey) do not (Raszeja, 1966).

In 1974, Bhatia conducted a number of experiments on phytagglutinins from a number of plants with the blood of a number of different animals. Three lectins were specific for guinea pig blood, while five others gave a combination of reactions, the patterns of which were distinctive for cow, chicken, sheep, rabbit, frog and rat bloods. Bloodstains were not studied in these experiments.

Tumosa (1976) looked at the reactions of the red cells of 37 different species with the "anti-A_1" lectin from *Dolichos biflorus,* and found that only those of the barasinga, chimpanzee, rhinoceros and wallaroo reacted. It has been appreciated for some time that the ABH blood group receptors of human red cells are actually widely distributed surface structures in many different living things. The matter is discussed in more detail in appropriate parts of section 19. In this respect, Tumosa (1977) has found that a variety of animal bloods are fully reactive with anti-A, anti-B and with anti-H.

Chowdhuri *et al.* (1975) looked at the phytoprecipitin activity in 50 different plant seed extracts against human and a variety of animal sera. 25 plant extracts showed phytoprecipitin activity, and many of these were nonspecific. Two extracts were specific for horse serum, one for chicken serum, one for goat serum and another for dog serum. Four lectins reacted with human and monkey sera.

16.6.7 Gamma-globulin deviation

In 1957, Ambrosi attempted to take advantage of the fact that antibodies are present in the γ-globulin fraction of serum, proposing that a reduction in the γ-globulin fraction of the antiserum after incubation with homologous antigen could serve as an indication that the antigen-antibody reaction had occurred. The quantities of γ-globulin present before and after the reaction were detected by paper electrophoresis. Although this method is not, strictly speaking, purely immunological, it is no less appropriately presented in this section than in a separate one dealing with electrophoresis. Ambrosi said that a consistent reduction in γ-globulin was observed when small aliquots of antisera were tested following incubation with 1:500 dilutions of homologous bloodstain extracts. No reduction was seen with heterologous antigen. The absolute value of the reduction varied in the homologous case, but was never less than 14.7%. Schleyer (1962) said that Ambrosi's paper electrophoresis experiments had not been carried out very rigorously, and that Hilgermann, working in his (Schleyer's) laboratory in 1960, had utterly failed to confirm Ambrosi's results.

16.6.8 Fluorescent antibody technique

In 1968, Gajos reported that he had conducted exploratory experiments on the applicability of fluorescent-labelled antibody technique to medico-legal immunology. The results were not very satisfactory, one of the problems being that many fabrics contain fluorescent "brightening" substances which have similar emission maxima. He believed further work would be required to render the technique applicable to

forensic problems, such as that of species of origin determination.

16.7 Immunoelectrophoresis

Immunoelectrophoresis was first described by Grabar and Williams in 1953, who applied the technique to the separation and characterization of human serum proteins. Since that time, it has become a standard method of immunological investigation (see Grabar and Burtin, 1964 and section 2.4). An important development was Scheidegger's (1955) introduction of a micro-immunoelectrophoretic method, which was widely adopted and modified by many workers. Immunoelectrophoresis has not been widely employed as a means of species determination. Muller and Fontaine (1960) explored the possibility, but said that the method was not inherently superior to other gel methods, and that the specificity of the test was a function of the specificity of the antiserum. Schleyer (1962) gave a detailed description of a method which he had devised in collaboration with Schneider. He agreed with Muller and Fontaine in noting that the specificity of the antiserum was the critical feature in determining the specificity of the test.

16.8 Cross Reactions of Antisera—The Problem of Closely Related Species

It has been recognized since the earliest experiments with precipitating antibodies that antisera to a particular protein cross-reacted with closely related proteins. This fact was clear from the studies on the reactions of anti-hen ovalbumin with duck ovalbumin (Myers, 1900; Uhlenhuth, 1900). Very soon after the precipitin test for medico-legal diagnosis of species was proposed, it was clear that antisera to a particular species cross reacted with the sera of closely related species, as for example, anti-ram sera with goat and cow blood (Uhlenhuth, 1901e) and anti-human serum with monkey bloods (Nuttall and Dinkelspiel, 1901a and 1901b; Frenkel, 1901; Stern, 1901). The cross reactions of anti-human serum have been a matter of particular concern to medico-legal investigators ever since that time. In some countries, which have indigenous monkey and other primate species, and where these bloods may be present in exhibits submitted for examination, the problem is a serious one. In this country and in Europe, it is improbable that such cross reacting primate bloods would be encountered.

A number of approaches have been taken to find ways of differentiating the bloods of closely related species, particularly of humans and primates. Among these are selective absorption of antisera with the cross-reacting antigens (Weichardt, 1905), so-called cross-immunization ("kreuzweise immunisierung") (Uhlenhuth, 1905), and various techniques for carefully estimating the quantity of precipitate obtained. By cross immunization is meant the preparation of antibodies to the proteins of a particular species by immunization of a member of the closely-related, cross-reacting species.

Welsh and Chapman (1910) indicated that they could immunologically differentiate the ovalbumins of closely related species by carefully determining the weight of the precipitates under carefully controlled conditions. Berkeley (1913) could not confirm Uhlenhuth's contention that specific precipitins to human proteins could be prepared in monkeys. They were unable to raise precipitin antisera to humans in two species of monkeys, although it is now known that this can certainly be done. Fujiwara (1922b) said that human and monkey bloods could be differentiated by the complement fixation test using anti-human serum absorbed with monkey blood.

In more recent times, a good deal of work was done on the problem by the South African forensic scientists, the differentiation of human and primate bloods being a serious problem in their practice. In 1952, Taylor published a detailed paper on the subject, in which the standards of potency and specificity for antisera as stated by many authorities were reviewed. In addition, the various techniques for attempting to render antisera specific for a single species were reviewed, and a number of experiments conducted. He had some success with anti-human sera prepared in baboons and vervet monkeys, but none of the antisera could differentiate human blood from that of the chimpanzee, the most closely related primate. Shapiro (1954) flatly stated that Taylor's experiments had conclusively shown that human blood could not be differentiated from that of the most closely related primates. Gradwohl (1956) tested anti-human serum with the sera of several monkey species and a small baboon species with negative results. The antiserum did react to chimpanzee, gorilla and orangutan sera though. An anti-chimpanzee reacted with gorilla, orangutan and human sera, as did an anti-orangutan preparation. He did not think there was any way to distinguish these species by means of the precipitin test. Vagnina (1955) discussed the problem of the differentiation of a closely related species, and said that Dr. Boyden had told him that it was indeed possible to distinguish closely related species by precipitin tests, but not using the methods employed by most medico-legal investigators. A photoelectric turbidity measuring system had to be employed, and antisera selected with very great care. Boyden and DeFalco (1943) and Boyden *et al.* (1947) described their technique in detail. An antigen was titrated to its endpoint with very carefully selected antiserum, the turbidity being measured in each dilution from the prozone, through the equivalence zone, and into the postzone with the Libby photronreflectometer. Curves can then be constructed in which tube number (in order of increasing antigen concentration) was plotted against turbidity units. The areas under these curves, which vary both in area and in shape with homologous vs heterologous antigens, are proportional to the summated turbidities in the titration series. The summed turbidity value is sometimes referred to as "relative area". This type of analysis is sensitive to very small differences in antigen structure, and could differentiate between closely related antigens, including human and chimpanzee serum. A representative set of results from Boyden

(1958) is shown in Fig. 16.4. The turbidity determinations were shown to correlate well with other methods of quantitative determination of precipitin reactions, such as gravimetry and total antibody nitrogen. Gravimetry, the accurate measurement of the mass of the precipitate, suffers from the disadvantage that combining valencies of antibodies may differ. Determination of total antibody nitrogen is accurate, but more complicated than turbidimetry (Kwapinski, 1972).

In 1955, Coetzee proposed an interesting, if complex technique which was capable of differentiating human from chimpanzee serum. The method employs sensitized tanned red cells as indicator cells (see section 16.3), and is based on the principle that homologous antigen should be able to annul its antiserum, while heterologous antigen (even if closely related) should not be able to do so to the same extent. Another way of putting it is that the failure of species X proteins to annul anti-species Y antiserum shows that X and Y are, in fact, different. The test demonstrates non-identity, although it could be used to establish identity if enough experiments were carred out. The test was arranged as follows: serial dilutions of human serum in one set of tubes, and serial dilutions of chimpanzee serum in another, were incubated with serial dilutions of anti-human globulin (AHG) serum. After a time, tanned red cells, sensitized with human serum globulin, were added to all the tubes, and agglutination read after a suitable interval. A set of results is shown in Table 16.1. In a similar way, beef and horse meat extracts could be readily distinguished. In 1958, Coetzee showed that the technique was applicable to bloodstain extracts, and that human and chimpanzee bloodstains could be differentiated from one another (i.e., chimpanzee serum could be shown to be non-identical with human) as had been done in the case of the sera.

Lichter and Dray's studies (1964) indicated that anti-human sera prepared in chimpanzees and in Rhesus monkeys could distinguish a number of human-specific serum proteins. Gempel *et al.* (1960) could differentiate human and monkey sera using an anti-human serum prepared in the monkey. The antiserum was apparently of very low potency, however, and the reaction was very slow. Using anti-human and anti-monkey sera prepared in rabbits, and absorbed with heterologous serum, it was possible to differentiate between human and monkey sera on the basis of the number and structure of precipitin lines in Ouchterlony gels (see section 2.2.2). Bloodstains were not studied in the course of these experiments. Sivaram *et al.* (1975) indicated that monkey blood could be distinguished from human blood using a very similar sort of analysis of the Ouchterlony diffusion precipitin bands, but employing a very potent anti-human globulin serum.

Grobellar *et al.* (1970b) utilized the AHG inhibition test (section 16.2) to differentiate between human and primate sera. AHG serum was prepared in goats, and with selected examples of the reagent, no inhibition was observed with primate sera at dilutions greater than 1:50 while human serum gave inhibition at dilutions of 1:1000. The test was

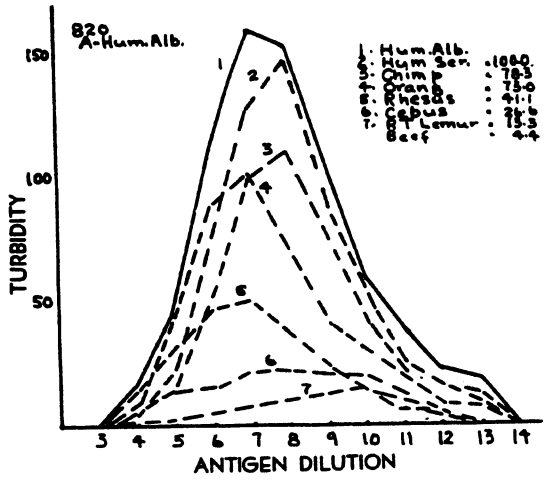

Figure 16.4 Quantitative Precipitin Analysis by Reflectrometry. Reactions of an anti-human albumin serum with a series of primate sera. From "Comparative Serology: Aims, Methods and Results" by Alan Boyden, in: *Serological and Biochemical Comparisons of Proteins*, ed. by William H. Cole. Copyright © 1958 by Rutgers, The State University. Reprinted by permission of Rutgers University Press.

Table 16.1 Results of the Application of the Coetzee Method to Human and Chimpanzee Sera (after Coetzee, 1955)

Dilutions of human/chimpanzee sera (reciprocal)	Dilutions of anti-human globulin serum (reciprocal)											
	3	6	12	24	48	96	192	384	768	1536	3072	C
4	c	c	c	c	c	c	c	—	—	—	—	—
8	+	c	c	c	c	c	c	—	—	—	—	—
16	+	+	c	c	c	c	c	c	—	—	—	—
32	+	+	c	c	c	c	c	c	—	—	—	—
64	+	+	+	c	c	c	c	c	c	—	—	—
128	+	+	+	+	c	c	c	c	c	c	—	—
512	+	+	+	+	+	+	c	c	c	c	c	—
1024	+	+	+	+	+	+	+	c	c	c	c	—
2048	+	+	+	+	+	+	+	+	c	c	c	—
C	+	+	+	+	+	+	+	+	+	+	+	—

Key to abbreviations in figure: + = agglutination with both human and chimpanzee sera; c = agglutination only with chimpanzee serum; — = no agglutination; C = control (normal rabbit serum).

recommended for medico-legal species diagnosis in South Africa.

Čarny (1969) recommended immunoelectrophoresis as a means of discriminating between the bloods of closely related animals.

16.9 Serum Protein Structure and Phylogeny—Taxonomic Serology and Immunology

Structural similarities in the proteins of different species, and their corresponding immunological cross reactivity are a reflection of phylogeny. This fact was recognized almost simultaneously with the discovery of precipitins for blood proteins. The undisputed pioneer in this field was G. H. F. Nuttall. In 1901, he was preparing antisera to scores of animal bloods, and recognized the fundamental biological importance of immunological cross reactivity as a delicate measure of protein structural similarities (Nuttall, 1901b). In the course of several years time, antisera were prepared by Nuttall and his collaborators against hundreds of animal bloods. These were tested with homologous and heterologous sera in a systematic fashion. The results were obviously complex, and took up dozens of tables. They cannot be readily summarized. All the material is collected in Nuttall's classic work, *Blood Immunity and Blood Relationship,* which appeared in 1904. The book additionally provided a detailed review of immunological investigations up to the time, and included a chapter on medico-legal species differentiation by the precipitin test.

More recently, quantitative techniques have been employed in comparative immunology and serology. These were reviewed by Boyden (1958). The reflectometric procedure for assessing the quantity of precipitate formed in the precipitin reaction was discussed in the foregoing section. Boyden said that with appropriate quantitative precipitin technique and careful selection of antisera, there was no reason for being unable to differentiate very closely related species in forensic medicine, including human and chimpanzee blood. He said that the usual technique employed in medico-legal investigations, the use of antiserum against whole serum rather than a specific protein, and the use of a single concentration of antigen and antibody for the test, was quite crude. He did not find particularly astonishing the fact that a number of medico-legal authorities had reported their inability to differentiate closely related species by the precipitin test. The use of quantitative immunological reactions to establish taxonomic relationships was discussed in the review as well, and some data presented. Readers interested in this subject are also directed to Leone (1964), which contains articles by many specialists on all the varied aspects of taxonomic serology and immunology.

Sensabaugh (1975) pointed out the intimate connection between immunological cross reactivity of those proteins which have evolved rather slowly, and the medico-legal differentiation of species of origin. Depending on the discriminatory capability of the technique being employed, the phylogenetic relationships between related species impose intrinsic limits on the ability to differentiate related bloods. Medico-legal tests would be far more satisfactory in these terms, he said, if monospecific antisera to the more rapidly evolving proteins were employed, thereby taking advantage of greater differences in the structure of similar proteins, even in very closely related species.

In the full paper on the subject, Sensabaugh (1976) noted that cross reactions between the proteins of closely related species are an inevitable consequence of evolution. Cross reactivity can be turned to advantage in medico-legal investigations, because of the known relationship between degree of cross reactivity and species relatedness. Proteins must differ from one another in sequence by about 40% of their residues in order to be completely non-cross reactive to an antiserum to one or the other. In discriminating between primates, anti-Ig gamma chain and anti-Ig kappa chain sera were found to be much better than antisera to albumin or transferrin. Sensabaugh noted further that different examples of commercial "anti-human" serum contained antibodies against different serum proteins, and that unless such characteristics of an antiserum are known, it is difficult to make the most productive use of a species test or indeed to select the most suitable antiserum.

Bauer (1969, 1970a, 1970b and 1970c) has carried out extensive studies of human and animal serum proteins using immunological methods with the objective of determining phylogenetic relationships among the species. Particular proteins could be placed into what he called "immunological evolution groups" based upon their cross reactivity, a direct measure of the speed with which they have evolved.

SECTION 17. Other Methods for Species Determination of Blood and Bloodstains, Body Fluids and Tissues

17.1 Differential Denaturation of Hemoglobin with Alkali

Although the absorption spectrum of hemoglobin in various species does not differ greatly, the rate at which the molecule denatures in alkali is decidedly species-dependent. Krüger observed this phenomenon in 1887. Magnanimi (1898) applied the principle to bloodstains, and thought it had forensic applicability. Zeimke (1901c) confirmed many of the previous findings, likewise recommending the method for medico-legal examinations. Krüger had another extensive paper on the subject in 1925, although he had no interest in forensic blood investigations. Schleyer (1962) quoted the Russian authors Blumenfeld and Krasovickaja as having said in 1955 that human blood could be readily distinguished from a number of animal bloods by following the rate of alkali denaturation of hemoglobin spectrophotometrically. Analogous results were obtained with bloodstain extracts. Their observations were generally in agreement with those of the earlier investigators. Human hemoglobin denatured at the fastest rate (1–2 min), followed by cat Hb (6–7 min) and dog Hb (12–14 min). Fowl, goat, ox and sheep hemoglobins do not significantly denature in alkali after an hour. Schleyer conducted a number of experiments with this technique in 1961, and summarized his results in the 1962 review. It could be confirmed that human Hb denatured faster than that of any animal hemoglobin tested. He said, in addition, that the pattern of denaturation might actually be more characteristic of the species than the rate. Schleyer thought that the technique was primarily applicable to fresh blood or to very fresh bloodstains. The alkali denaturation curves are characteristic for whole blood dilutions, but not for pure solutions of hemoglobin. Human hemoglobin is not denaturable by alkali at blood dilutions greater than 1:950.

It must be noted that fetal hemoglobin (Hb F) is quite resistant to alkali denaturation. This fact was used as a basis for differentiating Hb F from adult hemoglobin (Hb A) prior to the development of electrophoretic techniques (see section 8.3.1). The presence of Hb F in a bloodstain could cause a grave error if the alkali denaturation technique for species differentiation were being applied. Hb F is further discussed in section 38.

17.2 The fibrin plate method

The fibrinolytic system was discussed in section 8.1.2 (and see Figure 8.1). The work of Mullertz and Lassen (1953) on the fibrinolytic system had indicated that human serum contains substantially larger amounts of proactivator than most animal sera. On the strength of this observation, Szöllösy and Rengei in 1959 and 1960 devised a species of origin test based on fibrinolysis (Rengei and Szöllösy, 1959; Rengei, 1960; Szöllösy and Rengei, 1960). A fibrin plate was prepared using bovine fibrinogen in buffer in the presence of a small amount of thrombin. This mixture was allowed to clot and the surface then dried in a 37° incubator. A small quantity of streptokinase was mixed with a buffer extract of a bloodstain, and a drop of this mixture applied to the surface of the fibrin plate. Streptokinase is an artificial activator of proactivator, and its addition to the serum in the bloodstain sets in motion the fibrinolytic machinery. After a suitable incubation period, a marked lytic area develops in the fibrin plate if the extract was from a bloodstain of human origin. The authors claimed absolute specificity for the method, as well as a sensitivity of 0.3 μg human serum protein. Positive results were obtained with bloodstains on various substrata, including iron and glass, which were up to 8 months old, even ones which had been kept at 56°. It was noted that human milk and tears would give the fibrinolytic reaction as well. In 1962, Kumano utilized the technique, and said that it was sensitive and specific. He got a positive result with a 30 year old bloodstain, and on stains which had been exposed to 100° heat for an hour, or washed. Putrefied liquid blood could not be diagnosed satisfactorily by this method.

Schleyer (1962) presented a description of a method which he had found to work well. The method was found not to be species-specific, however, if non-human sera were less dilute than 1:100. At serum dilutions of 1:1000, only human blood gave a positive reaction, and Schleyer noted that with this method, as with the precipitin test, it was important to employ dilute (1:1000) bloodstain extracts to insure specificity. Mohri (1963) confirmed the results of previous workers, and Morioka (1965) said that putrefaction destroyed the fibrinolytic activity of blood steadily over the course of a month. Mikami *et al.* (1966) recommended the technique for species determination in small fibers of bloodstained material, to which they said they could consecutively apply a catalytic test, the fibrin plate test, and a mixed agglutination technique for the determination of the blood group. Akaishi (1965) noted that the test was sensitive to a whole blood dilution of 1:25,600. Hirose (1976), in his studies of the effects of rust on dried blood (see section 5.1 and Table 5.2), said that the precipitin test remained positive for 51 days after the mixing of the blood with the iron only if pH 9.4 buffer solution was used for extraction. The fibrin plate test was barely positive after 51 days using pH 9.4 buffer as extraction medium, and it became negative at 7 days if saline was used.

17.3 Hemoglobin Separation by Chromatographic and Electrophoretic Methods

Some efforts have been made to apply paper chromatography or gel electrophoresis to the diagnosis of species by separation of the hemoglobins. Fine *et al.* (1956) got separation of the hemoglobins from distantly related species using gel electrophoresis, but the hemoglobins of most mammals tended to migrate about the same distance. These observations were largely confirmed by Depieds *et al.* (1960). Fiori (1957) tried paper chromatography of hemoglobins, and tested a large number of different solvent systems. He could not get satisfactory differentiation of human hemoglobin from that of other species. Vidoni and Marenghi (1957) tried to distinguish blood species on the basis of the patterns obtained by paper chromatography. Schleyer (1962) said that he and Schneider were unable to reproduce these results. Santini (1960) applied circular paper chromatography to the separation of human and animal hemoglobins. He said that a specific, reproducible R_f of 0.72 could be obtained for human hemoglobin using this technique. Berg (1967) said that the hemoglobin electrophoresis and chromatographic methods for species determination were unreliable, and that other techniques should be used.

17.4 Isoenzyme Patterns

In 1962, Samico *et al.* reported that aqueous extracts of stains of human, cat, ox, sheep, goat, pig, turkey, chicken, rat, monkey, possum and toad bloods could be differentiated on the basis of their esterase profiles following starch gel electrophoresis and specific staining for esterase activity. Thick starch gels were employed for the separations, and staining effected with α-naphthyl acetate or α-naphthyl butyrate and Fast Blue RR salt. The banding patterns were different with each animal tested, and the results were better with the butyrate ester. The single band from human bloodstains was due to cholinesterase activity. This band persisted in bloodstains for 210 days if extraction was carried out for 30 min with water, but if extraction were done at 37° for 24 hrs, the band persisted to 310 days.

Madiwale *et al.* (1972a) tested blood samples from bullock, buffalo, sheep, goat, rabbit and human sources for lactic dehydrogenase and malic dehydrogenase activity band patterns by polyacrylamide disc gel electrophoresis. The patterns were reproducible, and different for the various animals. Bloodstain extracts gave similar results (1972b), and the method was considered very sensitive, only about 1 $\mu\ell$ of blood being required.

Some of the isoenzymes which are under the genetic control of polymorphic loci in human beings are found in certain animal bloods. The various human isoenzymes are discussed in Unit VI. In cases where animal blood isoenzyme patterns are consistent, and differentiable from human patterns, electrophoretic enzyme determination might offer a method of helping to identify the species of origin of bloods. Gallango and Suinaga (1979) studied the red cell enzyme UMPK (see in section 37.12) in a series of vertebrate bloods. Herr (1979) showed that a number of differences existed in the red cell acid phosphatase (see in section 29) enzymes of several animal species as compared with the human isozymes. Herr and Konzak (1980) suggested that these ACP patterns, as well as those of red cell PGM and AK (see in sections 27 and 28), might be helpful in differentiating species of origin.

17.5 Species Diagnosis in Other Body Fluids and in Tissues

It is not often necessary to carry out species tests on body fluids other than blood. In theory, the immunological tests could be applied for the purpose to any body fluid in the same way as they are used for blood, provided that specific antisera were available. The preparation and use of antisera to semen has been discussed in a previous section (10.3), as has the preparation and use of antisera to saliva (11.4). There do not seem to have been extensive studies on the species-specificity of these antisera. Most of the concern was with cross reactions with other body fluids, since the objective of the immunological tests was primarily identification of the body fluid.

Popielski *et al.* (1963) said that the precipitin test could be carried out on extracts of urine stains provided these were subjected to a protein concentrating step in advance. Pathologically albuminous urine can be tested more easily.

Tissue samples are sometimes encountered, and must be tested for species of origin. The species of muscle tissue may be conveniently determined by the precipitin test, as has been known since the early work of Uhlenhuth (1901f).

The subject of tissue antigens is complex, and beyond the scope of the present discussion. The species of origin of tissues can be determined based on their presence, however, if appropriate antisera and techniques are available. Milgrom and Campbell (1970) reported on a case in which a piece of intestine and a piece of mesentery, found in a sewer in Niagara Falls, N.Y., were submitted for species determination. It was known from previous work (Milgrom *et al.*, 1964a and 1964b) that tissue contains species-specific antigens which were called "BE antigens", because of their resistance to boiling in water, and their solubility in ethanol. A rather complicated mixed agglutination technique, previously described by Tönder *et al.* (1964), for determination of these antigens was employed in the case. A rabbit antibody to the tissue antigen is first attached to the tissue antigen. Test cells are then prepared by sensitizing sheep red cells with rabbit anti-sheep erythrocyte antibodies. These cells are then treated with a goat anti-rabbit γ-globulin antibody, and the agglutinates which form are broken up. The test cells then have, in effect, one free end of the goat anti-rabbit γ-globulin antibody which can combine with the rabbit antibody attached to the tissue antigen, giving the mixed agglutination reaction. The tissue submitted in the case was determined to be pig intestine using the technique.

It may be noted in conclusion that the entire subject of species of origin determination was reviewed by Pfeiffer (1938), by Boyd (1946) and by Schleyer (1962).

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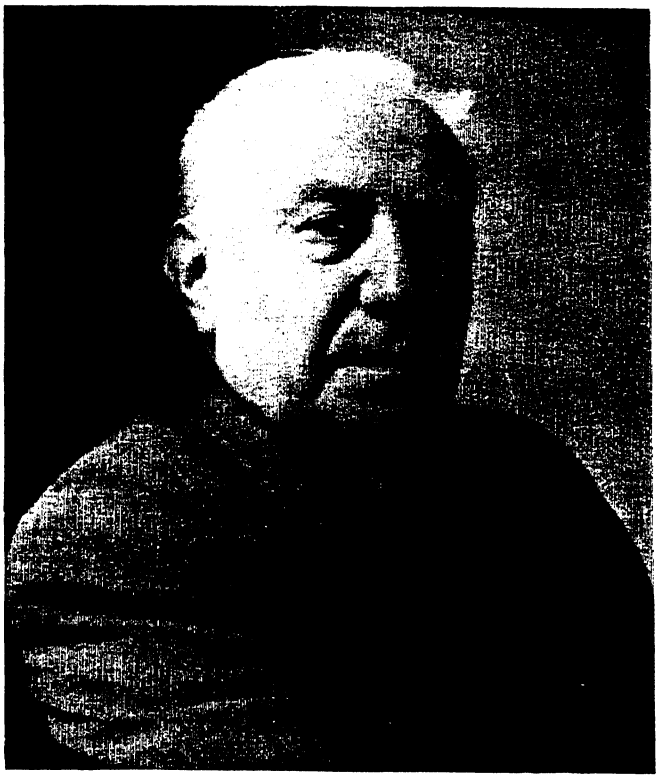
§[3] *Forensic Serology News* (*Forensic Serol. News*) see Note 1 to References for Unit VI

UNIT V.
BLOOD GROUPS

Karl Landsteiner (1869–1943)
Courtesy National Library of Medicine



Ludwig Hirszfeld (1884–1954)
Courtesy Col. Frank R. Camp, Jr. and Army Medical Research Laboratory, Fort Knox, KY

Fritz Schiff (1889–1940)
Courtesy Col. Frank R. Camp, Jr., and Army Medical Research Laboratory, Fort Knox, KY

Alexander S. Wiener (1907–1976)
Courtesy National Library of Medicine

Prof. Leone Lattes (1887–1954)
Courtesy Edizioni Minerva Medica and National Library of Medicine

Prof. Dr. Otto Prokop (1921–)
Courtesy Vergesellschaft Otto Spatz and National Library of Medicine

Dr. Ruth Sanger (1918–) and Dr. R. R. Race (1907–)
Courtesy Col. Frank R. Camp, Jr., Dr. R. R. Race and National Library of Medicine

SECTION 18. INTRODUCTION TO THE FORENSIC APPLICATIONS OF GENETIC MARKER SYSTEMS TO IDENTIFICATION AND DISPUTED PARENTAGE PROBLEMS

There are two major application areas of forensic or medicolegal serology and biochemistry: (1) disputed parentage testing; and (2) identification testing of bloodstains and secretions, usually in criminal matters. Although both these areas make use of the same genetic marker systems, they tend to be separate subspecialties in practice. It is exceptional, at least in this country, to find laboratories engaged in both kinds of testing. In parentage and affiliation testing applications, any genetic marker system for which the mode of inheritance has been firmly established can be employed, at least in theory. In blood and secretion stain analysis, there is an additional dimension: the blood or body fluid materials in the stains or spots must be identified before genetic markers and typed. Analysis, therefore, consists of identification tests followed by individualization tests (genetic marker system typing). Typing of the various systems in dried blood and secretion materials, and in post mortem tissues and fluids, presents problems that are not encountered with freshly taken samples. This book was prepared with stain analysis work foremost in mind, and it is organized accordingly. Units II through IV have discussed identification issues. Units V through VIII are devoted to individualizing markers. Emphasis has been given to blood and body fluid stain analysis, but parentage testing applications are discussed in connection with the various systems. In this section, the general principles and considerations involved in parentage testing and in stain identification analysis are discussed briefly as an overall introduction to the units on genetic marker systems. Genetic marker systems may be divided into five major classes: blood groups, isoenzymes, serum group systems, hemoglobin, and HLA. They are discussed in the units which follow. A sixth class that would include "other" genetic markers, such as the polymorphic proteins of saliva and hair, could be added for completeness.

Professor Dodd, in her presidential address to the British Academy of Forensic Sciences (Dodd, 1980), has given an excellent and thoroughly readable overview of both the civil and criminal aspects of forensic serology.

18.1 Disputed Parentage Testing

Disputed parentage cases usually involve disputed paternity. The mother is assumed to be the genetic mother in these cases.The legal issue is usually the support of a dependent child. Disputed affiliation cases can also arise in connection with immigration matters and citizenship claims, inadvertent baby mix-ups, kidnapping, disputes over the inheritance of money or property, and divorce cases.

In theory, any genetic marker system for which the inheritance patterns are straightforward and well established can be employed in disputed affiliation cases. It must also be clear that the genetic marker system is expressed in the child according to its own genotype. Probably over 60 systems could be listed as potentially applicable at the present time (Chakraborty *et al.*, 1974; Lee, 1975; Joint AMA-ABA Guidelines, 1976). As a practical matter, far fewer systems are normally used in casework. Some systems are much more informative than others. Some require reagents which are not widely available. Others requre special equipment or special training and expertise which every laboratory does not have. Cost is a consideration as well, since it must usually be borne by the defendant. It is clear that laboratories must be highly skilled in the typing of all the systems they use in casework, and such skill is usually developed through a combination of background knowledge and considerable practical experience. Lee (1975) reviewed the status of parentage testing, and gives comparisons of the various combinations of systems used in different countries. Polesky and Krause (1977) discussed the capabilites of American laboratories.

Genetic marker systems employed in disputed parentage tests must follow well known and established rules of inheritance. Thus, a child cannot have a gene which both parents lack, and must inherit one of a pair of chromosomes from each parent (if the system is controlled by a single locus, the chromosome will carry one allele; if the system is controlled by a series of linked loci, the chromosome will carry a haplotype). Further, a child cannot have a pair of genes unless both parents have the gene, and a child must inherit a genetic marker allele for which either parent is homozygous. Apparent violations of these rules are the basis for exclusions of parentage. Apparent violations of the first two rules, i.e. (1) a child has an allele which is absent in both parents, or (2) in a multiple allelic system, a child lacks both alleles which are found in the alleged father, are called direct exclusions (or "first order" or "primary" exclusions). With some extremely rare exceptions, these can be accepted with great confidence. Such exclusions are based upon the presence or absence of genetic markers demonstrable by direct examination. Apparent violations of the second two rules, i.e., (1) a child is homozygous for a marker allele which is not present in both parents, or (2) a child lacks a marker for which the alleged father is homozygous, are known as indirect exclusions (or "second order" or "secondary" exclusions). They are based on the inference of homozygosity detected by a negative reaction in a particular test. These exclusions are interpreted with great caution, particularly if

there is a single second order exclusion (one system). Each system has its own rarities and peculiarities, and one must be familiar with them and take them into consideration when interpreting the results of a parentage investigation.

The probability (PE) that a particular system will exclude a falsely accused father *a priori* can be calculated, and depends on the gene frequencies of the particular system in the population of interest. For most systems, the PE can be calculated quite easily (see Walker, in AABB, 1978). For some of the more complex systems, like Rh, Gm and HLA, the calculations are correspondingly more complicated. Tables of values have been published for many systems, however, for easy reference (e.g. AABB, 1978). The accuracy of the PE value depends on the accuracy of the gene frequencies used to perform the calculation. The cumulative probability of exclusion (CPE) for a series of genetic marker systems can be computed from the individual PE values according to: $CPE = 1 - (1 - P_1)\ (1 - P_2)\ (1 - P_3)\ \ldots\ldots (1 - P_n)$, where P_1 is PE for the first sysem, P_2 is PE for the second, etc., and P_n is PE for the n^{th} system, and where n systems have been used. A list of genetic marker systems that are applicable in forensic serology is shown in Table 18.1. The table shows the approximate PE for each system for Black and White populations. To the extent that data were available, the values are applicable to most U.S. populations. HLA (section 46) is the most powerful system by far. The CPE for all the systems in Table 18.1 would exceed 99%.

If a panel of systems has been employed for paternity testing, and no exclusions have been found, it may be of interest to calculate the probability of paternity. This value can be calculated by a number of different methods, some quite simple and some quite complicated (see Walker in AABB, 1978). The value will depend on which systems were used in the tests, and genotypic and phenotypic distributions. In some countries, this information is routinely calculated. In this county, the rules of evidence vary in different jurisdictions. In some states, blood grouping evidence is inadmissable in paternity cases unless it is exclusionary. In other states, courts will admit inclusionary evidence and estimates of the probability of paternity. Many genetic marker systems have rare alleles. These are seldom encountered, but can be very informative in parentage cases if found in a child and a putative parent. The incidence of rare alleles at 43 enzyme loci was discussed by Harris *et al.* (1974).

Sometimes, special situations arise that call for somewhat different approaches. PE values for cases involving relatives were discussed by Salmon and Brocteur (1978). Asano *et al.* (1978a and 1978b) discussed procedures and interpretation when all the parties to the case are not available for testing (e.g. deceased putative father).

Dodd and Lincoln (1978 and 1979) presented results on over 1,500 cases of disputed parentage investigated by blood grouping.

18.2 Blood and Body Fluid Stain Individualization

In criminal cases, individualizing genetic marker systems are used for comparisons of stains or spots with the known blood or body fluids of people known or thought to be involved. Firm conclusions can be drawn only in cases of nonidentity, that is, when the person suspected of having deposited the stain is excluded because he or she lacks a genetic marker found in the questioned material.

If samples are compared and found to be identical in one or more systems, it means only that the person tested is included in the subset of the population having the particular set of types found. If the frequencies of occurence of the types for the systems employed are known for the population in question, then the expected frequency of occurrence of the type or set of types can be calculated, and may be informative. The figure obtained in these calculations depends on the gene frequencies for the systems. The less accurate the gene frequency estimates, the less accurate will be the resulting estimate of frequency of occurrence of certain types. In general, the gene frequency estimate improves as more and more people are typed. In a population as heterogeneous as in the United States, it is sometimes difficult to decide how the "population in question" should be defined.

The ability of a genetic marker system to distinguish between individuals in a population is clearly related to how well the types are distributed. The probabilities of identity and discrimination can be calculated for genetic marker systems, and this subject was discussed in section 1.2.8. The principles are also covered in Selvin *et al.* (1979). In Table 18.1 is shown the DI for most of the systems. DI is a measure of the power, or value, of a system in distinguishing between individuals selected at random from a population. Comparisons of DI, and combined DI values for several systems, can help examiners and laboratories in choosing the panel of systems they will use in particular cases or in all cases. There are other important considerations in choosing systems as well.

In general, fewer systems can be used in stain analysis that in the typing of fresh materials. Techniques for typing stains are not worked out for every system. There are perhaps 20 systems that could theoretically be used in stain analysis. It is unlikely that so many systems would ever be applicable at a practical level because of the many other constraints imposed by casework samples. In many cases, the age of the stains is a problem. In others, the sample size is very limited. There are cost considerations (in comparison with the benefit to be derived) in adding new systems to the routine analysis scheme. In individual laboratories, resources may be rather scarce, and this will be a decisive factor as well. Nonetheless, a greater degree of individualization of stains is possible now than ever before. As new systems are added to the list of those applicable to this kind of work, the situation promises to improve even further in the years ahead.

Table 18.1 Genetic Marker Systems in Human Blood

System	Number of Common Phenotypes	Discrimination Index		Reference(s) to Typing in Bloodstains	Probability of Exclusion of a Falsely Accused Father		Reference(s) to Typing in Parentage Cases
		Caucasian	Black		Caucasian	Black	
Blood Groups				See in Unit V			See in Unit V
ABO	4 (Note 1)	0.62	0.64	See Section 19	0.13	0.17	Wiener, 1943
Rh	7 (Note 2)	0.79	0.69	Bergagna and Pereira, 1967; Lincoln and Dodd, 1968b; Martin, 1977	0.27	0.18	Wiener and Wexler, 1958
MNSs	9	0.83	0.78	Pereira, 1963a; Fiori et al., 1963; Lincoln and Dodd, 1968b and 1975a,b	0.31	0.32	Sussman, 1976
Kell	2 (Note 3)	0.15	0.04	Douglas and Stavely, 1969; Lincoln and Dodd, 1975a,b	0.03	0.005	Boorman et al., 1977
Duffy	3 (Caucasian) 4 (Black) (Note 4)	0.62	0.56	Lincoln and Dodd, 1975a,b	0.18	0.04	AABB, 1978
Kidd	3	0.61	0.55	Lincoln and Dodd, 1975a,b	0.18	0.15	
Red Cell Isozymes				See in Unit VI			See in Unit VI
PGM	3 (Note 5)	0.52	0.47	Cullford, 1967; Bark et al., 1976; Sutton and Burgess, 1978	0.15	0.12	Monn, 1969c; Herbich and Pessendorfer, 1969
ACP	5 (Note 6)	0.66	0.54	Brinkmann, 1971; Wraxall and Ernes, 1975	0.22	0.15	Fuhrmann and Lichte, 1966; Fiedler, 1967; Speiser and Pausch, 1967
AK	2 (Note 7)	0.13	0.04	Cullford and Wraxall, 1968	0.04	0.007	Prokop and Göhler, 1976; Dykes and Polesky, 1978; Boorman et al., 1977
ADA	2 (Note 8)	0.18	0.10	Cullford, 1971; Brinkmann and Dirks, 1971	0.05	0.03	Wust, 1971; Bauer and Herbich, 1972
ESD	3	0.35	0.27	Blake and Sensabaugh, 1974 and 1975; Perkin and Adams, 1976	0.08	0.08	Prokop and Göhler, 1976; Dykes and Polesky, 1977
GLO	3	0.61	0.62	Wraxall and Stolorow, 1976; MPFSL, 1978	0.18	0.16	Brinkmann and Püschel, 1978; Jaklinski and Koziol, 1979
CA_{II}	3 (Black) (Note 9)	–	0.33	Hughes, 1978	–	0.08	–
Gd	See Section 33.1			Cullford, 1971	–	–.	–
PGD	2	0.09	0.13	Cullford, 1971; Brinkmann, 1971	0.01	0.03	Brinkmann, 1971; Dykes and Polesky, 1978
GPT	3	0.63	0.48	Welch, 1972b	0.18	0.13	Radam and Strauch, 1972; Welch and Dodd, 1974
PEPA	3 (Black) (Note 10)	–	0.21	Cullford, 1971; MPFSL, 1978; Parkin, 1978	–	0.06	–
GALT	3 (Note 11)	–	–	–	0.066	–	Eriksen and Diesing, 1980
Serum Groups				See in Unit VII			See in Unit VII
Gm and Km	Many (Note 12)	–	–	Planques et al., 1961; Görtz, 1969; Nielson and Henningsen, 1962; Kipps, 1979	0.27	0.39	Ellis et al., 1973; Sebring et al. 1979; van Loghem and Nijenhuis, 1979
Hp	3 (Note 13)	0.62	0.72	Cullford, 1971; Stolorow and Wraxall, 1979; Blake and Sensabaugh, 1978b	0.18	0.15	Galatius-Jensen (1962); Giblett (1963); Dykes and Polesky, 1978
Gc	3 (Note 14)	0.57	0.33	Nerstrom and Skafte Jensen (1963); Tumanov and Il'ina (1974); Wraxall and Stolorow, 1978; Zajac and Grunbaum, 1978	0.16	0.08	Chakraborty et al., 1974; Dykes and Polesky, 1978; Hirschfeld and Helken, (1963); Bütler et al., (1963); Holzhausen et al., (1964); Reinskou, (1966b); Bütler, Kühni, et al., 1978; Hoste, 1979
Tf	See Section 42				0.01 (Note 15)	0.05	Mauff et al., 1975; Dykes and Polesky, 1978; Thymann, 1978; Hoste, 1979
Pi	4-6 (Note 16)	0.36	0.06	–	–	–	–
Other							
Hemoglobin (Hb)	See Section 38	–	0.2	Pollack et al., 1958; Huntsman and Lehmann, 1962; Cullford, 1964 and 1971; Wiggins, 1978	–	0.04	Chakraborty et al., 1974
HLA	Many	–	–	Newall, 1979; Hodge et al., 1979	0.8	0.8	AABB, 1978; Teresaki et al., 1978

1. 6 if A_1 and A_2 subgroups are distinguished.
2. There are many less common phenotypes.
3. Three phenotypes can be distinguished with anti-K and anti-k, but KK individuals are rare; a number of further phenotypes can be distinguished if anti-Kp^a, anti-Kp^b, anti-Js^a and/or anti-Js^b are used.
4. Fy (a-b-) is common in the Black population, but exceedingly rare in Caucasians.
5. Three phenotypes, determined by two alleles, are detected by common electrophoretic methods; ten phenotypes, determined by four alleles, are detected by isoelectric focusing. The DI is substantially increased if the subtyping in done and so is the theoretical PE in parentage cases. DI for Europeans would be about 0.77.
6. The C phenotype is rare, as are the RA and RB types.
7. AK 2 is comparatively rare.
8. ADA 2 is comparatively rare.
9. CA_{II} is not polymorphic in Caucasians.
10. PEPA is not significantly polymorphic in Caucasians.
11. Heterozygotes for 'Duarte' and 'Los Angeles' can be distinguished; some workers consider $GALT^D$ and $GALT^{LA}$ together as "$GALT^2$" as was done in the paternity study cited.
12. The number of phenotypes which can be distinguished depends upon which antisera are available for use; antisera for many of the specificities are very rare.
13. A modified kind of Hp 2-1, called Hp 1-2M, occurs relatively frequently in Black populations. Hp 0 occurs in Black populations as well. See in Section 40.
14. The DI is significantly increased by Gc[1] subtyping (section 41.3); the PE for a falsely accused European father is also significantly increased (section 41.6.1).
15. The PE for a falsely accused European father is increased to 0.13-0.15 by Tf C subtyping.
16. There are at least 12 phenotypes of Pi, but most are relatively rare; DI values have been estimated based upon European and African population distributions.

SECTION 19. THE ABO AND SECRETOR SYSTEMS

19.1 Origins and Earlier Studies

The ABO blood group system was discovered in 1900 by Karl Landsteiner. In a footnote to a paper, devoted primarily to other matters, he said:

> The serum of healthy humans not only has an agglutinating effect on animal blood corpuscles, but also on human blood corpuscles from different individuals. It remains to be decided whether this phenomenon is due to original individual differences or to the influence of injuries and possible bacterial infection. I observed this behavior as especially pronounced in the case of blood from severely ill patients. This phenomenon could be related to the dissolving capacity of serum for blood corpuscles in the case of various diseases, as it was described by Maragliano (10th Congress of Internal Medicine, 1892)

The full account of Landsteiner's observations appeared in 1901. The essential finding was that the sera of healthy persons agglutinated the red cells of certain other healthy persons. The data allowed the sera of the individuals tested to be placed into one of three groups: in one, called "A", the serum reacted with the cells of another group, called "B", but not with the cells of group "A"; the serum of group "B" reacted with group "A" cells; in the third group, called "C", the serum agglutinated the cells of both groups "A" and "B", but the red cells of group "C" were not reactive with sera from either group "A" or "B". Landsteiner said that there must be at least two agglutinins present, one in the group "A" serum, another in the "B" serum, and both together in the serum of group "C". There was a body of opinion at the time which held that the phenomena were a function of certain disease states, but Landsteiner said that this thinking was not in accord with his findings. In some placental sera examined, the agglutinins appeared to be absent. It was noted that sera dried for two weeks on linen still gave the observed reactions, and that this fact might very well be exploited for medico-legal purposes. Landsteiner concluded by noting that these characteristics of human blood permitted an understanding of the consequences of various kinds of transfusions. The phenomenon of agglutination of human cells by human sera was called isoagglutination, and the agglutinins in serum were referred to as isoagglutinins, after the suggestion of Ehrlich and Morgenroth. In 1902, von Decastello and Sturli conducted further studies on the phenomenon, and could fully confirm all of Landsteiner's observations. In addition, it was found that the blood of 4 out of 155 persons behaved differently from the previously described three groups, i.e., the serum contained no isoagglutinins, and the cells were agglutinated by the sera of all the other groups. The isoagglutination patterns showed no dependence on any pathological condition. Differences in the titer of isoantibodies in different individuals were noted as well, along with individual differences in agglutinability of cells with a serum of constant strength. In some newborn blood samples, the isoagglutinins were not observed. In cases where they were observed, they were of lower titer than in adult bloods, and it appeared that there was variable development of receptors on the cells of newborns as well. Halban and Landsteiner (1902) conducted a number of immunohematological studies comparing maternal with neonatal blood, and obtained results very like those of von Decastello and Sturli with respect to isoagglutination.

In 1907, Jansky published a paper in a rather obscure Bohemian journal, written in the Czechoslovakian language, in which he examined isoagglutination using the cells of 99 persons with the sera of 30 others. The tests involved over 3100 individual agglutination tests, and he recognized the existence of four isoagglutination blood groups from his data. Peculiarly, he did not mention or cite Landsteiner's work, though he cited other German literature in the field, including von Decastello and Sturli's paper. Jansky classified the isoagglutination groups according to Roman numerals, Groups I through IV. In 1910, Moss examined blood from 100 persons and noted four isoagglutination groups as well. He, likewise, classified them according to a Roman numeral designation. Unfortunately, Moss group I corresponded to Jansky group IV, and conversely, (Moss, 1910a and 1910b). The adoption of one or the other systems of nomenclature by various different authors and institutions led to quite a bit of confusion in the literature for quite a few years. Von Dungern and Hirszfeld [sometimes spelled Hirschfeld] conducted extensive experiments on the isoagglutination groups beginning around 1910, and concluded that the four groups could be accounted for by the presence or absence of two isoagglutinogens, which they called A and B. The agglutinins were designated α and β (von Dungern, 1910; von Dungern and Hirschfeld, 1910a, 1910b and 1911). Guthrie and his colleagues in this country used upper case letters to designate agglutinins and corresponding lower case letters to designate agglutinogens (Guthrie and Huck, 1923). Lattes (1932) said that such usage was not defensible, because it was clear by then that the agglutinogens were inherited as Mendelian dominant characteristics. There were periodic calls in the literature for standardization of the nomenclature at the time (e.g. Verzár, 1927; Aldershoff, 1927). In this country, the American Immunological, Pathological and Bacteriological Societies called for the universal adoption of the Jansky system in 1921. In 1928, Kennedy published a survey of the nomenclature usage in American

hospitals. At that time, 72% were using the Moss system exclusively, 16% the Jansky system, and the rest two or more systems simultaneously. The 1921 recommendation of universal adoption of the Jansky system had not resulted in any significantly increased use of the system, the survey found. Finally, more than half the hospitals surveyed did not favor a standardized "compromise" system, and about ⅔ felt that such a system would only lead to additional confusion. The matter was finally settled in 1930 when the Permanent Standardization Commission of the League of Nations Health Organization reported the work of its Laboratory Conference on Blood Groups (League of Nations Health Organization, 1930). A resolution to adopt essentially the scheme of von Dungern and Hirszfeld was adopted at an April 1928 session of the Permanent Standardization Commission held in Frankfurt-am-Main. The test sera for groups A and B were to be called anti-A and anti-B, respectively. A comparison of the Moss, Jansky and International systems appears in Table 19.1. The use of α to denote anti-A, and β to denote anti-B is still encountered, and does not lead to any confusion.

In 1931, Kennedy published a paper with the stated purpose of making available in the more accessible literature the information contained in the original paper of Jansky (1907). A careful analysis of the data was carried out, and it was argued that Jansky had been the first person to observe and categorize all four blood groups from a single set of isoagglutination tests all in the same paper. He said that Shattock (1900) had actually been the first investigator to publish an observation of isoagglutination in humans. Most authorities think that Shattock was actually seeing rouleaux formation, and in any case, his experiments were carried out with other things in mind, and the notion of isoagglutination groups did not occur to him. Moreover, he was studying blood from sick patients. Zinsser and Coca (1931) published a few remarks on Kennedy's paper. They said that Shattock had undoubtedly been looking at rouleaux formation. More to the point, they seemed anxious to emphasize that the credit for the discovery of the blood groups blonged to Landsteiner, without detracting from Jansky's contribution. Kennedy had not made an explicit issue of the matter of primacy, though one could gain the impression from reading his paper that he regarded Jansky's report of all four groups in the same paper as more significant than the separate reports of Landsteiner, and of his collaborators, von Decastello and Sturli. Kennedy said that, as far as he could tell, Guthrie and Huck (1923) were among the few American workers who read Jansky's paper, and the only ones who had actually analyzed the data. It is quite clear, however, that Moss (1910a and 1910b) had seen at least an abstract of the paper after his own were in press. In an added note, he gave Jansky due credit, and noted the differences in their Roman numerical designations of the blood group.

There is no longer any question about the significance and priority of Dr. Landsteiner's contribution. Landsteiner himself did not regard the description of all four groups in one and the same paper as the principal issue. Von Decastello and Sturli were in fact his collaborators, and their report, describing what was later called group AB, was considered a logical extension of the previous work. Speiser (1961) reproduced an interesting letter from Landsteiner to Sturli on this point. The letter, written February 12, 1921, was rendered in English in Prokop and Uhlenbruck (1969):

12.2.21

The Hague
van Slingelandstr. 39, Holland
Dear Dr. Sturli,

I was very pleased to receive your card. I should like to ask you this: Isoagglutinins, which we have already studied, have achieved great importance in America, as they do many transfusions and for this purpose a blood group determination is always made. I do not know if you are aware of this. You will find something about this, for example, in the first number of the Journal of the American Medical Assoc. of this year. The Americans always say that I have found only 3 groups, as if the few cases of the fourth group were the main thing. Only recently you and Decastello were cited as the authors of this fourth group, earlier it was usually Moss or Jansky who have not done anything new at all. It would be important for me to be able to say, when the occasion arises, that when you were working with me as my pupil or collaborator, you undertook with Decastello the continuation of my work. That was the way things happened. It does not harm, or rather it does not make any difference to your position and I would appreciate it, as other people could not then accuse me of an error as they have until now. I myself saw at the time the necessity for more numerous investigations and found, however, that it was desirable that you should do these. I should be glad if you would let me have a line about this. Have you seen the new book on Immunity by Bordet? . . . .

Yours, Landsteiner

In 1930, Landsteiner received the Nobel Prize for Physiology or Medicine "for his discovery of human blood groups". In his Nobel lecture, he mentioned the medicolegal applications of blood grouping. Until the blood groups were discovered, he noted, ". . . .forensic medicine knew no way of distinguishing between blood stains of different persons. Since the isoagglutinins and the corresponding agglutinogens will also keep for a considerable time in a dried condition, the problem can in certain cases be solved, in particular when the bloods in question, e.g. that of the accused and that of the victim, belong to different groups. . . . . .according to a report by Lattes, who was the first to use it in forensic cases, [the test] has proved useful in a number of cases, and has been the basis of court verdicts and of the acquittal of accused persons." Landsteiner's contributions to immunology and serology extend far beyond his discovery of the blood groups. Landsteiner was born in 1868, and died in 1943. He spent the last 21 years of his life at the Rockefeller University in New York. His scientific life was

Table 19.1 Historical and Present ABO Nomenclature

Moss	Jansky	International
I	IV	AB
II	II	A
III	III	B
IV	I	O

extraordinarily active, 346 publications having appeared between 1892 and 1943. A full bibliography may be found in the *Journal of Immunology* **48**: 1-16 (1944), and some biographical information about him appears in the introduction to the Landsteiner Centennial held at the New York Academy of Sciences (*Ann. N.Y. Acad. Sci.* **169**: 1-293, 1970).

Landsteiner (1901) had indicated that his observations on what later became known as blood groups A, B and O could be explained by the presence or absence of two agglutinins in human serum. Hektoen (1907) supposed that there were three agglutinins and three agglutinogens, and he arrived at this conclusion from a number of absorption experiments. While noting that O cells absorb no antibody, he appeared to be saying that he did get absorption of both α and β by both A and B cells, a most peculiar result (except see section 19.7.2). He also observed a blood which belonged to group AB. In 1910, Moss attempted a systematic explanation of the four blood groups which, as noted above, he designated I through IV (Moss, 1910a and 1910b). He too thought that there were three agglutinogens and three agglutinins. Letting agglutinogens be designated A, B and C, and their corresponding agglutinins by a, b and c, there were two possible arrangements, he said, which would explain the agglutination data: (1) Group I A,o; Group II B, a + c; Group III C, a + b; Group IV O, a + b + c; or (2) Group I A + B + C, o; Group II B + C, a; Group III A + C, b; and Group IV O, c. In these representations O stands for no agglutinogen and o stands for no agglutinin. In Moss nomenclature, groups I through IV represent what we now call AB, A, B and O, respectively. Thus, according to either scheme, AB serum agglutinates no cells, but AB cells are agglutinated by A, B or O serum; A sera agglutinate AB or B cells, and A cells are agglutinated by B or O serum; B sera agglutinate A or AB cells, and B cells are agglutinated by A or O serum; and lastly, O sera agglutinate A, B or AB cells, but O cells are not agglutinated by any sera.

Von Dungern and Hirszfeld took up studies on this subject around 1910, and put forward a systematic explanation of the blood groups based on the original notions of Landsteiner and of von Decastello and Sturli. The groups were explained by the presence or absence of two agglutinogens, A and B, and two agglutinins α and β. The groups were designated according to the agglutinogens possessed by the red cells: group A has A cells, β in serum; group B has B cells, α in serum; group AB has A and B on cells, and no serum agglutinin; and group O lacks agglutinogens, but has both α and β in serum (von Dungern and Hirschfeld, 1910b and 1911). It was noted that different human sera may vary considerably in their agglutinin titer, and that red cell agglutinogens from different persons can likewise exhibit very different activities toward their corresponding agglutinins. Extensive studies on the occurrence of agglutinins for human red cells in animal sera were carried out as well. Selective

absorption experiments by many workers, including Koeckert (1920), Schütze (1921), Hooker and Anderson (1921) and Dyke (1922b), helped to establish the acceptance of the two agglutinogen-two agglutinin scheme. Group A cells absorbed only the α from group O serum, while group B cells would selectively absorb the β. Group AB cells absorbed both agglutinins from group O serum, or α and β separately from B or A sera. Hooker and Anderson (1921) prepared heterologous antisera against human red cells in rabbits, and, by appropriate selective absorption of the antisera, could render some of them group specific.

While the four group scheme was fairly widely accepted by 1920, apparent exceptions to the results expected on the basis of the theory had been observed. There were cases where agglutination between a serum and cells was expected but failed to occur, other cases in which cross-agglutination was observed between persons belonging to the same group, and cases where O cells had been observed to agglutinate. The experiments which may have attracted the most attention in this regard were those of Guthrie and his collaborators. Based on extensive observations, they postulated at first one additional agglutinogen-agglutinin pair (Guthrie and Huck, 1923; Huck and Guthrie, 1924), and later several more agglutinogen-agglutinin combinations (Guthrie and Pessell, 1924a and 1924b; Guthrie *et al.*, 1924). They pointed out that the number of theoretically possible combinations, even using two antigens and two antibodies, was not four but nine, without violating the principle that agglutinins never occur in the serum of a person whose cells have the corresponding agglutinogen (Landsteiner's Rule). Thus, one could imagine (using present-day nomenclature) O$\alpha\beta$, Oα, Oβ, Oo, Aβ, Ao, Bα, Bo and ABo, where "o" indicates no agglutinins. They had in fact observed a blood in which the cells were of group B, but the serum had no agglutinins, i.e., behaved as an AB serum. Coca and Klein (1923a and 1923b) accepted the interpretation of Guthrie and coworkers that there were additional agglutinogen-agglutinin combinations and described one themselves which they called "X". Other workers confirmed the observations, and accepted the interpretation as well (e.g. Simson, 1926; Bunker and Meyers, 1927). Landsteiner and Witt (1924) at first thought that these observations might indeed indicate an additional pair of factors. They noted that two different types of group AB blood could be distinguished, one of which contained an agglutinin for certain group A cells, and the other of which did not. They later changed their opinion as to how this observation should be interpreted, as will be discussed below in connection with A subgroups. The Italian workers, Lattes and Cavazutti (1924) and Mino (1924) did not agree at all that it was necessary to invoke the existence of additional agglutinogen-agglutinin combinations to explain the observations of atypical isoagglutination. These could be understood, they thought, on the basis of quantitative differences in cell receptors and in agglutinin content of sera, as well as of varying avidity of agglutinin for receptor, while retaining the basic four group hypothesis. The matter will be discussed somewhat more in connection with subgroups.

19.2 Inheritance of the ABO Blood Groups

Langer in 1903 and Hektoen in 1907 noted in passing that mothers and their children could have the same blood groups. If they entertained the notion that the blood groups were inherited, they were not explicit on the point. In 1908, Epstein and Otterberg reported the blood groups of two families at a meeting of the New York Pathological Society, and suggested that the blood groups might be inherited. The clear and unequivocal demonstration of the heredity of the blood groups, however, came from the work of von Dungern and Hirszfeld (von Dungern, 1910; von Dungern and Hirszfeld, 1910b). The first experiments were carried out in dogs with immune isoagglutinins, but they soon looked at a collection of data from 71 human families, including 342 people, in Heidelberg. The data were interpreted to mean that the agglutinogens, and not the agglutinins, were inherited. A blood group agglutinogen could not be found in children if it was lacking in both parents. If both parents have an agglutinogen, most of the children have it, but a few may not. And in a family where the parents are AB and O, A and B children are possible. The hereditary factors determining the presence of the agglutinogen were believed to be dominant, those determining their absence, recessive. The factors behaved strictly according to Mendelian principles, and it was thought that A and B were determined at separate, independent genetic loci. This work formed the basis of hundreds of studies by many workers, including themselves. According to the von Dungern and Hirszfeld notion that the genetics of the system could be explained on the basis of two, independently inherited allelomorphic pairs, A being dominant to non-A, and B being dominant to non-B, nine genotypes and four phenotypes could be represented as shown in Table 19.2, where A and B represent the genes for agglutinogens A and B, and a and b represent the recessive genes for their respective absence. Hirszfeld (1928) reviewed in detail the experimental foundations for scheme, as well as numerous family and population studies with which it was consistent.

In 1924, the mathematician Felix Bernstein published the first of his several papers in which he formulated a theory of inheritance for the ABO groups based upon statistical genetic considerations, and inferences drawn from the rather extensive body of population data which existed by that time. He introduced the notion of multiple alleles at a single genetic locus for the system. There was precedent for multiple allelic systems in *Drosophila,* but not in human genetics. According to Bernstein, there are three genes, A, B and R, which can give rise to the genotypes RR, RB, BB, RA, AA and AB. The agglutinins corresponding to A and B were called α and β in conformance with the accepted usage, and he said that an agglutinin ρ would correspond to R. According to this scheme, a child inherits only one blood group

Table 19.2 Blood Group Genetics According to vonDungern and Hirszfeld

Genotype	Phenotype
aabb	O
AAbb, Aabb	A
aaBB, aaBb	B
AABB, AABb, AaBb, AaBB	AB

genetic factor from each parent, instead of two as postulated by von Dungern and Hirszfeld. Genotypically RR people belonged to group O, RB and BB people were of group B, RA and AA people were group A, and AB people were of group AB. Bernstein analyzed a considerable amount of the available population data using a statistical genetic model, and found that his model fit the observations much better than the two allelic pair idea. If p, q and r are taken to stand for the gene frequencies of genes A, B and R, respectively, then the occurrences of the genotypes AA, AB, AR, BB, BR and RR in the population are given by p^2, 2pq, 2pr, q^2, 2qr and r^2, respectively, where $p + q + r = 1$ and $p^2 + 2pq + 2pr + q^2 + 2qr + r^2 = 1$ (Bernstein, 1924 and 1925).

Bernstein elaborated upon his ideas somewhat further in 1930 (Bernstein, 1930a and 1930b). His work is considerably more involved both in bulk and in computational complexity than has been indicated here. Much of it consists of detailed statistical and probabilistic analyses of the then-existing population data to demonstrate the correctness of his genetic hypothesis in comparison with the two allelic pair idea, and to answer various objections that had been raised to his ideas.

The alternative theories of Furuhata (1927 and 1929) and of Bauer (1928 and 1929) may be mentioned for the sake of completeness. Furuhata's theory postulated two pairs of alleles, but said that they were closely linked. He thought, further, that the genes exerted control over the presence or absence of the agglutinins as well as of the agglutinogens. The chromosomal composition could be ab, Ab or aB, but never AB. The idea was similar to Bernstein's in that there were three "hereditary units", and to von Dungern and Hirszfeld's in that two pairs of genes were involved. He would not allow that AB could come about in one and the same chromosome, and thus in a single gamete, as the result of a crossover. For all practical purposes, Furuhata's theory turns out to yield results identical with that of Bernstein, except that the explanatory mechanism is different, of course. Bauer's idea was similar to Furuhata's, except that he allowed for crossing over in certain cases, giving rise to an

AB chromosome. Lattes (1932) noted that Kirihara and Haku in Japan had had this idea before Bauer.

Predictions of the blood groups of children from particular matings in large populations based on the two allelic pair hypothesis on the one hand, and on the multiple allelic hypothesis on the other, clearly favored the latter. There were a few observations in the literture, however, which appeared to contradict Bernstein's notion, these being reports of children having resulted from matings in which one or both of the parents was excluded by the theory. Most authorities eventually came to believe that such discrepant results were probably explainable on the basis of grouping errors or of unsuspected illegitimacy. Schiff and Boyd (1942) pointed out that the number of apparent exceptions to the rules of heredity dropped markedly after 1925, in part because of the fact that Bernstein's hypothesis had not been published, and investigators could not go back and re-examine all their groupings, and in part because of improvements in technique. There were 19.5 "exceptions" per 1000 families (13.2 per 1000 children) in the 1910–1925 period in material consisting of 973 families with 2270 children. In 1926, in 928 families with 2213 children, the comparable figures were 1.1 per thousand families and 0.47 per 1000 children. Comparably low figures were found in the data for 1927–1930 and 1935–1937 as well. These "exceptions" fall into two categories: A or B children being attributed to parents, neither of whom had either A or B; and O children attributed to parents, either of whom was AB, or AB children attributed to parents, either of whom was O. The possibility of errors in blood grouping cannot be overlooked either. In 1919, Pemberton, in his discussion of systemic transfusion reactions, noted that there were 12 such cases out of 1032 transfusions at the Mayo Clinic. In 9 of these cases, it was possible to do the blood grouping over again, and errors in the initial report of the blood group (primarily clerical) were found in every case. Lattes (1932) noted that there would always be a certain minimal number of apparently aberrant cases, because of errors and because of unsuspected illegitimacy. Furthermore, quantitative variations in antigen content, and the existence of weak subgroups of certain types, could lead to errors in routine group determinations on occasion. Such errors today would be entirely unacceptable, and these comments, it must be kept in mind, were made over 40 years ago.

It is clear that, at least by 1930, most of the major authorities had come to accept the Bernstein multiple allelic hypothesis as the correct explanation for the inheritance of the blood groups. With possible minor modifications, because of the possible occurrence of rare alleles in the system, the basic multiple allelic hypothesis is widely accepted today.

19.3 Subgroups in the ABO system

19.3.1 Subgroups of A

19.3.1.1 A_1 and A_2. In 1911, von Dungern and Hirszfeld noted that not every example of anti-A serum from B persons agglutinated every example of A cells. Their studies were carried out primarily with three anti-A sera. Two of these agglutinated a greater number of A cells than did the third. A small number of bloods, which reacted with the first two sera, did not react with the third one at all, and these were said to possess the characteristic "little A". In addition, if this third anti-A serum were absorbed with particular A cells, an agglutinin remained in the serum which would agglutinate certain A cells, but not others. It appeared, therefore, that there could be two types of agglutinins in the sera of group B (or O) people directed against A cells. Similar observations were made by Guthrie and Huck (1923) and by Coca and Klein (1923b), although they interpreted the results as indicating the presence of an additional agglutinogen-agglutinin combination in addition to A/α and B/β, and by Schütze (1921). Landsteiner and Witt (1924) made the same observation and initially agreed with the interpretation that indicated the presence of an additional pair of factors. Lattes and Cavazutti (1924) and Mino (1924) did not agree with this interpretation (although the observations were fully confirmed). The observations could be explained, the Italian investigators said, on the basis of quantitative differences, i.e., on the basis of differences in the number of receptors on the cells, and in the variability of titer and avidity of the agglutinins. In 1926, Landsteiner and Witt extended their observations and could, by suitable absorption experiments, establish quite unequivocally that the isoagglutinins which act on A cells could be separated into two qualitatively different fractions, which were called α and α_1. Similarly, A and AB cells could be distinguished on the basis of their agglutinability with α and α_1, the receptor for α_1 being named A_1. It was also found that the sera of AB people could sometimes contain α_1, the A_1 then not being present in the cells. In the same year, Landsteiner and Levine (1926b) suggested that the factor on A cells which is not recognized by α_1 be called A_2. They did not think at the time that A_1 and A_2 were probably controlled by separate genetic factors, and they noted too that their data indicated the presence of a receptor on O cells, which was not common to all bloods, but bore some similarity to A_2. The A_1–A_2 nomenclature has persisted to the present time. In 1930, Thomsen *et al.* put forward the idea that Bernstein's three-allele hypothesis for the inheritance of the ABO system should be modified to include two A factors, namely A_1 and A_2. This idea was supported by a number of studies on families (Friedenreich and Zacho, 1931). Thus, the system could have 10 genotypes, which give rise to six distinguishable phenotypes if anti-A, anti-A_1 and anti-B grouping reagents are used (Table 19.3). It is generally thought that A_1 blood has two antigenic receptors, A and A_1, while A_2 blood has only the A receptor. Anti-A from the serum of a B person generally has both anti-A and anti-A_1. Race and Sanger (1975) have noted that it is somewhat unfortunate that the term "anti-A" is used in two senses, i.e., to mean the antiserum on one hand, and to mean one of the antibodies contained in the antiserum on the other.

A number of different methods have been used for the discrimination of A_1 and A_2, and these are discussed by Prokop and Uhlenbruck (1969). The topic merits some dis-

cussion though, in part because the resulting classification can depend on the method used, but particularly because, as will be discussed below, there are some cases which do not clearly fall into one of the two categories. Von Dungern and Hirszfeld (1911) used a saturation method for the preparation of anti-A_1 serum, in which B-serum was simply saturated with A_2 cells, and the remaining antibody constituted the desired reagent. Thomsen *et al.* (1930) used a saturation technique as well, but in this case a B-serum was absorbed with a particular amount of unknown blood cells, and the titer of the remaining antibody against A cells was then determined. The process is repeated several times, and the data can be plotted to yield so-called "saturation curves". These can then be compared with the results obtained using measured amounts of known A_1 and A_2 cells. The finding that pepsin possesses A blood group characteristics has been exploited for the production of anti-A_1 serum (Ottensooer and Zurukzoglu, 1932; Schiff, 1933a). Prokop and Uhlenbruck (1969) noted that not all examples of pepsin are suitable for use with B sera in the production of anti-A_1 serum, and that the specificity of the reagent does not persist beyond a few days, at which time the pepsin treatment must be repeated. It is best, with all these methods, to choose a B-serum which had a high anti-A_1 titer to begin with. The data of Olbrich and Walther (1941) indicated that the anti-A_2 titer of a B-serum tends to be higher as the anti-A_1 titer is higher also. So-called "irregular" antibodies can be used as anti-A_1 reagents. These are anti-A_1 which occur in the sera of A_2 and A_2B people (Lauer, 1928; Friedenreich, 1931). The anti-A_1 titer varies with the temperature, the antibodies being detectable in more of the sera at lower than room temperature. Additionally, anti-A_1 is more often found in A_2B than in A_2 sera. Taylor *et al.* (1942) found α_1 in about $\frac{74}{283}$ (26.15%) of A_2B and in about $\frac{62}{3871}$ (1.6%) A_2 bloods in the U.K. Juel (1959) reported 12 A_2B bloods out of 40 (30%) as having anti-A_1 at 5°, and 9 (22.5%) at 18°. Lenkiewicz and Sarul (1971) said that they found about 14% of A_2 bloods and 51% of A_2B bloods to have anti-A_1 at 10° in a large number of cases examined in the Warsaw area. Prokop and Uhlenbruck (1969) cite various values from several authors, the numbers ranging from about 21 to 40% of A_2B bloods and from about 2 to 8% of A_2 bloods. Other techniques have been proposed for discriminating A_1 from A_2, based on blood group substances in saliva (see in subsequent section) and on

Table 19.3 ABO System Genetics with A_1 and A_2 Subgroups

Genotype	Phenotype	Reaction With		
		Anti-A	Anti-A_1	Anti-B
A_1A_1 A_1A_2 A_1O	A_1	+	+	−
A_2A_2 A_2O	A_2	+	−	−
BB BO	B	−	−	+
A_1B	A_1B	+	+	+
A_2B	A_2B	+	−	+
OO	O	−	−	−

the use of various animal sera. Perhaps the simplest technique is the use of A_1 specific phytagglutinins, one of the most common being from *Dolichos biflorus* (Bird, 1951 and 1952). Phytagglutinins are discussed more fully in subsequent sections.

It should be pointed out that Lattes in 1932 still did not accept the notion of qualitatively different subgroups of A as having been established. The data could be explained, he thought, on the basis of avidity differences in antibodies. The issue is still apparently not fully settled (Juel, 1959; Mäkelä *et al.*, 1969; Race and Sanger, 1975).

19.3.1.2 Subgroup A_3. In 1936, Friedenreich described an example of A cells which reacted more weakly than A_2 cells (Friedenreich, 1936a and 1936b). This type of behavior was characteristic of certain families, was apparently rare, and no transitions could be observed between this weak A and A_2. Six unrelated people, out of about 4000, were found to have the weak A cells, and were investigated along with members of their families. Since the results showed that the cells were of a distinct type of A, they were designated A_3. A_3 cells reacted with anti-A reagents at least as weakly as do A_2B cells (which react more weakly than A_2 cells), if not more so. Sera which gave strongly positive reactions even with A_2B cells gave only weak reactions with the A_3 cells. It was noted that these A_3 cells formed a few rather large, fragile agglutinates amongst a large number of free cells. This characteristic agglutination picture was later referred to by Dunsford (1959) as "mixed field agglutination." With weak antisera, the A_3 would be missed altogether, i.e., grouped as O. With sera of too high titer, the A_3 might be misclassified as an A_2, but Gamelgaard (1942) noted that, because of the characteristic and unusual agglutination pattern, this should not be much of a problem. The sera of A_3 people contain a normal quantity of β-agglutinin, and no unusual α_1-agglutinins, except for a trace of "cold α_1-agglutinin" in a few cases. The absorption capacity of A_3 cells for anti-A was found to be intermediate between A_2 and A_2B. Three A_3B bloods were noted, their absorption capacity being less than that of A_2B. Friedenreich suggested that the A_3 characteristic was almost surely the result of an additional allele at the ABO locus, recessive to both A_1 and A_2. Gammelgaard (1942), in his extensive and important study on the weak A receptor types, looked at 170 persons of type A_3 and 33 of type A_3B. The agglutination picture with A_3, as originally described by Friedenreich, was confirmed. The absorption characteristics, and the fact that the weak A_3 agglutination does not decrease as rapidly upon serum dilution as does A_2B agglutination with anti-A reagents, were also confirmed. In the material studied by Gammelgaard, the incidence of A_3 was about 1:1000 A persons. The A_3 cells appeared to consist of a spectrum of red cells of varying receptor strength, from relatively agglutinable to non-agglutinable. Subsequent studies on A_3 have not unequivocally proven, but have not excluded the possibility that A_3 is an allele of the system (Race and Sanger, 1975). Race and Sanger also note that the diagnosis of A_3B is not an easy one, and that family evidence is really needed for confirmation. Dguchi *et al.* (1978) could isolate a population of A_3 cells by affinity chromatography on a Sepharose column, to which was bound lima bean anti-A lectin, which were completely inagglutinable by anti-A. Studies with eel serum anti-H showed that these cells had a large number of H sites, but only about 9% of the A sites that would be found on an A_1 cell. The A structure was believed to be the same on those A_3 cells as on A_1 cells, but the density of A sites too low to permit agglutination by lima bean anti-A lectin.

19.3.1.3 Further subgroups of A. The other weak A receptors which have been described are principally weaker than A_3. They have been given a variety of designations, and the relationships between the different ones are not always clear. They are all quite rare. In 1935, Fischer and Hahn described a blood from a patient which had a very weak A receptor. The cells were agglutinated by some group O sera, but only weakly or not at all by group B sera. Sheep immune anti-A agglutinated the cells weakly. The cells were designated A_x. They absorbed less anti-A than either A_1 or A_2 cells, but the anti-A which was absorbed could be more easily eluted from the A_x cells than from the A_1 or A_2 cells. The patient's serum contained anti-B, an anti-A_1 activity at 6–8°, and an anti-A_2 activity at "ice bath" temperature. In 1940, Gammelgaard and Marcusson described a blood with a very weak A receptor, which was not A_3, and which they called A_4. They supposed that it was due to an additional allele in the ABO system. The investigated family of 64 persons had 24 A_4 members. The incidence of A_4 was put at 1:60,000. The original designation was changed by Gammelgaard in 1942 to A_5, because some other cases had been found which were better referred to as A_4. These were slightly weaker than A_2B but did not give the characteristic A_3 agglutinates. A trace of A substance could be identified in the saliva of some of the A_4 persons, but not enough to try to distinguish between secretors and non-secretors (see in subsequent section). The A_5 cells were weaker than A_4, and this was the reason for the redesignation. Gammelgaard speculated that Fischer and Hahn's A_x was most likely an A_4. Gammelgaard described one odd blood, which he called A_x, and which did not have the characteristics that would indicate it should be called "A_6", that is, the cells did not agglutinate with "anti-O" serum (see below) nor was there a decreased secretion of A substance in saliva, as is observed A_1 through A_5.

In 1948, Jonsson and Fast reported a blood in which the cells had a very weak A, but it differed somewhat from previously reported variants, and was called A_6. Some of the A_6 sera had an α_1-agglutinin, but no A was found in saliva. Dunsford (1952) reported a blood which behaved very much like the A_4 of Gammelgaard. Further examples of A_4 were reported in an English family, one of whose members also had a very rare Rhesus type in addition to the A_4 (Dunsford and Aspinall, 1952). Estola and Elo (1952) found a weak A blood that was similar to Gammelgaard's A_5, and this was called A_z. Grove-Rasmussen *et al.* (1952) reported a case of a weak A receptor, which they called A_o. Ellis and Cawley found another case of an A_o in 1958. Salmon *et al.* (1965) reviewed A_x and presented studies on 42 cases in 8 families.

By A_x in this case, they were referring to the original designation of Fischer and Hahn. For the most part, the results of the family studies indicated that A_x could be accounted for by a rare ABO allele, but in one case, an A_2B parent (the other parent was not A or AB of any kind) had six A_x children. The inheritance of many of the weak A forms is, therefore, not always that clear. As mentioned, some of the family studies can be explained quite simply by postulating the presence of rare allele. In other cases, though, such as the one of Salmon *et al.* just mentioned, and in others to follow, the inheritance does not seem to follow that simple pattern. Cahan *et al.* (1957) have described a family in which an A_x father had an A_2B son who, in turn had an A_x daughter. The wife of the A_2B son was an A_1, and in 1962, another child was born to these parents who was A_2B (Fisher and Cahan, 1962). Likewise, van Loghem and van der Hart (1954) found three A_4 children from an O x O mating. They noted that this "A_4" could be Fischer and Hahn's A_x or Grove-Rasmussen *et al.*'s A_o. Cahan *et al.* (1957) had suggested using the designation A_x for all the variants, except A_1, A_2 and A_3, until the situation became a little clearer. Thus, A_4, A_5, A_6, A_o, A_z and A_x could all be grouped under the "A_x" heading, to give an indication of the priority of discovery by Fischer and Hahn. Dunsford, in his 1959 review, rather agreed with this suggestion. Even in 1975, Race and Sanger, who were co-authors of the Cahan *et al.* (1957) paper, thought that this practice should be continued. It should be noted that Wiener (1953) was of the opinion that these weak A variants discussed so far in section 19.3.1.3 were not A bloods at all, but examples of blood group C (see below). This opinion was still held in 1973 (Wiener *et al.*, 1973).

In 1956, Wiener and Gordon described a somewhat different variant of A, which was called A_m. These cells were not agglutinated by anti-A from B-serum or from O-serum, nor by immune anti-A. The serum had normal quantities of anti-B, however, and A substance could be found in the saliva. The blood did not behave like other weak A variants, such as A_o, A_4, etc., and was not, they said, an example of blood group C. Examples of other family material showing the characteristic were described by Weiner *et al.* (1957), Salmon *et al.* (1958) and Hrubiško *et al.* (1966). A_m cells are not agglutinated by any kind of anti-A, i.e., they group like O cells. But the serum contains anti-B, and A substances can be secreted into saliva. Sometimes the serum contains an irregular anti-A_1 antibody, and the non-agglutinability of the cells can depend on the IgM content of the anti-A serum used (Hrubiško *et al.*, 1966). There is variation within the phenotype. Hrubiško *et al.* (1966) found two probable A_m homozygotes, and in their material, the A_m behaved as if it were due to a rare allele. On the other hand, Weiner *et al.* (1957) could show that their A_m person was genotypically A_1O, and that an A_mB person was genotypically A_1B. The A_m cells reacted with anti-H (see further on) and A and H substances were secreted. The cells would absorb anti-A. The A_mB cells did not absorb anti-A, but the saliva contained A, B and H substances. Weiner *et al.* postulated the existence of a Mendelian pair of modifying genes, called Yy, to explain these A_m cases. According to this idea, A_m people would be homozygous recessive at the Yy locus (i.e., yy), and the absence of Y would lead to impaired development of the A antigen on red cells, but not in saliva (see Race, 1957). A_m cells are very rare. Because of its unusual characteristics, A_m should not be considered in the same category with A_x, A_o, A_4, etc. (Dunsford, 1959; Race and Sanger, 1975).

19.3.1.4 So-called intermediate A (A_{int}, A_i). As noted in 19.3.1.1, it is possible to classify the vast majority of A cells as either A_1 or A_2. Cells are occasionally encountered, however, which appear to be intermediate between A_1 and A_2 and cannot be unequivocally placed in either category. This phenomenon was first noted by Landsteiner and Levine (1926b), and was further studied by Friedenreich and Zacho (1931). The cells react with anti-A_1 reagents, but also with so-called anti-A_2, usually more with one than with the other. They react with *Dolichos* anti-A_1 and *Ulex* anti-H lechtins as well. Dahr (1942b) described examples of the "intermediary A" blood. The diagnosis of A_i may vary with the technique used. Gmyrek (1962) pointed this out, noting that identification of A_i was best done on the basis of reactions with anti-A_1 and anti-H, rather than by the absorption methods. He also pointed out that the strength of A and H in a series of A bloods, if judged by the titer with anti-A_1 and anti-H, do not vary inversely in a linear fashion. He also noted that there can be identified a graded series of A types, in between A_1 and A_2 in reactivity. Grundbacher and Summerlin (1971) also made the point that the antigenic strength of A_1 and H on cells does not appear to vary inversely in these cases, as would be expected if H were a precursor substance for A (see below). They confirmed the fact that the incidence of A_i is much higher in Black people (36 of 114 A types) than in Caucasians (5 of 199 A types) in a Richmond, Virginia population. Wiener (1950) had found the same thing in New York, where 5 A_i were identified in 200 bloods from Black people, and only 3 were found among 846 Caucasian bloods. Race and Sanger (1975) examined two A_i samples and found that they reacted more strongly with anti-H than do A_2 blood cells. If A_i were truly intermediate between A_1 and A_2, the anti-H reaction would be expected to be intermediate as well, and they thought that A_i might, therefore, be a distinct class of some kind. Bird (1964) reported that Moharastrian blood donors (India) showed the A_i type in about 2% of all A cells. Brain (1966) found the incidence to be almost 14% of A cells in the South African Bantu.

19.3.1.5 Additional subgroups of A. A number of other variants have been described, and named, e.g., A_{end}, A_{el}, A_{bantu}. These are discussed and references given to the sources in Race and Sanger (1975) and in Prokop and Uhlenbruck (1969). They are all very rare.

19.3.1.6 Quantitative approaches. The red cell antigen-isoagglutinin reaction has been the subject of physico-chemical and quantitative studies. The ABO system has been looked at by a number of workers in an effort to understand the subgroups. The quantitative and physico-chemical

approaches will be discussed subsequently. Gibbs and Akeroyd (1959) used a technique for evaluation of the A receptor strength which had been developed by Wilkie and Becker (1955). The latter had devised the procedure for the study of B antigen-antibody reactions, based on the studies of the Filitti-Wurmser *et al.* group (see below). If one quantitates agglutination by measuring the number of cells remaining unagglutinated under a standard set of conditions with different concentrations of antisera, and plots the percentage agglutination against the log of the antiserum concentration, a sigmoidal curve is obtained. With suitable computational transformations, the sigmoidal curve can be fitted to a line, the slope of which is related to antigen strength. Gibbs and Akeroyd (1959) found that the slope decreased (antigen strength decreased) as $A_1 > A_1B > A_2 > A_iB > A_2B > A_3$. Grundbacher (1964 and 1965) devised a hemolytic assay for antigen strength, using an immune anti-A hemolysin serum which had been absorbed with B and O cells. Under carefully controlled test conditions, hemolysis, which can be readily quantitated colorimetrically, becomes a measure of antigen strength. The results of the application of this technique to the study of A_1, A_2 and A_i were mentioned in 19.3.1.4 (Grundbacher and Summerlin, 1971). Cohen and Zuelzer (1965) used an immunofluorescence technique, and found that there was a continuum of antigen strength, running from A_1 to the weakest forms. The data, they said, supported a quantitative basis for the differences in receptor strength, and they suggested that most of the weak forms be grouped under a single designation (they suggested A_w). Wiener and Karowe (1944) suggested that the difference between A_1 and A_2 might have a structural basis. If the antigenic receptor was the same in both cases, but if in the case of A_2 it was attached to the cell surface by a shorter "stalk", as it were, i.e., not as accessible to the antibody, this might explain the weaker reactivity. The strength of the receptor is thus explained by steric hindrance to antibody binding. Carrying the argument a step further, A_2B has a weaker reaction than A_2 because the neighboring B receptors are attached by the longer (A_1-like) stalks, and can thus interfere sterically with antibody molecule approach to the A_2 receptor. This view was expressed again quite recently (Wiener and Socha, 1974). The studies of Bar-Shany *et al.* (1970) were interpreted to mean that the A_1 and A_2 reactivity differences might very well have a steric basis. As expected, steric hindrance would give rise to greater differences in reactivity on red cell surfaces than in the soluble, secreted blood group substances. Economidou *et al.* (1967) used ^{125}I-labelled antibodies to determine the number of A and B receptors in A_1, A_2, A_1B and A_2B cells. The number of A sites decreased as $A_1 > A_1B > A_2 > A_2B$. B cells had more B sites than A_1B cells. The differences in equilibrium constant and dissociation rate with A_1 and A_2 cells using anti-A were consistent with slight differences in the molecular structure of the antigen.

Cartron *et al.* (1974) determined the A antigen site density on a series of samples representing a number of the "weak A" phenotypes, including A_3, A_x, A_{end}, A_m and A_{el}. They used a rabbit IgG anti-A which was labelled with ^{125}I. Site density was found to decrease in the order $A_1 > A_2 > A_3 > A_x > A_{end} > A_m > A_{el}$. While A_3 cells had about 30,000 sites per cell, A_{el} cells had only about 700, compared with 850,000 for A_1 and 240,000 for A_2.

19.3.2 Variants of B

Variants of B are rarer than those of A, and there is considerably less literature on them. Many of the weak B types have been observed in only one or a few kindreds. As with weak A types, the relationships between the different weak-B types, given a variety of designations by different authors, are not always clear. One of the earliest observations of weak B forms was that of Moskow (1935). He distinguished what he called B_1 and B_2. Following his work, the area was not very active for a number of years, but a number of papers began appearing in the mid-1950's. The variants have been well reviewed in Prokop and Uhlenbruck (1969) and in Race and Sanger (1975), and references to the individual observations may be found in these classic works. Race and Sanger (1975) classify the B variants into three categories, based on whether the individual secretes B substance or H substance in saliva or not, and on whether there is an anti-B in the serum. Table 19.4 gives a summary of the weak B variants.

19.4 Antibodies of the ABO System

19.4.1. Anti-A and anti-B

Ordinarily, human serum contains anti-A, anti-B, or both or neither, corresponding to the antigen(s) that is (are) absent. Absence of an expected agglutinin nearly always means that there is something unusual about the person's blood. The isoantibodies develop in most cases around the age of 3–6 months, and the titer increases for a number of of years, after which it declines steadily (Morville, 1929; Thomsen and Kettel, 1929). Infant serum contains some 10% of the IgM levels found in adults, and occasionally anti-A or anti-B is present in this fraction. It was thought for a long time that the isoagglutinins of newborns were primarily derived from the mother, but this need not always be the case, because isoagglutinins against maternal cells can be detected in some cases (Toivanen and Hirvonen, 1969a). Infant serum contains IgG antibodies derived from the maternal circulation. The initiation of IgM synthesis in the newborn precedes that of IgG by several weeks, IgM biosynthesis beginning within a few days of birth (West *et al.*, 1962).

The origin of anti-A and anti-B have occupied much attention, because their "natural" occurrence in human sera appears, on the face of it, to be in violation of the fundamental immunological principle: No antibodies without an antigenic stimulus. A major discussion of this subject was given by Thomsen in 1936. Furuhata (1927) thought that the isoantibodies were under direct genetic control, and that the genes controlling them were allelic to the genes responsible for the isoagglutinogens (see in section 19.2). This

Table 19.4 Summary of Some Weak B Types

Notation	Anti-B in Serum	B in Saliva	H in Saliva	Race and Sanger Category
B_v	+	(+)[1]	−	1
B_w; B_x; B_m	−[2]	+	−	2
B_3; B_x; B_{weak}	−	−[3]	+	3

[1] Some kind of B in saliva
[2] Or weak cold anti-B
[3] Or doubtful B in saliva

idea is no longer widely accepted, but the concept of genetic control of isoantibody biosynthesis has persisted in the Japanese literature for a long time, and the issue is not completely settled. Grundbacher (1976) recently determined the anti-A and anti-B agglutinin (and hemolysin) titers in 401 group O people from 74 families. The titers were generally higher in Blacks than in Whites. Whites also generally showed higher α than β titers, and antibody levels were generally higher in women than in men. In Blacks, the agglutinin titers tended to be equal, and sex differences were not apparent. Statistical analysis of data from related persons suggested that 20 to 30 percent of the variation could be accounted for by genetic variation. Schiff and Adelsberger (1924) believed that group A people had a small amount of B present, just enough to stimulate anti-B production, but not so much as to absorb the antibody formed. The stimulating agents were termed "isoreagins". Dupont (1934) first suggested that isoantibodies do arise in response to immunization, not with red cell antigens, but with closely related antigens from various things in the environment. The A-like and B-like antigenic stimuli could come from a number of different sources, e.g., bacteria inhabiting the gut, or on dust particles which are inhaled, or on various materials ingested as food. This notion has been called the "cryptogenic immunization" theory. There is some experimental support for the idea from the work of Springer *et al.* (1959). White Leghorn chickens form an anti-B-like agglutinin for human red cells when they are quite young, but this agglutinin does not form if the chicks are kept in a germ-free environment. The idea that some external stimulus is required for the production of these antibodies is more satisfying in terms of present-day immunology. Kabat (1956) was inclined to accept the view that a stimulus is needed. Burnet's clonal selection theory (Burnet, 1959) can explain isoantibody formation. In these terms, the antibodies are understood as an immunological tolerance phenomenon. The plasma cells forming molecules that can act as anti-A or anti-B (or anti-H) are influenced very early in embryonic life by the presence of an antigen. If an antigen is present, it has the effect of destroying those cells producing antibodies to it, i.e., the antigen is recognized as "self". Thus, in an A person, the anti-A machinery is shut down very early on, and only anti-B develops. There have been other theories proposed too, which are reviewed by Prokop and Uhlenbruck (1969). The work of Filitti-Wurmser *et al.* (see below) has strongly suggested that isoantibody formation does have some sort of genetic basis, a position with which a number of authorities agree.

A distinction is often made between "natural" and "immune" antibodies, the former being present in the serum of a person who has had no known exposure to the corresponding antigen. The best examples of so-called "naturally occurring" antibodies are the iso-anti-A and anti-B, although

"natural" antibodies to other blood group system determinants can be found as well. If it turns out that the anti-A and anti-B isoantibodies are, in fact, immune, i.e., the result of exposure to antigen then the distinction will have little meaning. The distinction is based on the fact that there are differences in the serological behavior of the two kinds of antibodies. Reepmaker and van Loghem (1953) have mentioned a number of these differences, which include: (1) the immune sera have a higher titer at 37° than at 4°; (2) the immune antibodies are more thermostable; (3) immune antibodies often give a higher titer in proteinaceous or viscous media; (4) immune sera usually react weakly with A_2 cells in saline; and (5) immune antibodies are more difficult to neutralize with soluble A or B substance. When a person who already has isoantibodies is exposed to antigen by injection, by a pregnancy with an incompatible fetus, etc., the term "hyperimmunization" is sometimes applied, and the antiserum referred to as "hyperimmune". A very good discussion of natural and immune isoantibodies may be found in Mollison (1972). Lopez *et al.* (1979) studied the reactivity of IgM and IgG anti-A and anti-B toward the "weak" forms of A and B cells. A_x and B_x cells were agglutinated better by IgM than IgG antibodies, but the two types of antibodies behaved similarly with A_3, A_{end} and B_3 cells.

19.4.2 Anti-H and "Anti-O"

An understanding of anti-H and anti-O is closely connected with the problem of blood group O. From the beginning, it was wondered whether blood group O could be positively characterized, that is, whether there was an "O receptor". Isoantibodies to group O are not found in human beings, and Bernstein thought that A and B were completely dominant over O. It has been known for quite a long time, though, that O cells can be positively characterized in some way, since sera, especially animal sera, were found which preferentially agglutinated O cells. Schiff (1927b) found that some cattle serum, absorbed with AB cells, preferentially agglutinated O cells. With one fairly potent serum, he got no reaction at all with a number of examples of A, B, and AB cells—a strange result, as Prokop and Uhlenbruck (1969) pointed out, in view of our present knowledge of H substance and its relationship to A and B substances (see further below). In any case, Schiff thought that this antibody was detecting the antigenic product of Bernstein's R gene. Other animal sera, such as dog, guinea pig, cat and chicken have agglutinins acting on O cells as well (Dahr, 1938). Hooker and Anderson (1921) produced anti-O sera in rabbits by immunization with human O cells. Matta (1937) did likewise in both rabbits and goats. Jadin (1934) found that Schiff's "anti-O" reacted with A and B cells, and thought that this would provide a way of distinguishing between homozygous and heterozygous A and B types, a possibility that was also pursued by Dahr (1938) and others around the same time. Moureau (1935b) found, however, that the "anti-O" reacted with A and B cells from persons who were known to be homozygous, and he said that the serum could not distinguish AA from AO nor BB from BO. It was found, too, that some AB cells could react with the so-called "anti-O", as well as with Eisler's Shiga immune serum. Eisler (1930) had found that serum from goats immunized with Shiga bacilli had agglutinins for the bacteria as well as for human red cells. Absorption with red cells did not remove the bacterial agglutinin, but absorption with the bacilli removed all the agglutinins. The common antigen was not identical to the Forssman antigen (Eisler, 1931). Thomsen (1932) thought that erythrocytes did have a specific agglutinogen O, which was the product of the O gene. The reactions of "anti-O" with other than O cells were explained by either heterozygosity or by different degrees of dominance. Moureau (1946) again denied the possibility of detecting the genotype of A or B persons using cattle "anti-O" serum, and said that this serum was detecting not a product of the O gene, but a heterogenetic antigen which was not specific to humans. Lambert (1941) did not believe that A and B people could be genotyped using the cattle serum, and said that the O element being detected with the serum decreased progressively in cells from type O to type AB. This finding was in complete agreement with Hirszfeld's so-called "pleiade theory" (Hirszfeld and Kostuch, 1938; Hirszfeld, 1947). According to this view, O is the primary evolutionary form, and the oldest antigen. A and B forms, and their so-called subgroups, are mutants of O in various mutational stages, the final one of which is called "complete". Mutation is occurring along two separate, parallel lines, one leading toward "A complete" and the other toward "B complete". The cells of people are classified according to how much of the "residual" O is present, and those having comparable amounts constitute a "pleiade". The subgroups represent different pleiades, and the notion of subgroups, Hirszfeld suggested in 1947, should be dropped, and replaced by this idea. At the end of the mutational line, where residual O substance is absent altogether, an "anti-O" in human serum is a possibility, but since this condition is so rare, "anti-O" antibodies are very rarely found in human sera. Lambert (1941) could confirm that the "anti-O" component of the goat *Shiga dysenteriae* serum is removed by absorption with the bacteria, as is also the "anti-O" of cattle serum. The O component of human cells is thus immunologically related to the bacterial antigen, and Lambert suggested that it was in fact a heterogenetic antigen, analogous to the Forssman antigen.

In 1948, Boorman *et al.* described a serum from a "Mrs. G.", who was A, Rh+, which reacted with all O cells, many A and B cells, a few AB cells, but not at all with cells of group A_1B. The serum was thought to be specific for O and A_2. The results obtained with 500 blood samples were in close agreement with what was predicted if the serum were reacting with the product of the O gene. The animal sera, on the other hand, were thought to be detecting a different substance, which corresponds to Hirszfeld's basic factor. In consultation with Dr. Morgan, it was proposed to call the basic substance being detected by the animal sera "H substance", and the reagents which detected it "anti-H". The "H" stands for "heterophile". Morgan and Watkins (1948)

conducted a number of experiments using "Mrs. G" serum and a few other sera which had become available. In addition, a cattle serum absorbed with A_1B cells was studied, along with a rabbit immune serum prepared by injecting rabbits with a purified human H substance. This last had been obtained by Morgan and van Heyningen (1944) from ovarian cyst fluid from group O women and purified by Morgan and Waddell (1945). This purified material inhibited the reaction of O cells with a rabbit antiserum prepared against it, and it also inhibited the reaction of absorbed cattle serum with O cells. The experiments of Morgan and Watkins (1948) indicated that certain of the human anti-O sera were, in fact, reacting with the O gene product. The serum could be inhibited in its reaction with O cells by soluble substances from human ovarian cyst fluids and erythrocytes. These soluble substances were suggested to be O gene products, and were said to be secreted only rarely. Those sera which detect H, and the cattle and immune rabbit and goat sera, are inhibited by the so-called "O substance" secreted in saliva and body fluids of all secretors regardles of blood group. The specific human "anti-O" sera, however, are only inhibited by the rare soluble substance which appeared to be the O gene product. The previously designated "anti-O" sera should, therefore, be termed "anti-H", and the designation "anti-O" reserved for reagents like "Mrs. G" serum. It was suggested that Hirszfeld's theory could be modified to accommodate this-information by considering H as the primary gene which gives rise to basic H substance. H substance is then an evolutionary precursor of O, A and B substances, and H gene is in the process of mutation to pure, or complete, O, A or B. Thus, most red cells come from transitional forms, and have variable amounts of H substance on red cells.

In 1949, Grubb obtained antisera from chickens by immunization with "O substance" from group O secretors, or with Shiga bacilli, which reacted preferentially with group O red cells. These had all the characteristics of anti-H reagents, and Grubb said that these should be considered as anti-H (Grubb, 1949 and 1950). Jonsson (1944) had discovered an eel serum with a high titer for O cells, and this was one of the best anti-H reagents available, according to Grubb's results. Eel anti-H was very useful in discriminating group O secretors from nonsecretors.

There are a number of plant seed extracts which have anti-H activity, such as those of certain *Ulex*, *Cystisus*, *Lotus* and *Laburnum* species (Renkonen, 1948; Cazal and Lalurie, 1952). There are usually called "lectins". The discussion of group substances will be concluded in section 19.9, dealing with the nature and biosynthesis of these materials. Our present understanding of the biochemical genetics of the ABO system (section 19.9) makes it quite clear that the *O* gene is, in fact, silent, in the sense that it does not make a product that is responsible for the synthesis of any ABO (H) substance. However, the gene may make a product, which is immunologically related to the products of the *A* and *B* genes (see in section 19.9.3).

The material in section 19.4.2 is excellently reviewed and enlarged upon by Watkins and Morgan (1955).

19.4.3 Isoagglutinins in body fluids other than serum

In 1919, Bond carried out experiments in which he looked for isoagglutinins in a number of body fluids, but, based on some observations he had made on blood, he was operating under the assumption that drying samples down, pulverizing them and then reconstituting them, or else simply exposing them to mechanical friction had some enhancement effect on the isoagglutinin content. The results with body fluids were not very clear-cut. Kirihara (1924) reported finding isoagglutinins is pleural and pericardial fluids, but not in cerebrospinal fluid. In 1928, Yosida conducted an extensive investigation of isoagglutinins in body fluids and secretions, and found them to be present in many cases. In most examples of tears, saliva, seminal plasma, and in pleural and pericardial fluids, isoagglutinins were found. They could be found in urine too, if it were first concentrated to ⅕ to ⅐ or its original volume (Yosida, 1928a and 1928b). Schwartzmann (1928) found isoagglutinins in vaginal secretions, and in ovarian and vaginal cyst fluids. In milk and colostrum were found isoagglutinins corresponding to those in the serum. Happ (1920) said that isoagglutinins occurred in human milk and corresponded to those in the serum. Heim (1926) agreed with this finding. Hara and Wakao (1926) reported that milk and colostrum can contain a nonspecific general hemagglutinin in some cases, and they found isoagglutinins corresponding to the serum in about half the samples examined. Hirszfeld (1928) found that isoagglutinins were present in the milk of group O mothers, but lacking in about half group A or group B mothers.

Yosida's observations on the presence of isoantibodies in the saliva (1928a and 1928b) have been confirmed, but with some modifications. Some of the Japanese investigators have tended toward the view that the isoagglutinins were inherited, or that the ability to secrete them in saliva was inherited. Prokop (1961) indicated, however, that "nonsecreting" parents could have "secreting" children, and that the presence of isoantibodies in saliva is much more frequent in group O people than in A or B people. In addition, the antibodies from O subjects can be of the cross-reacting type in some cases (Prokop, 1963). Putkonen (1930) had observed that isoagglutinins were most frequent in group O salivas as well. These observations which have been confirmed by others do not support a genetic basis for the phenomenon. Boettcher (1967c) found agglutinins in a significantly higher percentage of O salivas than in A or B ones, and a higher incidence of anti-B in A_2 than in A_1 saliva. Jakobowicz *et al.* (1966) got similar results. Schlesinger and Osińka (1964) found the very same thing, and in addition, they reported that a lower percentage of pregnant women showed aggutinins in saliva. Bell and Fortwengler (1971) found anti-A and anti-B in the whole salivas of most group O males. The titer of the agglutinins increased upon immunization with A and B substances. They thought that the agglutinin activity was due primarily to secretory IgA (see

below). There was not a good overall correlation of saliva with serum titer in the same person, and it was said that the secretory system is probably independent of serum titer. As Prokop (1961) showed, there are sometimes correlations between serum and salivary agglutinin titers, so that they do not seem to be completely independent, yet people with high serum titers can lack salivary agglutinins altogether. Matsuzawa *et al.* (1972) noted agglutinins in about ⅔ of the O, A and B subjects they examined, but said this percentage is much higher if the agglutination test is carried out in colloidal media, such as PVP.

The first report of isoagglutinins in tears appears to be that of Hegner (1916). He found the antibodies in 3 of 20 people suffering from relapses of typhoid fever, but there were no agglutinins in 20 healthy people who had been immunized against typhoid, although their serum showed normal isoagglutinin activity. Putkonen (1930) found isoagglutinins in the tears of 5 out of 21 O, A and B subjects, in whom he induced the lachrymal response with onion slices or with bromacetone. Prokop *et al.* (1963) looked for isoagglutinins in tears by placing strong test cell suspensions into the subjects' eyes, and examining the lower lid area for microscopic agglutination. Isoagglutinins did not always occur, but those which did corresponded to the serum content except that, as in saliva, O persons may sometimes have either anti-A or anti-B and not both.

Isoagglutinins have been found in cervical mucus as well. Gershowitz *et al.* (1958) reported that 17 of 77 women showed isoagglutinin activity in their cervical mucus, and 15 of these were group O. This work was expanded upon (Solish *et al.*, 1961) in studies on 128 subjects. When multiple samples were collected from the same subject, the percentage showing agglutinins in at least one of the specimens was 63.4. The occurrence of antibodies was much greater in persons of group O. The presence of the agglutinins was not dependent on the phase of the menstrual cycle, nor on the secretor status of the subject with respect to blood group substances. Parish *et al.* (1967) confirmed the presence of isoantibodies in cervical mucus, and found immune type anti-A hemolysins in some samples as well.

IgA is the most abundant immunoglobulin in saliva and colostrum, in which it is dimerized and connected to a smaller "secretory" component. The anti-A and anti-B of these fluids is most probably made up of secretory IgA, which has been detected in urine and nasal mucus as well. Secretory IgA differs from serum IgA in containing the "secretory piece" (Mollison, 1972; Zmijewski and Fletcher, 1972).

19.5 Quantitative and Physicochemical Approaches

Much of the work on the physical chemistry of the isoagglutination reaction was first carried out by the Filitti-Wurmser group in France, and reported in a lengthy series of papers beginning around 1947. It could be established that the isohemagglutination reaction is a reversible, equilibrium reaction, amenable to the usual kinds of mass action and kinetic treatment. Filitti-Wurmser and Jacquot-Armand (1947) carried out a series of experiments on the agglutination of B cells by anti-B (A serum), in which a known number of cells were incubated with a constant amount of antiserum, and percentage agglutination scored by determining the number of free cells remaining after the reaction had achieved equilibrium. The same yield of agglutination was obtained when the reaction was carried out at 37°, or in two stages, the first being at 4°, and the second at 37°. Similarly, the same yield was obtained when dilution was used as a means of dissociating the agglutinates. In studies on the temperature dependence of agglutination, it was observed that the maximum number of cells agglutinated at a particular temperature varied directly with the total number of cells present, and that the number of agglutinated cells present decreased as a function of temperature between 4° and 37°. The maximum number of cells agglutinated at 4° was designated N_4, and the same value obtained at 37°, all other conditions being identical, was designated N_{37}. It could be shown that sera differ in their ability to bring about agglutination as measured by the N_4/N_{37} ratio. Anti-B sera from A_1O, A_1, A_2 and O individuals differed in this property (Filitti-Wurmser *et al.*, 1950 and 1954). In a somewhat more complicated measurement, designed to give a measure of the dissociation constant for anti-B with B cells, differences between the four different sources of anti-B were also apparent (Filitti-Wurmser *et al.*, 1952). The heats of reaction for the various combinations were determined as well, and differences in this parameter were found to be significant. The reaction is exothermic (Filitti-Wurmser *et al.*, 1952 and 1953a). Measurements conducted with the ultracentrifuge indicated that $\beta(00)$, $\beta(A_1A_1)$ and $\beta(A_1O)$ differed in s_{20} (see in section 1.1.3.4) as well, corresponding to molecular weights of about 177,000, 300,000 and 500,000, respectively (Filitti-Wurmser *et al.*, 1953a). The MW of the $\beta(A_1A_1)$ agglutinin was later revised to about 200,000 (Wurmser and Filitti-Wurmser, 1957). Other physicochemical measurements were carried out as well, all of which indicated differences between the anti-B antibodies. It could be shown further that then $\beta(A_1O)$ isoagglutinin did not behave like an equal mixture of $\beta(A_1A_1)$ and $\beta(00)$ (Filitti-Wurmser *et al.*, 1953b). These studies were reviewed by Wurmser and Filitti-Wurmser in 1957 in a paper written in English. In sum, the experiments indicated that the same isoagglutinin, with the same blood group specificity, differed considerably in its properties depending upon the genotype of the person from whom it was derived. Furthermore, the isoantibodies from persons of a given genotype showed considerable homogeneity in the studies, while it is known that antibodies obtained by immunizing animals show a heterogeneous antibody composition in the resulting antiserum. Kabat (1956) levelled a number of criticisms at the studies of the Filitti-Wurmser *et al.* group, based on some consultation he had had with physical chemists. Reasons were given why some of the data might be deceptive

because of the conditions under which the measurements were done, and thus, why it could have been misinterpreted. Filitti-Wurmser *et al.* did not accept most of the criticism of their results, and replied to it in a paper in 1960.

As noted above (section 19.3.1.5), Wilkie and Becker (1955) devised a modified quantitative hemagglutination assay based on the work of Filitti-Wurmser *et al.*, but which was regarded as simpler. This procedure can be used to test relative antigen strength with a particular antiserum, as was done by Gibbs and Akeroyd (1959) and discussed above. If percentage agglutination in a hemagglutinating system is plotted against antiserum concentration, a sigmoidal curve, not unlike a pH titration curve, is obtained. Since the curves do not always have the same shape, and present difficult computational problems, the data are usually transformed to give a straight line. The linear transformation is accomplished by converting the original function, percent agglutination, to some other function, usually logit or probit, and plotting this against the log of serum concentration. From such lines, the 50% agglutination value can be read or calculated, and the probit transformation is often preferred because it gives a line with the least statistical error. Using this kind of analysis, antisera may be compared as to their relative strength with certain cells, or else a series of cells could be compared using a particular antiserum, as for instance, the comparison of a series of a subtypes with an anti-A serum. The "50% hemagglutinating dose", as it is usually called (abbreviated HD_{50}), may be compared as simple ratios of a standard value to unknown values. Antiserum concentration is often expressed as "mℓ undiluted antiserum/mℓ final dilution". The reciprocal of the HD_{50} can be taken as a measure of antiserum strength. If, for instance, 0.01 mℓ antiserum in 1 mℓ final volume gives 50% agglutination, then one mℓ of that serum may be said to contain 100 HD_{50} units. Another way of expressing relative differences between standard and test samples is to determine the difference in the HD_{50} values and take its logarithm to the base 2. A $\log_2$ difference of 1.0 may be understood as a difference of one full serum dilution, or, in more familiar serological jargon, as a "one tube difference" with the usual doubling dilution technique. it may also be noted that, as a consequence of the sigmoidal dependence of serum concentration on percent agglutination, the percentage agglutination is obviously most sensitive to changes in the agglutinin concentration in the region of 50% agglutination (i.e., the "steep" part of the curve). Experiments at constant antiserum concentration, where maximal sensitivity is wanted, should, therefore, be carried out at serum concentrations close to HD_{50} value. An excellent and understandable review of this material may be found in Solomon *et al.* (1965).

The studies of Salmon and collaborators should be mentioned here. They have employed quantitative hemagglutination methods, essentially those of the Filitti-Wurmser group, to the study of subgroups. The results have given rise to somewhat different designations of the subgroups. In a series of rare weak B variants, for example, the percent cells agglutinated by a constant concentration of a particular anti-B is compared to the same parameter for a "normal" B (Salmon and Reviron, 1964; Salmon *et al.*, 1964). The variants are designated by numbers, representing those percentages relative to an ordinary B, which is set at 100%. Thus, an ordinary B is B_{100}, and the variants are B_{80}, B_{60}, etc. This kind of analysis is thought to be more precise, and the designations more informative than the more descriptive usages. Similar analysis has been applied to weak A types, as for example the case of an $A_{1(80)}$ described by Salmon *et al.* (1965). For reviews of this work, see Salmon (1965 and 1969) and Salmon *et al.* (1973). It may be noted, too, that these quantitatively-based designations fit in well with Hirszfeld's pleiade theory, discussed in section 19.4.2.

19.6 The Bombay Phenotype

In 1952, Bhende *et al.* described the blood of three people in Bombay, which possessed a most peculiar set of ABO system characteristics. The serum agglutinated every sort of cell tested with it, and was shown to contain anti-A, anti-A_1, anti-B and anti-H. Although they were first thought to be of group O, their cells were not agglutinated by any of the usual reagents, and the cells did not react like cells of any of the four basic blood groups. They did not secrete A, B, H, or Le^b in saliva, but they did secrete Le^a. The Lewis system will be discussed separately. It was thought that these people had some new rare allele at the ABH locus. Ceppellini *et al.* (1952) speculated that the explanation for this phenomenon might lie in inhibitory genes, operating at a different locus. Ceppellini's idea was shown to be fully applicable to a family described in 1955 by Levine *et al.* (1955b). The proposita was an American of Italian descent. Her blood behaved like the Bombay bloods, but the pedigree clearly showed that she possessed an unexpressed normal B gene. It was suggested that the phenotype first described in the Bombay persons be called "O_h", and this usage has persisted. In 1955, Watkins and Morgan wrote with considerable foresight that the presence of H substance might be under the control of an independent allelic pair of genes, *H* and *h*, and that the Bombay type bloods were rare examples of homozygous recessivity at this locus. With the support of the biochemical studies which have since been done, this notion has indeed become the basis of the current understanding of the ABO system. The subject will be returned to in a subsequent section. Further examples of O_h phenotypes were soon found, and it became clear that they could be manifestations of A_1 or A_2 suppression, as well as of B or H suppression. In cases where it could be discovered which allele was suppressed (through family studies), a superscript was added to the O_h designation. The person described above by Levine *et al.* (1955b) would thus be an O_h^B. Lanset *et al.* (1966) demonstrated in a French family with O_h members that the suppressed allele could be detected on red cells by absorption-elution technique. In this way, they detected an O_h^B and three O_h^{AB} people in a family without recourse to family studies. These results and others have to mean that the ABH substances are present on the red cell, in accordance with the genotype, in

some form, even though agglutination with anti-A, anti-B and anti-H cannot be observed.

Rare examples of bloods have been observed which behave in some ways like O_h, but are not identical. These have been referred to by Race and Sanger (1975) as "Para-Bombay phenotypes." The first example of this kind of blood was described by Levine *et al.* (1961d) in a healthy, young Czechoslovakian woman with no history of pregnancy or transfusion. Her cells grouped as A_2, and the serum contained anti-A_1, anti-B and anti-H. Her red cells were anti-H negative, but Le(a+). Le^a was secreted in saliva, but A, B and H were not. This case was interpreted as one of an incompletely suppressed A by a suppressor gene, and it was suggested that the phenotype be called "A_h" by analogy to O_h. If a case of an incompletely suppressed B were found, Levine *et al.* (1961d) said that it should be called "B_h". Examples of B_h have since been described (see Kitihama *et al.*, 1967). Cases are known, too, where cells of phenotype O or A_x did not react with anti-H, but the group substances were secreted in saliva. These have been designated O_m^h or A_m^h. It has been suggested that persons with suppressed red cell antigens, but with group substances present in saliva, be designated instead as O_{Hm}, O_{Hm}^A, O_{Hm}^B and O_{Hm}^{AB}. Race and Sanger (1975) agree with this newer notation, and additional information on these kinds of cases may be found in their discussion.

19.7 Some Other Complexities in the ABO System

19.7.1 So-called "Cis-AB"

In 1964, Seyfried *et al.* reported on a family in which an A_2B daughter of an O mother (father not tested) was married to an O man, and had two A_2B children. These were not ordinary A_2B types, in that the B was weak, and the serum contained some kind of anti-B (not autoagglutinating). A and weak B were secreted in the saliva of these people. Paternity could not be excluded by other systems, and there was every reason to believe that the children were legitimate. Obviously, A and B had been inherited in this family from a single parent. Another family was reported in Japan (Yamaguchi *et al.*, 1965) in which an A_2 and a weak B antigen (called A_2B_3) had been inherited from a single parent. A note was added to this report by Professor Komai, saying that the B gene was weakened in these cases by a position effect, the B gene giving a weaker antigen when in the "cis" position (AB/O) than in the usual "trans" position (A/B). The slash in these parenthetical designations stands for the homologous chromosome pair, and it is clear that a designation like "AB/O" carries with it the assumption that the ABO locus is complex, i.e, that A and B genes are not precisely allelic. Professor Komai had expressed this view in 1950 with respect to both the ABO and the Rh systems. There is ample precedent in classical genetics for very closely linked genes in *Drosophila* and in other organisms. Loci of this kind have been termed "semi-allelic," "partially allelic" and "pseudoallelic." The meaning is the same whatever the term used. Such loci, when carefully studied, turn out to consist of a series of very closely linked "alleles," hereditary units which are adjacent to one another on the chromosome. Crossing over among these "pseudoalleles" does occur; that is the way in which they are detected. Crossing over at such loci is, of course, exceedingly rare. Another family exhibiting the inheritance of A and B from one parent was described by Yamaguchi *et al.* (1966). An A_2B_3 mother and an O father had three A_2B_3 children. These workers referred to this unusual condition, following Professor Komai's suggestion, as "cis AB," in contrast to the usual "trans" AB. A number of "cis AB" A_2B bloods have been reported. They seem to be more common in Japan than in Europe (see Yamaguchi *et al.*, 1970). Reviron *et al.* (1967 and 1968) reported the first cis AB blood that was A_1B. Their results indicated that the B antigen in the cis A_1B complex is part of the normal B antigen. The anti-B in such people is directed against the remaining part of the antigen. Boettcher (1966) discussed modifying the original Bernstein hypothesis in terms of looking at ABO as a pseudoallelic locus, although he did not use that term. One of the possibilities he put forth was that the "alleles" A, A_1, and B are really pseudoalleles. This idea can satisfactorily explain cis A_2B and cis A_1B types by recombination. Understood in terms of what we now know about biochemical genetics, we would say that the ABO locus is "polycistronic." This explanation is very satisfying in biochemical genetic terms. It would be even more satisfying if the relationship between the gene (cistronic) product and the ABO antigen were a little clearer. It is to be noted that the phenomenon can be explained by postulating a rare allele, which gives rise to an antigen having A_1 or A_2 with weak B determinants, but, as Race and Sanger (1975) have rightly noted, this latter explanation is the far less exciting of the two. Finally, Moullec and Chevrel (1959) had reported a family in which an AB with a weak B and some kind of anti-B in serum was segregating in four generations. It seems probable in hindsight that these persons were of the cis AB phenotype. Valdes *et al.* (1978) reported an interesting case from Illinois, in which an A_2B mother had two group O children. She was apparently cis-AB, but with a normal B antigen, and no anti-B in her serum.

Badet *et al.* (1978) have studied the transferase activity (gene product activity) in the sera of a number of cis-AB people and their families (see in section 19.9). The transferase activities differed from those of ordinary people, and from one family to another, but tended to be similar within a family. They favored the explanation that cis-AB is the result of a mutation leading to an enzyme which could transfer both Gal and NAc-Gal-NH_2.

19.7.2 Cross reacting anti-A- and anti-B-like antibodies in group O serum—blood group C

Absorption of A cells with group O serum, and subsequent elution, yields an antibody which reacts with B cells in addition to the expected anti-A. Hektoen seems to have been observing this phenomenon in 1907 (see in section 19.1).

Landsteiner and Witt (1926) described it in detail for the first time. Thus, A cells yielded, upon elution, not only the expected anti-A, but also an antibody that reacted with B cells, after having been placed into group O sera. Similarly, B cells will yield up the expected anti-B, but some antibody that reacts with A cells as well. Dodd (1952) studied this phenomenon, and suggested that certain of the antibody molecules in O sera might have both A and B specificities. It was also found that immune sera, i.e., those O sera having an anti-A or an anti-B as the result of a known frank immunization, are a richer source of the cross-reacting antibodies. Bird (1953) largely agreed with Dodd's explanation and termed the behavior "dual receptor" antibody structure. Bird (1953 and 1954) also drew attention to the often observed asymmetry of the reaction, i.e, that with some O sera, A cells may absorb and yield anti-B upon elution, whereas B cells will not absorb and yield up any anti-A that is detectable. Equal mixtures of anti-A and anti-B do not exhibit the cross-reacting behavior. In 1955, Rosenfield noted that in a large sample, most mothers of incompatible infants were of group O, and it could be shown that the cross-reacting antibody crossed the placenta more easily than do ordinary anti-A from B serum or anti-B from A serum (Rosenfield and Ohno, 1955).

There is no completely satisfactory explanation for these observations, and there is certainly not general agreement on an explanation. The different hypotheses that have been given to account for the observations can be conveniently grouped into four categories (Race and Sanger, 1975). The explanation noted above, that some of the antibody molecules in group O sera can have anti-A as well as anti-B specificities, was put forth by Dodd (1952) and supported by Bird (1953 and 1954). Dodd (1957) gave further experimental support for the view, indicating that the cross-reacting antibodies (which were called anti-AB) showed dual specificity with animal cells possessing A- or B-like receptors. Jones and Kaneb supported this view as well, using a different technique designed to detect a minor population of red cells in a mixture. This technique was first applied to Rh antigen studies, and later to ABO. As an example, suppose one wanted to detect a minor population of A cells in a mixture of many O cells. Detector cells consisting of a cells sensitized with anti-A, but not agglutinated, would be added to the mixture. Agglutination upon addition would mean that A cells had been present in the mixture (Jones and Kaneb, 1959). The technique could be used to titrate the cross-reacting antibodies in O serum without absorption and elution procedure by linking A cells to B cells with antibodies in group O serum (Jones *et al,* 1959). The technique was applied to the study of cross-reacting antibodies (Jones and Kaneb, 1960) and they appeared to be reacting with an antigen homologous to A and B. The asymmetric behavior of the cross reacting antibodies was also noted.

A second explanation of the observations postulates the existence of a third antibody in group O serum, usually called anti-C, along with the notion that A, B and AB cells have the corresponding C agglutinogen. Moss (1910a and 1910b) formulated an explanation of the ABO system with three agglutinogens and three agglutinins, but the view was gradually abandoned as subgroups of A were disclosed. Koeckert (1920) did not agree with the Moss scheme based on his experiments. A number of the Japanese investigators have accepted the idea of a third agglutinogen-agglutinin pair for quite some time (Furuhata and Matsunaga, 1950). They believed that the isoagglutinins, including anti-C, were inherited as well. In this country, Wiener has supported the idea. It must be noted that Wiener had a slightly different immunological conception of the system (and of other systems, too, such as Rh and MN) than do others, and he has employed nomenclature devices to keep the distinctions clear. An "agglutinogen" is a definable substance on the red cell surface, and these are designated by ordinary Roman capital letters. Agglutinogens are antigenic, but they may have multiple antigenic determinants, i.e., they may give rise to more than one kind of agglutinin if injected into an animal. The individual antigenic specificities associated with an agglutinogen are called "blood factors," and are denoted by upper case boldface Roman letters. Agglutinogens in Wiener's view, therefore, are or can be complex antigens. Blood factors are not definite substances, but may be particular intramolecular arrangements which, under a specified set of conditions, are bound by an antibody recognizing the constellation and causing agglutination. Since the antibodies are regarded as being against the blood cell factors, they are designated with the upper case bold face symbols (anti-**A**, anti-**B**, etc.). The *genes* which code for the agglutinogens are denoted by *italic type,* as are genotypes. Wiener's conception of the ABO system is summarized in Table 19.5. According to this, O serum does not contain "cross-reacting" antibody, but anti-C, which reacts with the C blood factor in A, B and AB cells. The scheme has further implications. One can imagine four additional ABO blood groups, as indicated in Table 19.6. At the time when the first major paper on the subject came out (Wiener, 1952), Wiener thought that an example of blood group C (first row of Table 19.6) had been observed by Dunsford and Aspinall (1952). They had called their blood sample an A_4 (it is discussed in section 19.3.1.3), but Wiener said that the blood possessed all the features of a group C, i.e, the serum contained anti-A and anti-B while the cells reacted like group O to ordinary grouping sera. Her cells were strongly agglutinated by all group O sera, however. Some agglutination was seen with potent anti-A reagents. As for the hypothetical blood groups, Wiener said that they should be readily recognizable if encountered. The difficulty with anti-C was in obtaining it separately from anti-A and anti-B (Wiener *et al.,* 1953c). In 1973, Wiener *et al.* said that sera containing anti-C and anti-A could be produced by frank immunization of O persons with blood group A substance originating from pigs. Because of the anti-A content, these reagents were said to be useful for testing cells which had C but not A. Cells having A and C would have to be tested with a serum prepared by immunization of O people with B substance. Jones and Kaneb

Table 19.5 ABO System as Conceived by Wiener

Blood Group	Genotype	Red Blood Cell Agglutinogen	Red Blood Cell Factors	Serum Isoantibodies
O	*OO*	–	–	anti-A, anti-B, anti-C
A	*AA*	A	A + C	anti-B
	AO	A + O	A + C + O	anti-B
B	*BB*	B	B + C	anti-A
	BO	B + O	B + C + O	anti-A
AB	*AB*	A + B	A + B + C	None

(1960) thought that if the cross-reacting antibody were, in fact, a third agglutinin anti-C, that it should behave symmetrically toward A and B cells, and they used the asymmetric behavior as an argument against blood group C. Bird (1954) noted, however, that the asymmetry could be explained on the basis of differences in titer of the various antibodies in serum, and could be accommodated by the C/anti-C hypothesis. At the time, he did not regard blood group C as having been established, however.

Kabat (1956) stated a third explanation, based in part on the experiments and discussion of Owen (1954). Since there are close similarities in structure between A, B and O substances, then an individual of group O might form some antibodies of anti-A and anti-B specificity which recognize molecular constellations common to both A and B. Kabat believed this to be the most attractive explanation, and the most satisfying to an immunochemist. Schiffman and Howe (1965) agreed with this explanation. Franks and Liske (1968) interpreted the results of their studies on mixed agglutination between red cells and buccal epithelial cells with cross-reacting antibodies, and its inhibition by soluble A and B substances, to mean that the cross reacting antibody was recognizing a common portion of the A and B antigens, and that it had a lower affinity for A and B sites than do anti-A and anti-B from B and A people.

The fourth explanation is based on the observation that antibodies are said to be able to be absorbed in a nonspecific way by an antigen-antibody complex (Ogata and Matuhasi, 1960). Thus, β can be taken up, and subsequently eluted, from A cells to which anti-A is already bound, and conversely for α. Ogata and Matuhasi say that an equal mixture of anti-A and anti-B behaves exactly like the anti-A,B of an O serum in their experiments. The existence of cross-reacting antibodies is denied (Ogata and Matuhasi, 1962). The non-specific absorption has been called the Ogata-Matuhasi phenomenon. The existence of blood group C is denied by these authors on the basis of experiments in which fluorescent A or B cells do not form mixed agglutinates in the presence of anti-A,B, which, they say, should occur if a third agglutinogen were in fact present. The non-specific uptake of blood group antibodies by cells coated with specific antibody has been confirmed by Bove *et al.* (1973) using ^{125}I and ^{131}I labelled antibodies, but the coating of the cells with specific antibodies did not increase the non-specific uptake. While this study shed considerable light upon the Ogata-Matuhasi phenomenon, it did not specifically deal with the phenomena being discussed in this section.

Dodd *et al.* (1967) have shown that the "cross-reacting" antibody obtained from O mothers who have had A children is different from the one obtained from O mothers who have had B children. This must indicate that there are two types of cross-reacting antibodies, and they said that these appeared to be directed at a common portion of the A and B antigens, which includes the C-2 substituent of the terminal sugar (see further on in the discussion of the biosynthesis of ABH substances in section 19.9). Lincoln and Dodd (1969) have reiterated this theme in their subsequent studies on the phenomenon.

Table 19.6 Additional Blood Groups Predicted by the C/anti-C Hypothesis

Blood Group	Red Blood Cell Agglutinogen(s)	Red Blood Cell Factors(s)	Serum Isoantibodies
C	C	C	anti-A + anti-B
A′	A′	A	anti-B + anti-C
B′	B′	B	anti-A + anti-C
A′B′	A′ + B′	A + B	anti-C

19.7.3 Acquired B

Although acquired B appears to be a rare event, it is an important one in that it illustrates the close similarity of bacterial cell wall structures to that of the ABH blood group substances. Red cells, almost always in group A_1 people, can "acquire" B under certain conditions of old age or disease. The cells then type as A, B weak.

In 1959, Cameron *et al.* reported on seven bloods they had been examining over the course of four years. The people were all genotypically A or A_1O, and suffered from a variety of diseases, often cancer of the colon or rectum, or they were quite old, or both. Their serum had the expected anti-B, and the secretors secreted A and H but not B. The cells were agglutinated, though, by some anti-B reagents. It was suggested that the B-like receptor on the cells was acquired, not inherited, and it was noted as being significant that none of the seven were of group O. Giles *et al.* (1959) reported a completely similar case in a patient with carcinoma of the colon who was A_1A_2. Marsh *et al.* (1959) described a group A cancer patient who had acquired B (which was referred to as "pseudo B"). The B reactions had all but disappeared when the cells of this person were examined postmortem. They noted that a "pseudo B" could be produced *in vitro* by allowing cells to react with a bacterial enzyme (T-activating enzyme). This treatment rendered the cells polyagglutinable, so that the presence of the B-like antigen had to be detected by absorption and elution.

In 1956, Springer had noted that certain extracts of higher and lower plants contained substances that were A-like, B-like or H-like, in that they could inhibit the isohemagglutination reactions in much the same way as the secreted blood group substances. *Escherichia coli* O_{86} was found to possess a high B-like activity. It soon became clear that the B-like activity in the *E. coli* O_{86}:B7 resided in an extractable lipopolysaccharide fraction (Williamson and Springer, 1959). The soluble B-like substance from *E. coli,* as well as from a variety of other microorganisms, could be found in the culture media. Furthermore, these soluble substances, as well as the partially purified lipopolysaccharide and protein-lipopolysaccharide fractions from *E. coli* O_{86}:B7, could coat human red cells, thus rendering them sensitive to anti-B. The treatment did render the cells polyagglutinable, but the B-like activity could be detected by absorption and elution, and the treatment had no effect upon the genetically determined blood group antigen present on the cells. Both O and A cells could be coated (Springer and Ansell, 1960; Springer and Ansell-Hahn, 1960). Stratton and Renton (1959) had noted, in reporting a case of

acquired B by an A_1 cancer patient, that red cells take up *E. coli* polysaccharide *in vitro*, and are thus rendered B-like. They suggested that this was probably the mechanism of the acquired B phenomenon. Springer and Ansell-Hahn (1960) noted that some coating of red cells by the *E. coli* substances had occurred *in vivo* in sick children, but they thought that there might be some plasma factors which inhibit such uptake in healthy people. It is known that *E. coli* O_{86}:B7 have an A-like antigen as well as the B-like one (Pettenkofer *et al.*, 1960; Gonano *et al.*, 1961), but there are no cases of "acquired A." It is also quite well established (Springer and Ansell, 1960; Springer and Ansell-Hahn, 1960) that the B-like substances can coat O cells as well as A cells *in vitro*. Marsh (1960) could produce a B activity in A or O cells *in vitro* with a potent T-activating enzyme of bacterial origin, prepared by Dr. Friedenreich. The B-producing activity was separable from the T-producing activity, and the two had different thermostability properties. There have not, however, been reported cases of acquired B in persons of group O. The majority of acquired B cases were of group A_1, with a few being A_2. Marsh (1970) noted that A_1 cells, transfused into an A_1 individual who had acquired B, and then recovered, had themselves acquired the B within 48 hours. The transfused cells were recovered, incidentally, by using cells of a different MN type. If O cells were transfused into the same subject, and recovered, however, there was no acquired B antigen.

Iseki (1977) gave an excellent discussion of the ABH-like substances in living organisms other than human beings. Many vertebrate red cells react with anti-A, anti-B and/or anti-H, as demonstrated by Tumosa (1977a).

It can be said that acquired B is most often associated with old age and/or disease, frequently carcinoma, that it usually occurs in A_1 people, and that it is very rare *in vivo*. To clinical serologists, and those who deal with fresh blood, acquired B is primarily an instructive curiosity, seldom if ever encountered. In forensic blood grouping, and especially in body fluid grouping work, however, "acquired B" has a somewhat different meaning (at least in terms of coming about *in vitro* rather than *in vivo*) and must be taken much more seriously. This matter will be taken up under the heading of medico-legal applications of the ABO system (section 19.10).

19.8 The Secretor System

19.8.1 Group specific substances in body fluids

The occurrence of soluble A, B and H group specific substances in body fluids was recognized clearly in the 1920's. It was later found that the presence of these materials in body fluids is under discreet genetic control. In most populations, the majority of people are "secretors," having substantial amounts of soluble group specific substances in their body fluids. The "non-secretors" do not have the large amounts of these substances in their body fluids. The secretion property has had broad implications for the understanding of the ABO system, as will be discussed in the section on the nature and biosynthesis of the ABH substances. Since body fluids other than blood are often encountered in criminal cases, the presence of group specific substances in them has important medico-legal ramifications as well. Further, since the secretor property is inherited, it may be used as an additional genetic marker in cases of disputed affiliation. The medico-legal aspects are discussed in a subsequent section.

Credit for the first recognition of group specific substances in body fluids other than blood belongs to the Japanese investigator Saburo Sirai in 1925. He was mentioned in this regard by K. -I. Yosida (1928b). The reference to Sirai's studies is: *Hokkaido Igaku Zasshi* 3(2):25-73 (1925). Schiff (1924b) had observed soluble A substances in the serum of A and AB people by noting that anti-A red cell serum from rabbits gave a precipitin reaction and a positive complement fixation test with these sera. Dervieux (1921 and 1923) had done some experiments in which he showed that an anti-human semen serum gave a stronger precipitation reaction with the seminal plasma against which the antiserum had been raised than with samples from other individuals (see in Section 10.5.1). Sussmann (1925) could not confirm this finding, nor several other assertions that Dervieux had made. He did notice some individual-specific serological differences in seminal plasma, though, and speculated on the possible presence of group specific substances. In 1926, Yamakami established that seminal plasma, as well as saliva and vaginal secretions, contained group specific substances. Seminal plasma from a group A individual could inhibit anti-A activity in either O or B serum, and had no effect on A serum. Group AB semen could inhibit both anti-A and anti-B activity. Yamakami said that saline extracts of four month old dried seminal stains had the same specific hemagglutination inhibition properties, and that this fact should be significant for medico-legal investigations. Landsteiner and Levine (1926a) tested spermatozoa for the group specific substances, and found that they were present, and corresponded to the red cell group. The observations on seminal plasma were confirmed by Krainskaja-Ignatowa (1929) and by Steusing (1930). Brahn and Schiff (1929) found group specific substances in saliva, in stomach and duodenal material, and in urine if it were concentrated and dialyzed. Yosida (1928a and 1928b) confirmed the presence of the group specific substances in a wide variety of secretions, including saliva, semen, tears and pleural and pericardial fluids. Likewise, Thomsen (1930a and 1930b) reported group-specific substances in serum, organ cells, especially tumor cells, leucocytes and in urine if it were concentrated. Brahn and Schiff (1929) detected the A and B substances in human milk as well. In 1930, Lehrs conducted extensive studies on the group specific characteristics of saliva, working under the direction of Professor Schiff. The saliva of 40 different individuals from all four blood groups was studied by the inhibition technique. On occasion, O saliva slightly inhibited anti-A and anti-B, but in most cases not at all. Inhibition of the A reaction by A saliva, and the B reaction by B saliva, was shown to be quite strong and

completely group specific. Saliva from AB persons inhibited both reactions. There were considerable differences in the group specific substance content of saliva from different persons, as indicated by the differing degree of inhibition observed by titrating the antiserum against constant amounts of saliva, or by titrating the saliva with constant amounts of antisera. In a few cases, inhibition was very weak (the people were probably nonsecretors). There seem to have been fewer non-secretors in the population he studied than would be expected on the basis of what we now know about their distribution in the population. There was little variation in the same individual over about 5 months time. Immunization with saliva produced the corresponding group specific immune agglutinin.

Another major study on group specific characteristics in body fluids was carried out by Putkonen in 1930. Studies were conducted on the presence of isoaggutinins in body fluids (see in section 19.4.3). The presence of isoagglutinogens was established in saliva, urine, tears, semen and in the amniotic fluid (corresponding to the blood group of the child). The tests were done quantitatively, either with doubling dilution titration of antisera at constant body fluids concentration, or conversely. Serum dilutions were carried out to 1:512, and body fluid dilutions were carried out to as much as 1:4000 in some cases. The group specific substance content varied among different individuals. Further, it tended to be high in saliva, semen and amniotic fluid, low in tears and urine. Some variation was noted in samples collected from the same individual at different times. In about 14% of A, B and AB people, group specific substances were absent. In AB people, a majority showed similar amounts of A and B, but in six cases out of 32, the A and B content was unequal. Gibb (1965), using an improved technique, noted that there is more group specific substance in A and B secretor urine that Putkonen had thought. There were difficulties in assessing group O.

Likewise in 1930, another major study was published by Schiff in Berlin. The presence of group substances in urine, saliva, gastric and duodenal juices, bile, milk and in a number of organs was confirmed. Schiff concentrated on studying the nature of the group substances as well. Most of the secretions and organs contained these materials in water soluble form. But alcohol soluble fractions could be found in red cells and in a number of organs. The water soluble group substances from saliva and urine particularly were thermostable and nondialyzable. They did not give positive reactions with simple chemical tests for protein, carbohydrate or "lipoid". Both water- and alcohol-soluble fractions of A substance inhibited A-specific hemolysis, but they showed differences in behavior in the complement fixation test. It was not clear whether the two fractions were chemically different, or whether the differences reflected the chemical environments in which they were located.

In 1937, Friedenreich put forward the idea that group substances in the body fluids were synthesized locally, in the cells of the organ secreting them. A "nonsecretor" individual was one in whom this synthesis did not take place. This notion contradicted Schiff's original idea that the group substances were present on red cells in alcohol-soluble form and that they were excreted into body fluids, being altered to a water-soluble form in the process. High concentrations of water soluble group substances were found in saliva, gastric juices, bile and seminal plasma, and in the mucous membranes and glands of the digestive tract in Friedenreich's studies. The parotid gland, liver and colon had relatively little group substance, and the lowest concentrations were found in brain and testis. The alcohol soluble antigen content of various organs was studied, and the results indicated that the alcohol soluble substances were quite distinct from the water soluble ones. Secretions do not have the alcohol-soluble substances. The content of alcohol soluble group substance in organs is independent of secretor status, and was said to be independent, therefore, of the secretor system. Friedenreich and Hartmann (1938) carried on these studies. They tested a variety of body fluids for antigen content (the highest doubling dilution of the body fluid which would inhibit an appropriate antiserum with a titer of 1:4), and they tested serum for group specific substances as well. The idea behind the experiments was to see whether there was enough A, B or H substances in circulation to account for their presence in body fluids by simple transfer, and there was clearly not enough. The inevitable conclusion was that the group substances in body fluids had to be synthesized by the cells secreting these fluids. A number of organs from corpses were investigated in this study, and a correlation was found between the group substance content of an organ and the content in its secretion. Studies on this subject were pursued by Grethe Hartmann, and the results communicated in a lengthy monograph (Hartmann, 1941).

19.8.2 Inheritance of the secretor characteristic

By 1932, it was clear from the work of Brahn and Schiff (1929), Putkonen (1930), Sasaki (1932) and others, that the secretion characteristic was a relatively consistent feature in an individual, but that there were individuals within all the blood groups in whom group substances appeared to be absent in body fluids. In 1932, Schiff and Sasaki established that the trait was indeed hereditary. "If one chooses an experimental arrangement which deliberately neglects minor differences, one obtains two sharply distinct types for the A and also for the B secretors and nonsecretors," they wrote. Similar behavior was found with AB people, and, using cattle serum absorbed with AB cells (which they called anti-O, but which we would now call anti-H—see in section 19.4.2), a secretor-nonsecretor distinction could readily be observed in group O salivas as well. In 50 families, it was clear that secretor children had to have at least one secretor parent, and that nonsecretor parents did not ever have secretor children. In 51 pairs of twins, of whom 21 were identical, 7 discordant pairs were found, and these exclusively in fraternal twins. The property of secreting was dominant, and designated "S", while its allele was called "s". *SS* and *Ss* persons are secretors; *ss* persons are nonsecretors. The original German words for secretor and nonsecretor

(*Ausscheider* and *Nichtausscheider*) have sometimes been translated as "eliminator" and "noneliminator" in the older literature. Among the 369 people studied, 30.9% were nonsecretors. The gene frequencies were 0.444 for *S*, and 0.556 for *s*. Additional family studies over the years which followed fully confirmed the mode of inheritance of the secretor characteristic postulated by Schiff and Sasaki. Wiener (1943e) had collected together the data of a number of other workers, including Schiff and Sasaki, for 185 families with 486 children. Of these, 18 families showed the informative nonsecretor x nonsecretor mating, and all 42 children were nonsecretors. Andersen (1951) studied 154 matings with 370 children, this material containing six nonsecretor x nonsecretor marriages, and fully confirmed the mode of inheritance. Prokop and Uhlenbruck (1969) collected the data from 252 additional families and these results were also in complete accord with the postulated inheritance pattern. They gave the references to the family studies of others. They also reported another study by Kerde in 1961 on 105 additional families with 3 informative matings. The designations *S* and *s* were originally applied to the secretor genes. This usage persisted in the literature for about 20 years. In 1947, a new blood group factor was discovered by Walsh and Montgomery in Australia, and found to be associated with the MN system. The new anti-body was named anti-S by Sanger and Race (1947) after some consultation with Prof. Fisher and Dr. Ford (see Section 21.2). Race and Sanger (1950) said that in assigning the symbol "S" to the new MN-associated factor, they had simply overlooked the fact that the designation was being used for "secretor". The duplication apparently caused no confusion, but in 1955, Levine *et al.* called the secretor alleles "*Se*" and "*se*" (Levine *et al.*, 1955b), Race and Sanger adopted this usage in 1958, and it has persisted since that time. It is now known that the *Se/se* locus is linked to the Lutheran blood group system locus. The linkage was discovered by Mohr (1951a and 1951b) who thought, however, that *Lu* was linked to Lewis. The relationship of the Lewis system to secretion will be discussed in the section on the Lewis system. The *Lu:Se* linkage has since been confirmed (Greenwalt, 1961; Lewis *et al.*, 1977).

19.8.3 Further studies on group substances in body fluids

19.8.3.1 Saliva. Considerable attention has been focused on group substances in saliva because it is so easy to obtain. Once it had been recognized that the secretor characteristic had a definite genetic basis, there was interest in using it as a genetic marker. A number of the studies which have been carried out indicate that the secretor characteristic may not be quite as simple and straightforward as it first seemed to be, at least in some cases. It is quite likely too that variations in findings from different laboratories may be attributable, at least in part, to differences in technique.

Wiener and Kosofsky (1941a and 1941b) conducted a series of quantitative studies on A and B group substances in saliva. They showed that the inhibition characteristics of secretor salivas were very much dependent on the antiserum being used. This fact was observed with anti-A as well as with anti-B reagents. There was little variation in group substance content from secretor saliva of the same person over time, and not very much difference from one secretor to another. Little difference was found in the amount of A secreted in saliva by persons whose cells were of subgroups A_1, A_2 and A_3. Gammelgaard (1942) was not in agreement with these findings, saying that the amount of A substance secreted in saliva decreased in going to weaker and weaker A subgroups. Secretion was so low in A_4 individuals, he said, that secretor status could not be reliably determined.

McNeil *et al.* (1957a) raised the point that there are some people of blood groups A or B who secrete A or B but no H (as detected by *Lotus tetragonolobus* lectin), while others of group A_1, and especially of group A_2, secrete H but fail to secrete an A which will inhibit the anti-A reaction with A_1 cells. Such persons were termed "aberrant secretors". A substantial number of them were noted among couples in which the women had experienced spontaneous abortions caused by blood group incompatibility (McNeil *et al.*, 1957b). McNeil *et al.* (1960) pointed out that a number of A_2B people do not secrete detectable amounts of A_1 substance in saliva, but do secrete B. Bhatia and Randeria (1970) found the same thing in their study of 194 individuals representing all the blood groups. They used the term "aberrant secretor" to apply to secretors of A and/or B without any H secretion. It was noted that the detection of H in these people depended to a great extent on the anti-H reagent being used. The studies were extended (Randeria and Bhatia, 1971). The lectins from *Psophocarpus tetragenolobus* and *Erythrina subrosa* were very poor anti-H reagents in the inhibition test. The lectins from *Ulex, Laburnum* and *Cystisus* were better, but the best reagents were those in human or animal sera. There was more A substance in A_1 than in A_2 secretor saliva, and more in A_2 than in AB saliva. Bhalla and Bhasin (1976) found 13 aberrant secretors among 122 subjects in India, among whom were A people who secreted H but not A in saliva (A−H+), as well as B−H+ and B+H− B persons. AB people were found who were A+B−H+ and A+B−H−. Secretors were categorized as "strong" if they had inhibition titers of $\geqq$ 1:16, "weak" if the titers were $\leqq$ 1:8. It was suggested that a scale be used to score the number of aberrant secretors in populations. Aberrant secretor index (or ASI) was defined as (Number of aberrant secretors/Number of all secretors) X 100. *Ulex* anti-H was used in the study, and it was noted that antisera of the same specificity would have to be used if different populations were to be compared, since results can and do vary with different reagents. Clarke *et al.* (1960) studied the secretor characteristic of a number of sib pairs, and thought that the amount of ABH secreted by secretors was, at least in part, inherited. The distribution of A/H in a series of A secretors was found to behave like a distribution that would be characteristic of a trait controlled by polygenic inheritance. They said that "aberrant secretors" repre-

sented arbitrarily chosen extremes in a continuous distribution of A/H or B/H in populations.

There are differences in the behavior of secretor salivas, depending upon the reagents used to detect the group substances. The point has been made by a number of authors (Wiener, 1943c; Boettcher, 1967b; Randeria and Bhatia, 1971; Bhalla and Bhasin, 1976; Masis, 1964). Many investigators have employed lectins, and the differences in H titer with *Psophocarpus* and *Erythrium* reagents as against those from *Ulex*, *Laburnum* and *Cystisus* (Randeria and Bhatia, 1971) were discussed above. Plato and Gershowitz (1961) found differences in the behavior of various types of secretors toward *Ulex europaeus* and *Cystisus sessilifolius* lectins. The amount of H substance secreted by secretors decreased in the order: $O > A_2 > A_1 > B > AB$. A_1 saliva showed similar behavior with the two lectins, but A_2 and O salivas were more reactive toward *Ulex* lectin, while B and AB salivas were more reactive toward *Cystisus* lectin. They said that the two lectins were not detecting the same H specificity. Hakim and Bhatia (1965) tested human anti-A serum and lectins from *Phaseolus lunatus* (lima bean) and *Dolichos* with secretor salivas, and found differences in specificity. A_2 secretor saliva showed better inhibition of the lima bean reagent, while A_1 secretor saliva better inhibited human anti-A_1. Boettcher (1967b) found that the mean titer of A_1 saliva with anti-A or with *Dolichos* lectin was significantly greater than that of A_2 saliva. With *Ulex* lectin, the order was $O > A_2 > A_1 > B$, different from the order for red cell agglutinability. Boettcher said that the rates of production of secreted group substances controlled by the *H/h* and the *ABO* loci were independent, and that aberrant secretors were people in whom the two rates were significantly different. Boettcher (1967a) showed that the inhibition titer of A_1 and A_2 secretor salivas with *Dolichos* lectin was proportional to the amount of precipitate obtained from those salivas with this lectin. Precipitation could, therefore, be used as a quantitative measure of secreted substances. Using *Ulex* lectin, it could be shown that secreted substances from A_1 secretors have a higher ratio of A/H determinants that those from A_2 secretors. Potapow (1970a) said that extracts of the seed hulls from the seeds of *Evonymus sacrosaneta* and *Evonymus alata* make excellent anti-B reagents in inhibition tests, especially if trypsin-treated B detector cells are used.

Other investigators have used precipitin tests as well. Baer *et al.* (1961) tested the H in secretor saliva with a precipitating serum prepared by immunizing chickens with ovarian cyst fluid from O women. Prokop and Geserick (1972) said that A secretors could be detected by a precipitin test with anti-A_{HP} (*Helix pomatia* agglutinin), while B secretor saliva gave precipitin reactions with saturated extracts of *Evonymus europaea* seeds. Agglutinins from animal sources are sometimes called "protectins", as distinct from those of plant origin (lectins). Active anti-A reagents have been obtained from several snail species, including *Helix pomatia*, *Helix aspersa* and *Cepaea memoralis* (Prokop *et al.*, 1965; Grace, 1969; Grace and Uhlenbruck, 1969). An application of the *Helix aspersa* anti-A_1 reagent to the A subtyping of bloodstains is discussed in section 19.10.4. Grundbacher (1973) showed that lectins from *Cystisus sessilifolius* and *Laburnum alpinus* gave a precipitin reaction with the saliva of all secretors. *Ulex* lectin gave this precipitation as well, but comparative immunodiffusion experiments indicated incomplete identity between the *Ulex* precipitin band and those of the other two lectins (the technique is discussed in section 2.2.2). The lectin from *Lotus tetragonolobus* gave two precipitin bands with secretor saliva, and one band with that of nonsecretors. The new antigen being detected in secretor saliva was called "L" (for *Lotus*), and was different from, though related to H and Le^a. A full paper on the L antigen appeared in the following year (Napier *et al.*, 1974). The *Lotus* extract was detecting H as well as L in saliva, and L was not identical to A, B, Le^a, Sd^a or SC_1. It was found by immunoelectrophoresis to be part of a cathodically migrating glycoprotein, and sugar inhibition studies indicated that the terminal sugars of L were the same as those of Le^a and H substances. L is absent from the saliva and red cells of persons of the Bombay phenotype, but all other red cells absorb the lectins from *Lotus tetragonolobus*.

Pereira and Kabat (1974) extensively purified the lectin from *Lotus tetragonolobus*, and the purified preparation had three fractions, all with the same specificity. They determined the specificity of the lectin in terms of the structures of the terminal oligosaccharides of the blood group receptors (see in section 19.9.2). The pure lectin reacted with H, Le^a and A_2, but not with A_1 or B substances in precipitin reactions. It was specific for type 2 chains containing L-fucose on C-2 of the galactosyl residue of β-D-Gal (1→4)-D-GlcNAc, whether a second fucosyl residue was attached to the NAc-D-$GlcNH_2$ residue or not. Similarly substituted type 1 chains did not react.

Holburn and Masters (1974) used ^{125}I-labelled glycoproteins (obtained from Dr. Winifred Watkins) for a radioimmunoassay of A activity in serum and in the saliva of A secretors. A activity was found in all sera examined, there being more in A_1 than in A_2 sera, and the amounts being higher in secretors than in nonsecretors. Sturgeon *et al.* (1973) used an automated system, and could readily discriminate secretors from nonsecretors. Significant differences in ABH concentrations within various ABO types were noted, however.

It has been known for some time that the group substance activity in the secretions of the three salivary glands is not equal. Hartmann (1941) showed that the parotid gland was poor in water soluble group specific substances, while the submaxillary gland was relatively rich in them. Wolf and Taylor (1964) fully confirmed these findings in the saliva of secretors collected from the separate glands. Parotid saliva has the lowest group substance concentration—it can be zero—followed by submaxillary saliva, with sublingual saliva being the highest. Milne and Dawes (1973) found the same results in quantitating A substance in the saliva from the separate glands of A and AB secretors. Sublingual saliva, and the secretions of the minor mucus glands contained

significantly more group substance than whole or submandibular saliva. It was estimated that sublingual and minor mucus glands contribute some 70% of the group substance to saliva, the remainder being contributed by the submaxillary gland. Parotid saliva contained negligible amounts of A substance.

Prodanov (1979) investigated the inhibitive titer of A, B and H substances in adults and in newborns. The inhibiting strength of the A and B antigens in the saliva of newborns was significantly lower than that seen in adults, while the relationship was just the reverse for H substance.

19.8.3.2 Seminal plasma and spermatozoa. Group specific substances in secretor seminal plasma were described by Sirai (1925) and by Yamakami (1926). Landsteiner and Levine (1926a) found group substances on washed spermatozoa. Fernandez-Collazo and Thierer (1972) showed that sperm cells from secretors absorbed anti-A and anti-B to a greater extent than do red cells. Potent, immune anti-A and anti-B did not agglutinate sperm, nor have any cytotoxic effect on the cells in the presence of complement. The absorption of antibody could still be observed following ten washings of the cells in saline. Rangneker and Rao (1970) said, however, that the ABH antigens were readily washed out of secretor sperm cells with saline. The absorption test was negative after two washings. Kerék and Eliasson (1975) studied the amount of group specific substance present in secretor semen using the "split ejaculate" technique. This technique is based on the observation that the accessory glands of the male reproductive tract discharge their secretions in a characteristic sequence and pattern, and by collecting and separately analyzing sequential fractions of the ejaculate, one can sometimes draw inferences about the accessory gland origin of a seminal constituent (Lundquist, 1949). In three A and two O secretors, it was found that the various fractions exhibit very similar hemagglutination inhibition activity toward appropriate antisera or lectin, suggesting that all the fluids which contribute to human seminal plasma possess group specific substances, and in approximately equal quanitities.

Parish *et al.* (1967) have noted that sperm cells will take up soluble A and B substances from seminal plasma. Spermatozoa from A and O nonsecretors could be "coated" with A substance simply by exposure to A secretor seminal plasma. Boettcher (1968) confirmed that sperm cells absorb ABH substances from aqueous solutions. No antigens were detected on the sperm cells of nonsecretors using the inhibition technique. Boettcher found that the inhibition titer of sperm cells correlated with that of the seminal plasma from the same person, consistent with the notion that the cells acquire their antigens from the seminal plasma by absorption. Karsznia *et al.* (1969) detected no group specific inhibition by sperm cells from nonsecretors, and said that they had had no luck in demonstrating the antigens on nonsecretor cells by mixed agglutination technique (see in section 19.10.3.5). However, Ackerman (1969), using cellular microcataphoresis, demonstrated that the mobility of spermatozoa is altered by treatment with antibody, a result consistent with specific antibody binding. This behavior was observed regardless of the secretor status of the sperm donor. Sato and Ottensooer (1967) studied the levels of H substance in O secretor seminal plasma and found them to be from 3 to 70 times greater than in O secretor salivas. There was not a very great variability in seminal H content from the same person on different occasions, but variation was greater than that found in saliva. Secretor semen contained relatively more A and B than did secretor saliva as well. Similar findings were reported by Dorrill and Whitehead (1979). A and B substances were 2 to 3 times higher in semen than saliva, while H substance was about 20 times higher, on the average. There were differences in the A:H and B:H ratios between the two fluids as well.

Karamihova-Tsacheva (1966) examined the inhibition titers of 200 seminal plasma specimens from secretors of groups A, B and AB. Anti-H was unavailable. A sort of bell shaped curve distribution was found in plotting inhibition titer against the number of persons exhibiting the particular value. The curve peaked at around 1:256. The technique employed was that of Wiener and Kosofsky (1941) (see in Section 19.8.4). The curve was broader for A than for B. In AB people, the titers were most often equal for A and B, but there were examples of A>B and of A<B. Interestingly, a prozone effect was seen with two B secretor samples. But for the titration technique, these would have been classified as nonsecretors (see also in section 19.10.5.2 "Elution"). The average inhibition titer for anti-A was 1:256, and for anti-B, 1:64.

Davie *et al.* (1979) presented quantitative studies on the ABH substance content of seminal samples from 225 people. On the average, there was about 4 times more H than A in A secretors, and about 6 times more H than B in B secretors. A:H and B:H ratios were almost always less than one, this in marked contrast to saliva. 65 of the samples were tested for Lewis substances as well. In seven, there were detectable levels of H substance, yet the fluids were Le (a+ b−), that is, they typed as "nonsecretors" on the basis of Lewis (see section 19.9.3 and Table 19.8).

Waissbluth and Langman (1971) found that salivary total protein concentration was significantly lower in group O individuals than in members of other blood groups. Serum protein concentration did not show this variation. Salivary protein concentration was higher in males than in females as well, and tended to be high in group A people. There were no clear differences in the concentrations of immunoglobulin classes in saliva, though IgA tended to be higher in secretors than in nonsecretors.

In 1972, Herrmann and Uhlenbruck showed that the A activity in group A secretor semen was due to a heat stable glycoprotein, specifically precipitable with snail agglutinins anti-A_{HP} and anti-A_{HH}. Double diffusion tests indicated the identity of the antigen and human ovarian cyst A substance. These results indicate that the seminal protein has a terminal N-Ac-D-galactosamine residue (see section 19.9.2). For the background on the snail anti-A agglutinins, see Prokop *et al.* (1965), Grace (1969) and Grace and Uhlenbruck

(1969). The A specific glycoprotein was partially purified by Uhlenbruck *et al.* (1973). These workers found too that rabbit antiserum against pooled human seminal plasma (regardless of group) did not precipitate the A glycoprotein from semen. The antiserum did behave, however, as a kind of "incomplete" agglutinin for A red cells, in that pronase treatment of the cells was required to give a significant agglutination titer.

19.8.4 Inhibition tests for group substances in body fluids

It is common to use saliva for the determination of secretor status. Many authors have commented on the technique for carrying out the inhibition test. Even though other methods are used, as noted in the above section, the inhibition technique is still in very wide use. There are a number of variations of the procedure which deserve brief comment, and the considerations apply, at least in a general way, to all body fluids as well as to saliva. Hartmann (1941) titrated the body fluids against a constant concentration of antiserum, and the $-\log_2$ of the highest dilution giving negative agglutination was taken as a measure of the antigen content of the fluid being tested. Thus, if a series of doubling dilutions of A secretor saliva were made, the first tube would be $½^0 = 1$, the second $½^1 = ½ = 2^{-1}$, the third $½^2 = ¼ = 2^{-2}$, and so on. If agglutination were observed in the 1:32 tube, but not in the higher saliva concentrations, after inhibition and addition of test cells, the strength of the sample's group substance would be called 4, i.e., $-\log_2 2^{-4} = 4$. The constant concentration of antiserum was selected by titrating it with test cells, and choosing the highest dilution which still gave 3+ agglutination. The next tube in the series gave 2+ agglutination.

Wiener and Kosofsky (1941a and 1941b) discussed the inhibition technique as well, and carried out many control tests to carefully standardize the procedure. If doubling dilutions of antiserum were tested in series with doubling dilutions of secretor saliva, the "inhibitive titer" increased as the serum dilution increased, as expected. The "inhibitive titer" was the reciprocal of the highest dilution that showed negative aggutination, i.e., completely neutralized the particular concentration of antiserum. An illustration of the type of results they obtained is shown in Figure 19.1. The experiment was actually carried out with B secretor saliva and a particular group A serum, but the behavior illustrated is applicable generally. The table has been modified for presentation here, but still illustrates the point. It was found that the change in inhibitive titer as a function of serum dilution was different for different antisera with the same blood group specificity. In some cases the differences were of large magnitude. Two points are noteworthy based upon these studies. First, if the reciprocal of the highest dilution of body fluid which completely inhibits agglutination at a given serum concentration is taken as a measure of the quantity of group substance present, the results obtained will depend very much on the antiserum chosen, and on the dilution of antiserum employed. Second, it is important to select conditions for carrying out the test which maximize the inhibition sensitivity. One does not want an anti-serum whose inhibitive titer changes drastically as a function of serum dilution, for in such a case, small dilution errors in the test could give rise to large errors in estimating the inhibitive titer. Further, it is better to use sera at dilutions where they contain relatively more agglutinin per unit volume to maximize sensitivity. Similar studies were conducted on a number of anti-A sera. Inhibition titers for group substances from either A_1 or A_2 secretor salivas were higher with A_2 than with A_1 test cells, as expected. But the differences in behavior between the subgroups were not great enough to

Figure 19.1 Determination of Inhibitive Titer for an Antiserum with Secretor Saliva Containing the Corresponding Group Substance

Dilution of Antiserum	Dilution of Saliva														Saline Control	Inhibitive Titer
	$1:2^1$	$1:2^2$	$1:2^3$	$1:2^4$	$1:2^5$	$1:2^6$	$1:2^7$	$1:2^8$	$1:2^9$	$1:2^{10}$	$1:2^{11}$	$1:2^{12}$	$1:2^{13}$	$1:2^{14}$		
neat	–	–	–	(+)	+	++	++								++++	2^3
1:2	–	–	–	–	–	(+)	+	+	++						++++	2^5
1:4	–	–	–	–	–	–	–	(+)	+	+	++				++++	2^7
1:8	–	–	–	–	–	–	–	–	–	–	(+)	+	++	++	++++	2^{10}
1:16	–	–	–	–	–	–	–	–	–	–	–	(+)	++	++	++++	2^{11}

make the authors think it should be considered an issue. Wiener (1943c) discussed this whole matter in his book as well.

Prokop and Uhlenbruck (1969) gave an inhibition technique in which a series of doubling dilutions of antiserum is prepared in four rows. The first row contains only saline. The second, third and fourth rows contain saliva in dilutions of 1:1000, 1:100 and 1:10, respectively. After suitable incubation, test cells are added and the tubes read after an appropriate waiting period. Wiener (1943c) noted that the 10-fold dilutions might be too far apart to insure maximal sensitivity.

Boorman and Dodd (1970) recommended a routine technique in which antisera or anti-H lectin is employed at a titer of about 1:64. With one volume of serum is incubated a volume of saliva for about 30 minutes at room temperature. During the waiting period, the anti-serum is titrated by doubling dilution technique. At the end of the incubation period, the antiserum-saliva mixture is titrated by doubling dilution technique as well, red cells of appropriate group being added, and the test being read after 2 hours. A typical secretor saliva would null the antiserum at all dilutions. If a comparison of different secretors is wanted, the saliva may be diluted out in tubes, and incubated with a constant concentration of antiserum at a titer of about 1:32 to 1:64. Saliva dilutions in this case were made with a 1 mℓ pipette, resulting in dilutions of 1:10, 1:50, 1:100, 1:150, etc., out to 1:500. Equal volumes of diluted saliva and antiserum are incubated for 30 min, after which test cells are added, and the tubes read after 1½ to 2 hours. Secretors generally show inhibition up to a point in the 1:100 to 1:500 range, while nonsecretors do not show inhibition above 1:10.

Issitt (1970) gives a one-step method in which antiserum is used at a dilution one tube stronger than that which gives good macroscopic agglutination with appropriate cells. One volume of antiserum is mixed with one volume of saliva (1:2 dilute), and the tubes incubated for 20 minutes. Appropriate test cells (5% suspension) are then added and the tubes read after 15 minutes following a brief, low speed centrifugation.

Race and Sanger (1975) recommended a technique very like Issitt's except that 2% cell suspensions were employed. They say that the antisera should be from donors who have not been "boosted" by frank immunization, and they said that they thought that most routine American antisera came from boosted donors. In cases where a closer look at the secretor property was wanted, the saliva is titrated.

A few other points about technique based upon important principles should be made. It has been known for quite some time that there is an enzyme present in saliva (and in feces) which can destroy the activity of blood group substances (Schiff and Akune, 1931; Schiff and Weiler, 1931; Schiff and Buron, 1935). The principle is heat-labile, however, while the group substances are not. Saliva is heated, therefore, immediately after collection, usually in a boiling water bath for a few minutes, to destroy this activity. It is usually centrifuged hard after heating to remove debris, and the supernatant fluid used for the secretor status tests. This supernatant can be stored frozen for a long time if necessary, and tested at a convenient time. Some authors dilute the saliva with saline before testing, usually 1:1, particularly if a test is being done which does not involve any titration of the saliva.

In testing for the A substance, most authorities have preferred to use A_2 test cells. As Wiener and Kosofsky (1941b) showed, the change in inhibition titer with serum dilution was more suitable for the inhibition test when A_2 test cells were used, giving a better sensitivity. Issitt (1970) and Race and Sanger (1975) both recommended that A_2 test cells be used in the inhibition test for the A group specific substance in secretor saliva.

19.9 Biochemical Studies on the ABO System

19.9.1 Early studies

The older studies on the chemical nature of the group substances are primarily of historical interest. Until the 1950's, biochemical methods were not sufficiently developed to make possible the elucidation of the structures. As will be appreciated by reading through this section, the rather complete basic knowledge that we now have on the chemical nature and biosynthesis of the ABO group substances emerges in large part from the studies of W. T. J. Morgan, Winifred Watkins, Elvin A. Kabat and Victor Ginsberg and their collaborators.

Landsteiner and van der Scheer (1925a and 1925b) and Landsteiner *et al.* (1925) found that partially purified alcoholic extracts of both horse and human red cells would give precipitin reactions with appropriate antisera. The extracts were antigenic, but their antigenicity was enhanced by the addition of foreign serum, and the antisera obtained were different as well. They did not think the active material in the alcoholic extract was protein in nature. Brahn *et al.* (1932) partially purified the blood group A-like substance from commercial pepsin. This pepsin was prepared from pig gastric juice, and pig gastric mucin is a rich source of a group A-like substance. Landsteiner and Chase (1936) obtained a preparation from commercial pepsin that was highly active serologically. Morgan and King (1943) obtained a fairly pure preparation of this material, which Morgan (1943) noted was polysaccharide-peptide in nature. It was not itself antigenic but, when complexed with the conjugated protein component of the O somatic antigen of *B. shiga,* and injected into rabbits, yielded an extremely potent anti-A serum. Group substances have been obtained from pseudo-mucinous ovarian cyst fluid (King and Morgan, 1944), human urine (Jorpes, 1934; Jorpes and Norlin, 1933 and 1934), human gastric juice (Witebsky and Klendshoj, 1941), horse saliva (Landsteiner, 1936), human saliva (Landsteiner and Harte, 1941) and several other sources (Bray *et al.,* 1946). A number of investigators noted the polysaccharide content of the preparations, and D-galactose

(D-Gal), D-mannose, D-glucosamine (D-GlcNH$_2$) and L-fucose (L-Fuc) were found in hydrolysates in some of the investigations. Reviews of this work may be found in Stacey (1946) and Kabat (1949 and 1956). Kabat's (1949) review gives a good overview of the state of knowledge at the time. As noted, any number of relatively pure preparations had been obtained, and Kabat and collaborators had shown by quantitative immunochemical studies, using precipitation as an assay technique rather than hemagglutination inhibition, that a substantial fraction of the preparations consisted of the activity of interest. Morgan and King's introduction of the phenol method for purifying the group substances (1943) had made possible better preparations than had been available previously. Kabat (1956) gave a detailed review of the procedures used in the preparation of bood group substances from erythrocytes as well as from body fluids. By 1956, it was clear that the composition of the H, A, B and Le^a substances showed striking similarities, and that the best preparations contained D-Gal, L-Fuc and D-GlcNH$_2$ and D-galactosamine (D-GalNH$_2$), the latter two most probably as the N-acetyl derivatives.

19.9.2 Chemical nature of the blood group substances

The majority of the detailed studies that eventually yielded the structures was carried out between about 1955 and 1970. The literature on this material is quite extensive, and a complete review would be repetitious and unnecessarily complicated. The details of the investigations may be found in Morgan and Watkins (1969), Marcus (1969), Grollman *et al.* (1970), Morgan (1970), Watkins (1972), Ginsburg (1972) and Hakomori and Kobata (1974). The chemistry of the Lewis substances is discussed in this section because it is closely related to the discussion of the A, B and H structures. The serology of the Lewis system will be dealt with in section 20, though there is necessarily overlap.

A number of approaches, both direct and indirect, were used in these studies. Direct analysis is possible only with highly purified preparations. Indirect approaches have been extremely useful. Watkins and Morgan made use of plant and eel agglutinins, specific for O and H substances. Specific agglutination of A or O cells by these reagents was found to be inhibitable by certain simple sugars and oligosaccharides, from which it could be deduced that the structures capable of giving inhibition were similar to, or identical with the structural parts of the antigens. Thus it could be shown that agglutination by eel serum anti-H was inhibited by L-fucose (Fig. 19.2) and better by methyl-α-L-fucoside, and that agglutination by anti-A from *Vicia Gracca* or lima bean was inhibited by N-acetyl-D-galactosamine (Fig. 19.3) or by methyl-α-N-acetyl-galactosaminide. In this way, an α-N-acetyl-D-galactosaminyl residue was implicated in the A structure and an α-L-fucosyl residue in the H structure. A useful plant or animal source for anti-B was not available when these experiments were done (1952–1953). As oligosaccharides were isolated by hydrolysis of the blood group substances, these could be tested for their ability to inhibit hemagglutination in these systems. Le^a hemagglutination

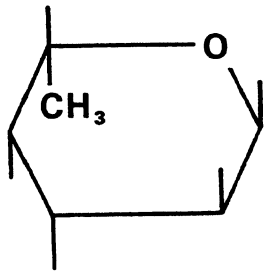


Figure 19.2 α-L-Fucose

was inhibited by a compound called "lacto-N-fucopentaose II" (Fig. 19.4), and that of Le^b by another compound called "lacto-N-difucohexaose I" (Fig. 19.5). Kabat and his collaborators looked at the inhibition of specific precipitin reactions between blood group substances and their immune sera (the natural isoagglutinins are not good precipitins). Inhibition of the B specific precipitation by melibiose (6-O-α-D-galactopyranosyl-O-glucose; Fig. 19.6) implicated the α-D-galactopyranosyl residue in B-specific sites. Group A specific precipitation was inhibited by an oligosaccharide containing N-acetyl-D-GalNH$_2$ released by weak acid hydrolysis. Still another approach was the enzymatic degradation of blood group substances (see, for example, Watkins, 1962), and inhibition of the enzymatic reaction by various carbohydrates ("substrate inhibition"). An enzyme from *Trichomonas fetus* which destroyed H activity was inhibited by L-fucose and D-galactosamine. Enzymatic hydrolysis of A substance by crude extracts of *T. fetus* or *Clostridium welchii* (type B) was inhibited by N-Ac-GalNH$_2$, while that of the B substance was inhibited by D-Gal or its α- or β-D methyl pyranosides. The enzymes which bring about the losses of A, B and H activity are, respectively, an N-acetylgalactosaminidase, a galactosidase and a fucosidase. Watkins (1972) said that no explanation had yet been found for the inhibition of the *T. fetus* H enzyme by D-Gal-NH$_2$. Enzymes from a number of other bacterial sources have been found which destroy the blood group specificities. Enzymatic

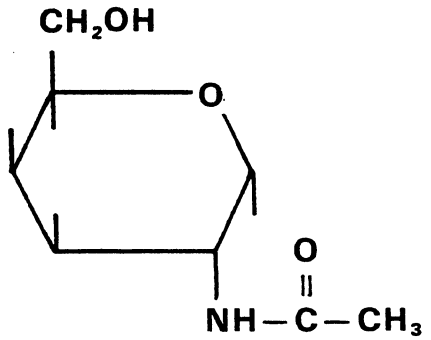


Figure 19.3 N-Ac-Galactosamine

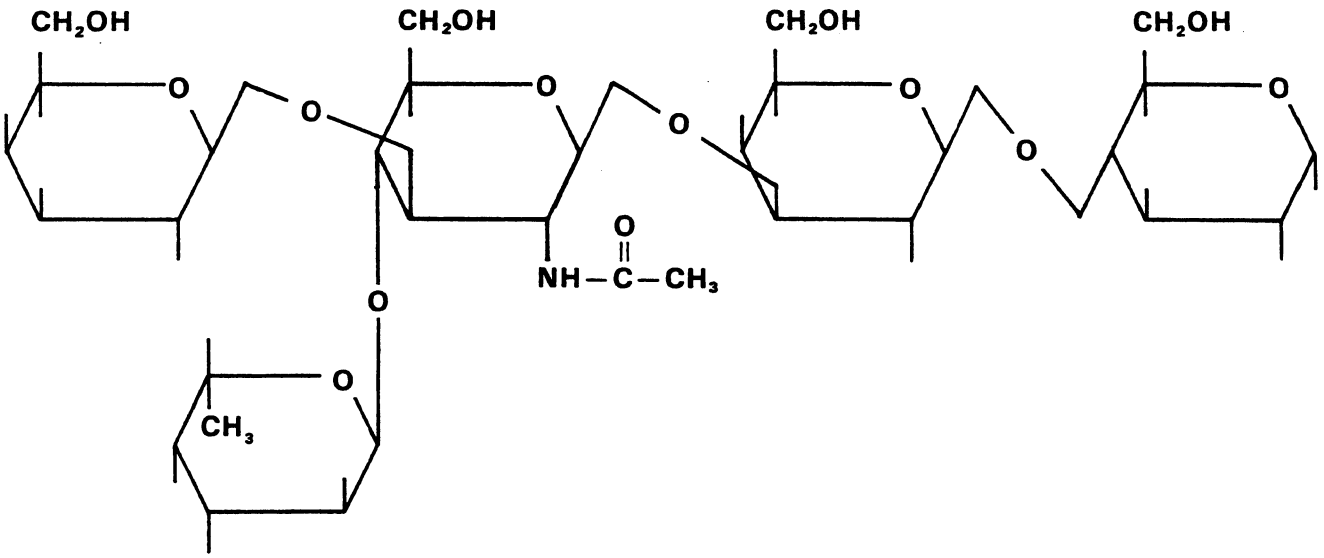


Figure 19.4 Lacto-N-Fucopentaose II

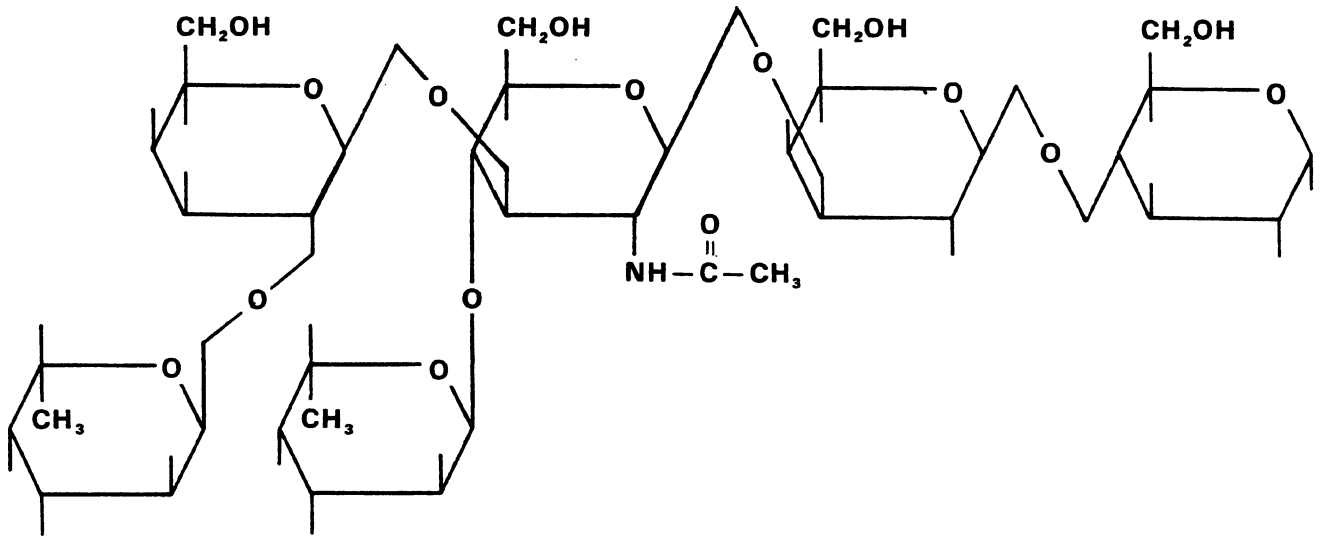


Figure 19.5 Lacto-N-fucohexaose I

degradation of the blood group substances was studied directly as well. Enzymes which specifically inactivated A or B specificities gave rise to increases in H specificity in the process. An A enzyme from *T. fetus* which causes conversion of A→H specificity simultaneously releases N-Ac-GalNH$_2$. Similarly, a B destroying enzyme released reducing sugar consisting of a major Gal component. A partially purified H enzyme from *T. fetus* liberates fucose in the course of destroying H specificity, and leaves as a product an antigen that reacts with horse anti-type XIV pneumococcal serum. Enzymes have been found that destroy Le^a activity, releasing L-fucose which was in α-1→4 linkage to N-acetyl-GalNH$_2$ in lacto-N-fucopentaose II, but these enzymes do not release the fucose joined in α-1→2 linkage to galactose in lacto-N-fucopyranose I. Direct analysis of the structures consisted of limited hydrolysis of the blood group substances (enzymatic and chemical), characterization of the products, and inferences about the original structure from the fragments obtained.

The sum and synthesis of many hundreds of studies has resulted in a fairly complete understanding of the chemical structures of the blood group determinants. The immunospecificity without doubt resides in the carbohydrate moieties of the glycoproteins which constitute the soluble group sub-

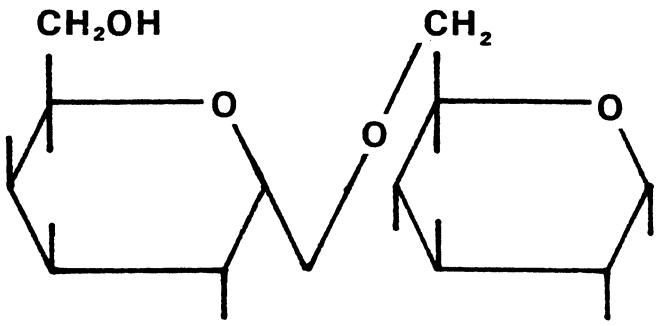


Figure 19.6 Melibiose

stances, and of the glycolipids which constitute the determinants of the red cell. There are two kinds of serologically active carbohydrate chain endings:

Type 1 β-Gal-(1→3)-GlcNAc

Type 2 β-Gal-(1→4)-GlcNAc

A, B and H determinants can be based on either type while Le^a and Le^b structures, in which L-fucose is substituted at the 4-position of NAc-$GlcNH_2$ are based on type 1 chains. The oligosaccharides characterizing the A, B and H substances are indicated in Fig. 19.7. The structures shown in Fig. 19.7(a), (b) and (c) have type 2 chains. Structures with type 1 chains, i.e., in which the linkage between the terminal N-Ac-$GlcNH_2$ and the subterminal β-D-Gal is 1→3, occur as well. The oligosaccharides characterizing the Le^a and Le^b substances are shown in Fig. 19.8. Obviously, structures with 1→4 linkages between the galactopyranosyl and N-acetylglucosamine residues cannot give rise to Lewis specificities since the C-4 of the N-Ac-$GlcNH_2$ is occupied by the glycosidic linkage and no substitution by a fucosyl residue can occur.

The details of the structural work are considerably more complicated than has been indicated. The issues of how the oligosaccharides are put together in the native glycoprotein group substances and the nature of the linkages of the sugar moieties to the amino acids of the peptide chain must await clarification. Composite structures, branched oligosaccharide chains, which include the known structures of several of the group determinants, have been proposed. These have been put forth, at least in part, to account for the many oligosaccharides isolated when the blood group substances are subjected to partial hydrolysis. It has been suggested that the linkage to the peptide chain may be through the N-Ac-galactosaminyl residue to serine or threonine. Goodwin and Watkins (1974) have isolated a number of oligopeptides from the A specific glycoprotein and determined their sequences. Their results were fully consistent with hydroxyamino acid-N-Ac-GalNH_2 linkages in the intact molecule. From the structures of the immunodeterminants that have been determined, it has been possible to characterize the specificities of the so-called "blood group enzymes" that were mentioned above. Some of these are indicated in Table 19.7.

A number of the A-like, B-like and H-like substances from animal sources have been purified and subjected to structural studies. Springer (1970) gave the structure of the B-active receptor from *E. coli* O_{86}. H substance from hog gastric mucosa has been characterized by Kochetkov *et al.* (1976). Newman and Kabat (1976) did extensive studies on the B-active substances from horse gastric mucosa. Pereira and Kabat (1976) showed that the glycoproteins from hog gastric mucosa or human ovarian cyst fluid can be purified by an immunoadsorbent technique in which purified lectins from *Dolichos* or *Lotus* are coupled to Sepahrose 2B support media. Etzler and Kabat (1970) purified and characterized the specificity of the *Dolichos* lectin, and Pereira and Kabat (1974) carried out similar studies on the *Lotus* lectin. It may be noted too that a carcinoembryonic antigen (CEA-M) from human hepatic metastasis is closely related structurally to H substance, and can be modified to possess A and B activities with appropriate enzymes (Bali *et al.*, 1976).

The structual studies on the receptors of the red cell membrane have developed more recently than those on the water soluble materials. The glycolipid structures are more difficult to isolate and purify than the glycoproteins, but considerable progress has been made. The studies are well reviewed in Hakomori and Kobata (1974). Glycolipids with H, A, B, Le^a and Le^b activity have been isolated and the oligosaccharide structures that have been determined thus far are in complete conformity with what is known of the glycoprotein materials of similar serological specificities. The sugar-lipid

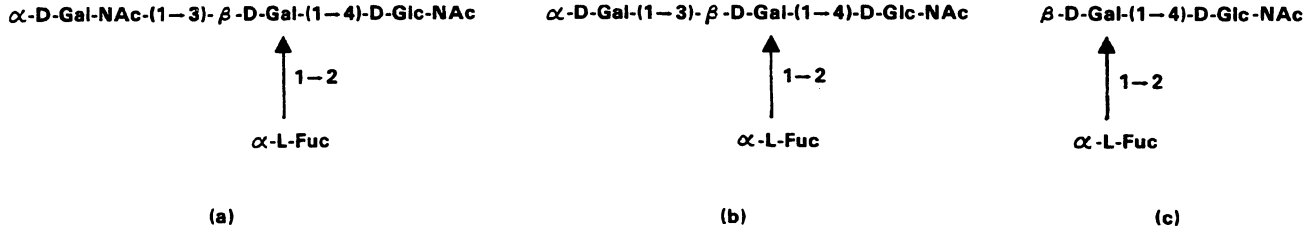


Figure 19.7 Composition of the Carbohydrate Fragments Responsible for Blood Group Activities A (a), B (b) and H (c).

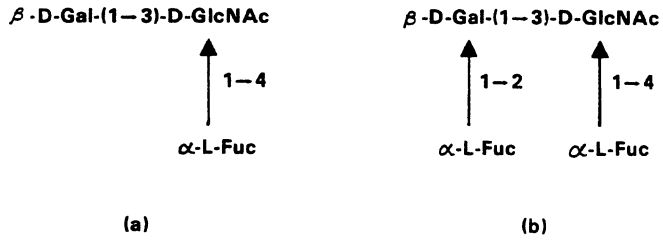


Figure 19.8 Composition of the Carbohydrate Fragments Responsible for Le^a Specificity (a) and Le^b Specificity (b)

linkage is thought to be through a terminal glucosyl residue to a ceramide. Kent *et al.* (1977) have studied the relationship between the glycolipid and glycoprotein blood group active substances in the red blood cell membrane. A procedure for the isolation of ABH blood group active glycolipids from red cell membranes is given by Hakomori (1978). Gardas (1978) purified an A-active glycolipid from red cells, and carried out extensive structural studies which enabled him to propose a structure for the nonreducing end of the chain. It is of interest that the A antigenic receptor sites for anti-A appear to be restricted to the "outside" of the red cell membrane. They are not detected in studies with radio-labelled anti-A on "inside-out" red cell membranes (Schenkel-Brunner *et al.*, 1979).

19.9.3 Biochemical genetics of the ABO, Secretor and Lewis Systems

The biosynthesis of ABH and of Lewis substances are intimately related, and involve the Secretor locus as well. The serological characteristics of the Lewis system will be discussed in section 20, but the biochemical genetics will be included here. The inheritance of the ABO characteristics seemed straightforward for a number of years. There was no particular reason not to suppose a direct relationship between the genes and the blood group determinants. The recognition that the genetic material carries coding information only for the synthesis of proteins (section 1.2.2), and the discovery that the immunospecific structures in the determinants were polysaccharides, required a closer examination of the system. The role of the secretor and Lewis loci had to be accounted for as well, insofar as the presence of Lewis and ABH substances in body fluids are concerned. The basic outlines of the scheme were put forth in the late 1950's, based on the structural work that had been carried out on the ABH substances, and on genetic considerations. These pathways, which have since been shown to be largely accurate, were proposed by Watkins (1958 and 1959), Watkins and Morgan (1959) and by Ceppellini (1959). Four loci are involved in the control of the biosynthesis of ABH and Le^a substances: the Lewis (*Le/le*), the *H/h*. the secretor (*Se/se*) and the ABO loci. Figure 19.9 summarizes the relationships. The genes *O, h* and *le* are regarded as inactive in terms of conversion of precursor substances. The precursor substance is a macromolecular glycoprotein with the peptides fully synthesized and carbohydrate chains incorporated, but not fully completed. There are a few people whose secretions are ABH−, Le(a−b−), but they have in their secretions a glycoprotein which is similar in many ways to the blood group active molecules. It is low in fucose, and cross reacts with horse anti-type XIV pneumococcal serum. This molecule is believed to be the precursor substance for the group substances. The *Le* gene gives rise to conversion of the precursor into Le^a. *H* gene acts to convert the precursor substance or Le^a into H. Both the *Le* and *H* genes cause α-L-fucosyl residues to be added to different sugars in the precursor substance. Le^b activity results from an interaction of *H* and *Le* genes. Gene *Se* is thought to activate gene *H* in tissues producing secreted glycoproteins, so that homozygous recessive *sese* people do not form any soluble H substance. H substance is considered the substrate for the reactions controlled by the ABO genes. The *A* gene controls the addition of α-N-Ac-D-galactosaminyl units to H, while *B* gene controls the addition of α-D-galactosyl units to it.

All the experimental evidence heavily supports the scheme outlined in Fig. 19.9. The *H* gene product has been identified in a number of sources as a fucosyl transferase. The enzyme is not present in the secretory organs of *sese* people, but the synthesis is carried out in the red blood cells regardless of secretor locus genotype. The product of the *Le* gene is likewise a fucosyl transferase but with a different specificity than that of the *H* gene. *A* gene product is an N-acetylgalactosaminyl transferase and *B* gene product is a galactosyltransferase. Type 1 and type 2 chains conversions to the various blood group substances are indicated schematically in Figures 19.10 and 19.11 respectively. It may be noted that the enzyme giving rise to the substances called "X" and "Y" in Fig. 19.11 occurs in human milk, submaxillary glands and gastric mucosa from secretors as well as nonsecretors. The gene giving rise to the enzyme is designated "X" by Hakomori and Kobata (1974) and "3-F" by Watkins (1972). Watkins does not give the substances names. Hakomori and Kobata (1974) designate the positional isomer of Le^a as X, that of Le^b as Y, as has been done in the Figure. No serological specificities were associated with X or Y for a long time, and it was thought that the gene locus controlling the transferase is not polymorphic, and the enzyme is therefore present in all individuals (Watkins, 1972). There is recent evidence that X and Y may have Lewis substance specificity,

Table 19.7 Some Enzymes Acting on Blood Group Specific Structures

Enzyme Source	Blood Group Specificity Affected	Probable Specificity	Effect
Trichomonas fetus	A	N-Ac-Galactosaminidase	Destruction of A; Enhancement of H
Clostridium tertium	A	N-Deacetylase	Destruction of A; No enhancement of H
Clostridium tertium	A	N-Ac-Galactosaminidase	Destruction of A; Enhancement of H
Helix pomatia	A	N-Ac-Galactosaminidase	Destruction of A; Enhancement of H
Trichomonas fetus	B	D-Galactosidase	Destruction of B; Enhancement of H
Clostridium maebashi	B	D-Galactosidase	Destruction of B; Enhancement of H
Coffee Bean	B	D-Galactosidase	Destruction of B; Enhancement of H
Trichomonas fetus	H	1,2- α-L-fucosidase	Destruction of H; Enhancement of Reactivity with Anti-type XIV Pneumococcal Serum
Bacillus fulminans	H	Fucosidase	Destruction of H; Liberation of L-fucose
Clostridium perfringens	H	1,2- α-L-fucosidase	Destruction of H
Aspergillus niger	H	α-L-fucosidase	Destruction of H
Trichomonas fetus	Le^a	1,4- α-L-fucosidase	Destruction of Le^a

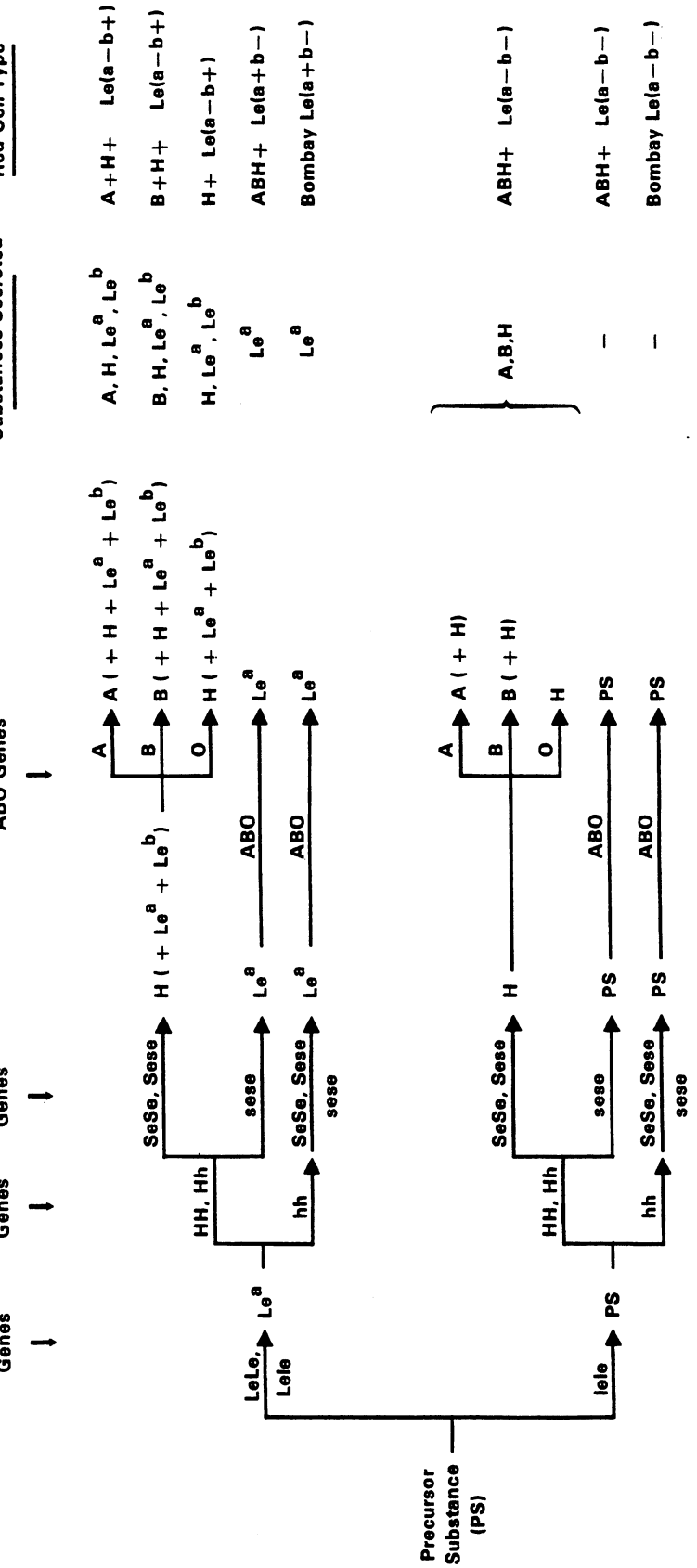


Figure 19.9 Overall Scheme for the Genetically Controlled Synthesis of A, B, H and Le[a]

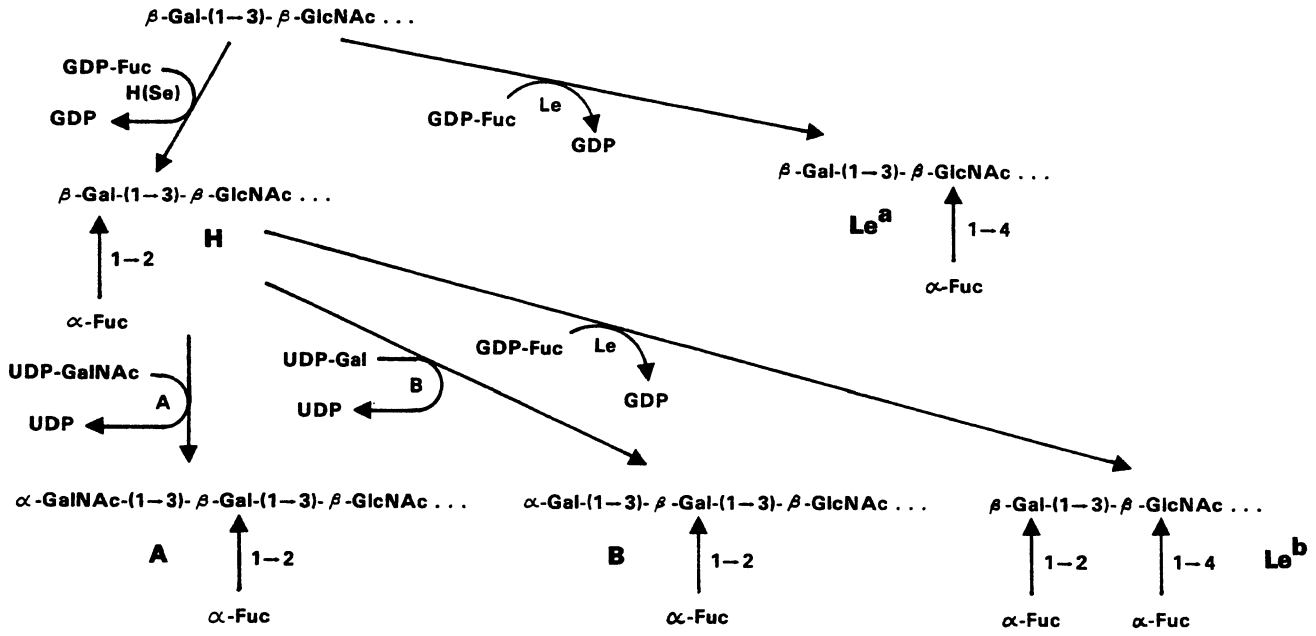


Figure 19.10 Conversion of Type 1 Chain to Group Substances

corresponding, respectively, to Le^c and Le^d (see discussion in section 20.5.1). Carne and Watkins (1977) reported on the purification of *B*-gene-specified α-3-galactosyltransferase. Badet *et al.* (1976) studied the correlation between agglutinability with anti-B sera and the serum concentration of the galactosyltransferase activity in B individuals in several populations. Different correlations were found in African and Caucasian populations. Koscielak *et al.* (1976) found that galactosyltransferase activity in the serum of persons of group B_m (see in Table 19.4) was normal, but that the red cell concentration was very much lower than in ordinary type B cells, perhaps accounting for the weakness of the B antigen in B_m on the basis of the number of H sites converted.

Tilley *et al.* (1978) noted that the α-3′-NAc-D-galactosaminyl transferase specified by the A_1 gene, is appreciably lower in recently delivered mothers than in ordinary adult or newborn serum. A similar, but less striking decrease was noted for H-specified α-2′-L-fucosyl transferase. Although newborns have fewer A and H antigens than adults, the levels of these enzymes in serum were found to be the same. Romano *et al.* (1978) found that O cells from adults, incubated with UDP-galactose and B serum, contained about 200,000 "generated" B sites per cell. The same experiment with group O cord blood cells showed 40,000–70,000 B sites generated per cell, indicating that infant blood cells were lower in the amount of substrate available to the transferase enzyme.

In section 19.6, a number of variant types of ABO expression were discussed. Mulet *et al.* (1979) have studied the biosynthesis of the B antigen in B_h people. The evidence indicates that the B in B_h people is made from H substances, and that the *H* gene is, therefore, not completely silent in these people.

Yoshida *et al.* (1979) have carried out experiments using an antibody to N-acetylgalactosaminyltransferase (anti-A enzyme antibody) with A, B and O sera, and have shown that the antibody cross reacts with B enzyme as well as with a protein found in group O persons, called "O-CRM". This enzymatically inactive, but immunologically cross reactive protein appears to be the *O* gene product. These experiments show that the genes responsible for A enzyme, B enzyme and O-CRM are allelic. Yoshida (1980) indicated that the genotypes of A and B blood could be determined using the anti-A enzyme antibody if the serum was tested for the O-CRM.

The relationships between genotypic combinations and the red blood cell and secretion phenotypes, as modified from Morgan and Watkins (1969), are indicated in Table 19.8. These relationships hold generally for European populations, but there are exceptions (see section 20.2).

19.10 Medico-Legal Applications

19.10.1 Introduction and disputed paternity testing

19.10.1.1 General introduction. The general principles and considerations concerning the medicolegal application of genetic markers in blood and body fluids were discussed in section 18.

In this section, the principal techniques for antigen grouping in bloodstains are introduced, and discussed in some

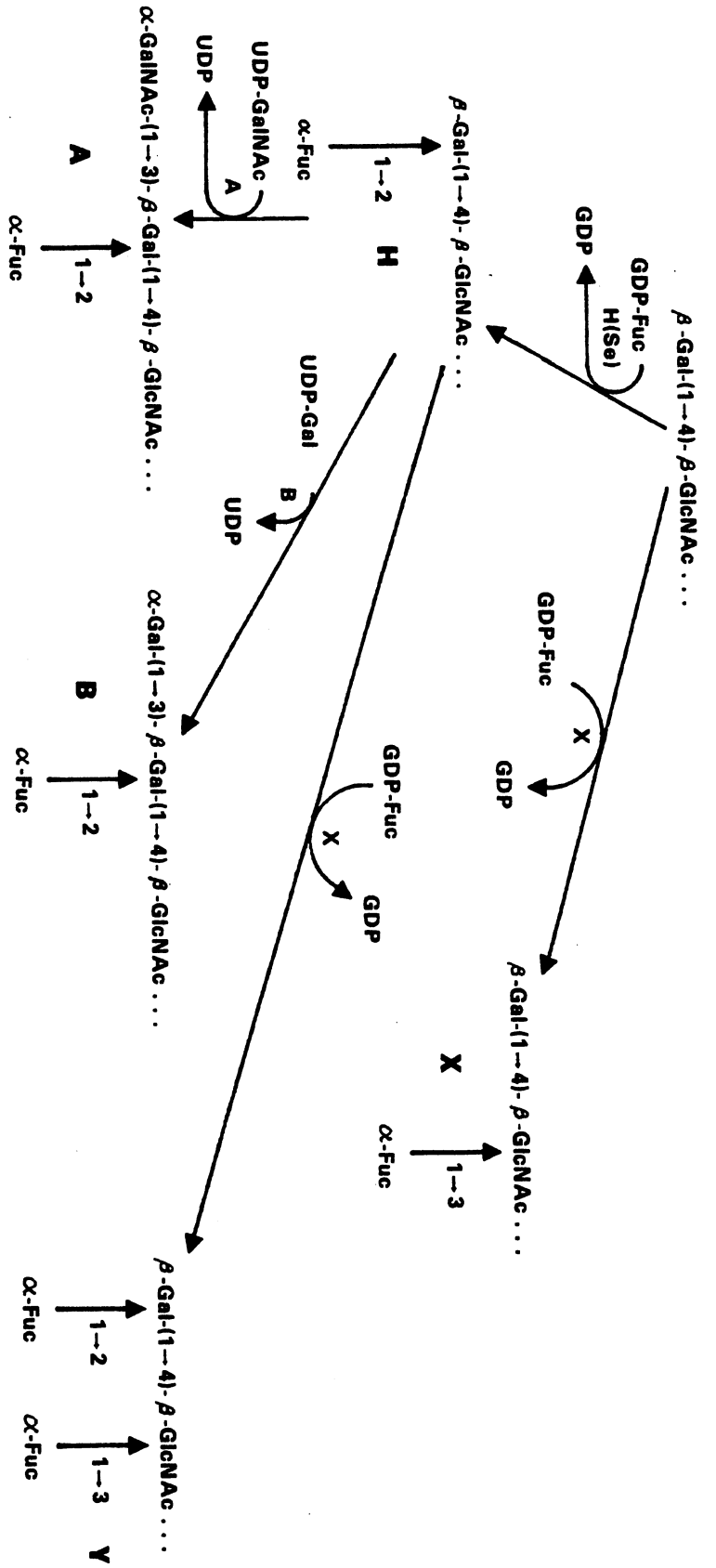


Figure 19.11 Conversion of Type 2 Chain to Group Substances

Table 19.8 Relationship of Genotype to Red Cell and Secretor Phenotype

Genotype Combination	Red Cell Antigens			Substances in Secretions		
	ABH	Le^a	Le^b	ABH	Le^a	Le^b
ABO, H_, Se_, Le_	+	−	+	+	+	+
ABO, H_, sese, Le_	+	+	−	−	+	−
ABO, H_, Se_, lele	+	−	−	+	−	−
ABO, H_, sese, lele	+	−	−	−	−	−
†ABO, hh, Se_ or sese, Le_	−	+	−	−	+	−
†ABO, hh, Se_ or sese, lele	−	−	−	−	−	−

† Bombay phenotypes

detail, since the time they were first employed with the ABO system. The techniques are not very different in principle for the other blood group systems.

19.10.1.2 Some early developments in the medicolegal application of blood groups. The development of medicolegal applications of the ABO blood groups (and later, other group systems as they were discovered) has taken place over the past 60 or 70 years since ABO was discovered. The acceptance of blood grouping as a means of connecting bloodstained materials to individuals and excluding other individuals, and as a means of excluding parentage, occurred at different rates in different countries. Lattes (1932) gives an excellent review of developments in disputed parentage cases. It appears that Germany was one of the earliest countries whose legal system accepted blood tests in disputed affiliation cases as a routine matter. According to Schiff and Boyd (1942), the first case went to Court in Berlin in 1924. Lattes (1932) said that there were 3000 cases tested in Berlin alone in 1924, doubtless because Schiff was there. Popoff (1927) mentioned that Kolzoff first applied grouping in a medicolegal case in Russia in 1921.

In this country, Ottenberg (1921a and 1921b) published a set of exclusionary tables for parentage cases, but the concept was not accepted by everyone. Buchanan (1922a) denied that blood groups could be used as a basis for excluding parentage, and criticized (1922b) Ottenberg's tables. Dyke (1922a) in England agreed with Ottenberg, and published a set of tables as well. In 1930, Dyke said, however, that an affiliation case had yet to be decided by an English court based upon blood grouping results. Wiener (1935) reported on an affiliation case in this country, tried January 18, 1933, in which he had excluded a defendant from paternity. The plaintiff had withdrawn her charges. A baby mixup case in Chicago in 1930 is also reported, in which the children were sorted out by blood grouping tests. As usual, the higher courts were slower to accept the results of the tests. In a New York case in January, 1934, Kings County (Brooklyn) Supreme Court Justice Meier Steinbrink ordered blood tests in a disputed paternity case entitled Beuschel v. Manowitz [271 NYS 277; 272 NYS 165]. The Court apparently used as a precedent the decision of the Supreme Italian Court of Cassation of February 13, 1931, in which it recognized blood grouping as affording a potential means of certain exclusion of parentage (Lattes, 1932). Justice Steinbrink's decision was, according to Wiener (1935), reported in the January 2, 1934, issue of the *N.Y. Law Journal.* Unfortunately, the plaintiff appealed the order, and the Appellate Division of New York Supreme Court reversed Justice Steinbrink's order (see *J. Am. Med. Assoc.* **104:** 344 for 1935). Soon afterward, laws were passed in a number of the States providing the courts with statutory authority to order blood tests in these kinds of cases. Both Wiener and Levine were instrumental in bringing passage of these laws about. The Committee of Medico-legal Problems of the A.M.A. (1952 and 1957) periodically issued recommendations on various aspects of the medicolegal use of blood groups, primarily disputed parentage. The recommendations included the systems that ought to be used, criteria for qualifying experts, model state laws, and summaries of court decisions. They also contained nomenclature recommendations which, in the case of Rh, were not always amicably accepted, as will be discussed in section 22. The most recent guidelines and recommendations were issued in 1976 (Joint AMA-ABA Guidelines, 1976). They recommended the use of 7 systems, ABO, Rh, MNSs, Kell, Duffy, Kidd and HLA in routine parentage investigations. These seven have a cumulative probability of exclusion of 91–93% (see in section 18).

The examination of bloodstains for ABO groups in criminal cases was first reported by Lattes (1916a). In this country, acceptance of this sort of determination appears to have come about in the 1930's. Gettler and Kramer (1936) reported a number of attempts to do blood grouping on case stains. Their results, in trying to follow Lattes' methods, were poor, and they said that the procedures were not very good, and probably should not be used. Landé (1938) at the N.Y. Medical Examiner's Office, reported that the had correctly diagnosed more than 90% of 109 dried blood and 93 seminal stains in a blind trial, and that some of the errors were corrected upon doing the tests a second time. Boyd and Boyd (1937) in Boston took exception to Gettler and Kramer's position. They presented their procedures, which were slight modifications of the ones in use in Europe. They used both human and immune antisera in the tests (inhibition procedure), and could correctly assign the groups of 120 two month old human bloodstains on cotton in a blind trial. They asked Dr. Wiener to send them 28 more stains, half on cloth and half on filter paper, as a further blind trial. All were diagnosed correctly, even a few A_2 stains, and Wiener agreed in a letter to Boyd and Boyd that stain grouping was reliable in competent hands. Wiener (1935) gave a thorough discussion of stain grouping methods, but did not report any criminal cases of his own, or of anyone else in this country. In the 2nd edition of *Blood Groups and Transfusion,* however, Wiener (1939) does mention a case in which he had testified. He had not examined the stains themselves because they were too small, but he had testified that another expert's diagnosis of group AB based on a negative Lattes test alone was completely unwarranted.

19.10.1.3 Disputed Parentage As can be appreciated from the foregoing section, the ABO (and secretor) systems have been applied to disputed parentage cases for many years. All laboratories which do paternity testing at the present time include the ABO system in their tests. A few may include the secretor system, although it is not very useful.

The probability of excluding a falsely accused White father with the ABO system is about 13.4%, while for a falsely accused Black father, it is about 17.4% (Chakraborty *et al.,* 1974; AABB, 1978) The ABO system has its peculiarities, many of which have been discussed in the foregoing sections. These can led to problems of interpretation if they should be encountered in a disputed parentage case (Tippett, in AABB, 1978). There is some indication that problems may be encountered with A_1 and A_2 subtypes in Black people

(Perkins and Morel, 1980). Some people who are genotypically A_1B may type as A_2B. Perkins and Morel (1980) indicated that their analysis of the population data for Black people suggested that as many as 1 in 5 "A_2B" individuals may have the A_1B genotype. Their analysis was prompted by a parentage case in which an apparent A_2B mother (and an O putative father) had an A_1B child.

The only exclusion that can be obtained in the secretor system is in a case where nonsecretor parents have a secretor child. The probability of excluding a falsely accused father with this system is thus quite low, about 3% for White or Black people.

19.10.2 Early studies on grouping bloodstains

Determination of the ABO groups of bloodstains may be said, in a sense, to be as old as the system itself. In Landsteiner's laureate account (1901) of the blood groups, he noted that the agglutinins in a blood sample dried on linen for 14 days would, upon being redissolved, still give group specific agglutination. Based upon this observation, he wrote: "Thus the reaction may possibly be used in some cases for the identification, or better, for the recognition of unidentified blood samples, e.g., for forensic purposes unless rapid variations in the agglutinating ability should be found, which would prevent this application". In 1903, Landsteiner and Richter published a full paper on the activity of agglutinins in blood dried out on glass slides and on linen. In all the tests (blood samples from six different people were used), the reconstituted stain showed the same agglutinating behavior as the original serum, except that in some cases the agglutinins had apparently become inactive. Thus, some samples which were distinguishable in fresh specimens were no longer so in the dried state, but no dried specimen gave a reaction different from what it had given in the fresh state. They noted that it might be possible in certain cases to observe an identity in agglutination behavior between a stain extract and the serum of a person, but not always. Since a person's serum does not agglutinate his own cells, they said that the agglutination of a person's cells by a stain extract excluded that person as the source of the stain. Failure of agglutination, however, could not be interpreted in terms of whose blood might have been responsible for the stain. Checking for agglutination of a suspect's blood cells with a stain extract became known in the literature as the Landsteiner-Richter test. Because of the known fact that many animal sera will agglutinate human red cells, it had to be established that the bloodstain was of human origin before the test was carried out. Although this was not a blood grouping test as such, it was most certainly based on the ABO groups.

Attention continued to be focused on the isoagglutinins for some years. Biffi in 1903 noted that a suspect could be excluded from consideration as the source of a bloodstain if his cells were agglutinated by the bloodstain extract (Landsteiner-Richter). He apparently thought too that agglutinable cells could be extracted from the stain and tested with suspect's serum with the same idea in mind. The testing of the agglutinins in the stain with test cells was carried a bit further though. The isoagglutinins extracted from the stain would behave somewhat differently in the isoagglutination reaction with examples of cells from a number of individuals. If only a few individuals were under consideration in the case as potential sources of the bloodstain, Biffi thought that an "individualization" might be possible by such a comparison. This idea was pursued by Baecchi for a number of years, as discussed below. Florence, writing in 1904, did not accept the idea that results obtained with cells gotten from stains should be treated like those obtained with fresh cells. He seemed to deride the idea of individualization of bloodstains by isoagglutination tests in a statement which, even today, is not without a certain irony: "Il ne s'agit plus de distinguer le sang de lièvre du sang d'homme, mais bien de dire que cette tache a été faite par le sang de Pierre, et non pas par celui de Paul ou de Francois". Florence accepted the idea that a positive Landsteiner-Richter test (agglutination) gave an unequivocal exclusion, as did Galli-Valerio (1905). Verdier (1906), who made the test the subject of his thesis, agreed as well.

In 1910, Baecchi took up studies on agglutinins in bloodstains. Part of his study was concerned with heteroagglutinins in stains, i.e., agglutinins in animal bloodstains that would agglutinate human red cells. This property had been proposed as the basis for a species test by Marx and Ehrnrooth, and is discussed in section 16.6.4. The studies that are of interest here had to do with isoagglutinins in bloodstains. Baecchi thought that the isoagglutinins were fairly stable, and that they retained their activity and group specificity in dried stains. The antibodies resisted heat inactivation and putrefactive change. Like Biffi, he tried to carry the investigations further than the Landsteiner-Richter test. If a stain extract agglutinated a person's cells, the stain could not have originated from that person's blood, since autoagglutination is a rare, pathological phenomenon. But if there were no agglutination, the results could not be interpreted in terms of individuality. It meant simply that the person could not be excluded. This point was made by Lattes (1913) and by Lecha-Marzo and Piga (1914) as well. Baecchi found what he regarded as individualizing differences in the agglutination behavior of a person's serum toward a series of test cells of the same type from different people, e.g., an A serum (β) tested against a series of different B cells. These differences, he thought, would still show up in certain stain extracts. Thus under particularly favorable sample conditions, and if a limited number of persons were involved, he thought that a set of "individual" reactions could be obtained. In a large percentage of cases, he said, an exclusion could still be obtained by these kinds of studies (Baecchi, 1910, 1912, 1913 and 1920).

In 1913, Lattes began his studies on isoagglutinins in bloodstains. He thought that one should proceed to devise the practical tests for medico-legal applications on the basis of fundamental principles. Apparently, not everyone in medico-legal circles was familiar with the blood group literature. Bohne (1913), in a paper he read at the 8th meeting of

the German Society for Legal Medicine, was still making reference to Landsteiner's original groups A, B and C. Lattes' 1913 paper dealt with two issues: the Marx-Ehrnrooth test for species of origin, and the detection of isoagglutinins in bloodstains. The former is discussed in section 16.6.4. Lattes did not agree with Baecchi on the "individual" reactions of bloodstain extracts. He admitted that there were differences in the agglutination behavior of a particular serum towards a series of different cells, but he did not think that this fact formed the basis for individual differences that could be used in legal medicine. He noted too that AB bloods have no isoagglutinins, and will give no agglutination test with A or B cells. Such a negative reaction is indistinguishable from one in which isoagglutinins had been present, but had been denatured or destroyed. Negative results in the test could, therefore, not be interpreted. Positive results with A or B cells or both, however, could be interpreted, and Lattes gave in the paper an early version of his procedure for the direction of isoagglutinins in bloodstains, a procedure which is still in use in some places, and is generally called the "Lattes crust test" in this country. Bloodstains belonging to groups O, A or B could be assigned to their proper group using this technique, and this he thought would be of considerable importance in some cases. Lattes carried out systematic experiments on the technique and, in 1915, published extensive reports on the results in both the Italian (1915a) and French (1915b) literature. The studies had primarily to do with standardization of the procedure. A major point was that the stain extract could be neither too dilute nor too concentrated. In the former case, activity would be too low and agglutinins missed; in the latter case, pseudoagglutination (rouleaux formation) could result giving a false positive result. Lattes reported on the application of the test in two cases in 1916. One was a private matter in which a husband was suspected by his wife of infidelity (the investigation exonerated him), but the other was a homicide case, in which it was a matter of deciding whether bloodstains on the suspect's clothing were his own, as he contended, or could have belonged to the victim, as the police thought. In this case, the suspect was O, and the victim A. The bloodstains grouped as O, and the suspect's version of the story was supported in what was probably the first case in the published literature in which bloodstain grouping played a role in a criminal inquiry (Lattes, 1916a). In the same year, Lattes (1916b) published further experiments on his technique, giving further refinements. Among other things, he noted that subjecting the agglutinated cells to dilution would usually distinguish between true agglutination and rouleaux, since the latter would disappear with dilution while the former would be unaffected.

19.10.3 Further developments — bloodstain grouping methods

19.10.3.1 Detection of isoagglutinins — Lattes Test. As is clear from the previous section, all the early efforts were focused on the determination of agglutinins in stains to determine the group. The procedure has undergone many modifications since its introduction by Lattes. It is now clear that the agglutinogens are considerably more stable than the agglutinins, and the test for agglutinins is used primarily to confirm the results obtained with methods which detect the blood group substances. AB bloods always give a negative Lattes test (except see below). O cells may be used as a control for nonspecific agglutinins. The possibility of naturally occurring antibodies in serum other than ABO isoagglutinins cannot be overlooked either.

Sensitivity has been an issue with the procedure as efforts have been made to determine the group characteristics in ever smaller amounts of stained material. It has been the experience of most investigators that the test is more successful on blood crusts than it is on stains in which the blood has permeated the substratum. This result may be a reflection of the concentration of isoagglutinins in the material actually taken for the test. Extraction procedures have been proposed to try and overcome these difficulties, but some have been cumbersome and not very practical. Lattes and Canuto (1926) said that stains impregnated in fabric can be extracted in a minimal amount of distilled water, and the extract dried down on a slide, dropwise, in a current of air in order to form a crust. Lattes (1932) indicated that this procedure required relatively large amounts of bloodstained material and was not very suitable for smaller stains. In 1927, Müller proposed a technique for extracting, and then concentrating the extract by means of evaportion under vacuum. He called the method "Agglutinin-Anreichungsverfahren" (agglutinin enrichment or concentration technique). About 70% of the stains tested could be grouped correctly in this way. 24 hour extraction times were used. Brunner (1927) carried out a number of studies on this procedure. Colloidal solutions, such as gelatin, albumin and gum arabic were added to prevent rouleaux formation, and stains up to 14 months old could be correctly grouped in some cases. Holzer (1931) reported, however, that he had been unable to get good grouping results with this technique, following Müller's prescriptions closely. Lattes (1932) regarded the procedure as unreliable as well. Serebryanikov (1927) in Odessa proposed a somewhat similar procedure, which was discussed by Popoff (1929). Gettler and Kramer (1936) said that they had obtained reasonably good results with the classical technique in blood crusts, but not in bloodstains on fabrics. Faraone (1942) recommended extraction of bloodstains at 45–50°, and said that the procedure improved the results, especially with older stains in which insolubility was a problem. Harley (1943) used an extraction technique as well. Saline was the extraction medium, and in cases where the bloodstain had permeated the fabric, about 0.5 cm^2 of material, finely shredded, was extracted for 24 hrs in the cold. The extract was collected in a capillary tube, centrifuged, and the supernatant tested with A, B and O cells. The reactions were read over a period of hours, up to 24 hours if necessary. Merkeley (1953) used this procedure in his studies. Nickolls (1956) mentioned that the test could be done with trypsin-treated test cells. Marcinkowski (1959) tested a concentrated extract from a stain, obtained by the

paper chromatographic procedure described in section 7.2 for separating bloodstain components from contaminating material, and concentrating them in the tip of the V-shaped filter paper strip. He said that the agglutinins could be detected. Fiori *et al.* (1963), however, said that they had had no success with this procedure. Ducos (1960) suggested that the sensitivity of the test could be increased by using papain treated test cells. Results could be obtained on three year old stains in this way, but some stains, even recent ones, failed to react. Funk and Tostiak (1965) recommended doing the test in 11% bovine serum albumin. Agglutination could occur as soon as 15 minutes after adding cells, or require as long as 24 hrs depending on the condition of the stain. Wiener (1963) recommended the addition of a solution of gum acacia to the test to increase sensitivity. The stain was extracted with saline and the extract placed separately into three tubes with A, B and O cells. These were kept at 4° for a half hour, then centrifuged. The supernatant fluid was removed, replaced with cold saline, and the tubes then read. Two drops of gum acacia solution (10 g in 90 mℓ water containing 1 g monobasic sodium phosphate, and sterilized) were added, and the tubes re-read after 2 hr at room temperature. A third reading after centrifugation was taken as well. Stains up to two years old could be grouped in this way, the agglutination coming up more strongly with successive readings.

The sensitivity of an agglutination test is a sensitive function of red cell concentration at a given antiserum concentration. While this fact is sort of intuitively clear, a good many workers appear to have overlooked it on connection with the Lattes test. Lund (1941) demonstrated experimentally the importance of red cell concentration to the sensitivity of an agglutination test. Many investigators used cell suspensions ranging from 0.5% to as much as 5% in concentration. Lund found that an agglutination test was some eight times more sensitive at cell concentrations of 0.0625% and about 32 times more sensitive at cell concentrations of less than 0.007% than with the usual 0.5 to 5% suspensions. Outteridge (1965a) made a point of these findings in his review. Kind (1955) recommended a simple tube technique in which 0.5% cell suspensions were put in contact with portions of stained material for 2 hours, then removed and read for microscopic agglutination. Outteridge (1965b) recommended a very similar technique, except that the test was performed in well slides, and 0.01% indicator cell suspensions were employed. Kissling and Neumann (1972) proposed a curious technique for the agglutinin test. They employed suspensions of test cells which were sensitized with the homologous antibody. Sensitization was accomplished by treating the cells with dilutions of antisera eight times more dilute than the titer value (3 tubes more dilute than the last tube giving agglutination). The procedure, they said, gave greater sensitivity. The idea for this approach was based on results obtained by Schulz (1970) in which he had found that such sensitized cells gave greater sensitivity in the mixed agglutination test (see in subsequent section).

Outteridge (1965a) made the important point that the extraction of stains for the agglutinin test with media that do not contain the test cells are not to be remommended, especially where extraction times are relatively long. Many substrata contain substances that absorb α and β agglutinins. Wool can be a particular problem in this way. For this reason, cloth controls are always done in carrying out the absorption tests (see in next section). In the course of a long extraction, the substratum material could absorb a substantial portion of the solubilized antibody, leaving little to react with test cells. If cells are present, they are at least able to compete with substratum receptors, should the latter be present, for liberated antibody. Another point, made by Moureau (1963) in his review, is that test cells should be carefully and thoroughly washed. The reason is that it has been known since the experiments of Schiff (1924b) (see in section 19.8.1), that serum can contain soluble blood group substances corresponding to the red cell type, and these could compete with the test cells for available agglutinin, thus decreasing the sensitivity of the test. The issue of non-ABO isoantibodies being present must be considered. Moureau (1963) recommended carrying out the test in duplicate with both Rh+ and Rh− test cells. This procedure would detect any saline agglutinating anti-D (anti-$\mathbf{Rh_o}$) in the bloodstain. The incidence of natural antibodies to Rh, MNSs, Kell Kidd and Duffy antigens is not very high, and most of them are not saline agglutinating. Giblett (1977) found that less than 1% of a very large series of blood donors in the Seattle area had such antibodies, but the possibility should not be overlooked if the Lattes test results are not in agreement with the results of the tests for ABO antgens. Most investigators have recommended the use of A_1 test cells for α in the Lattes test. In this regard, the occurrence of the so-called "irregular" α_1 agglutinins in A_2 and A_2B sera, which is not all that rare in some populations (see section 19.3.1.1), should not be overlooked (see the case discussed in section 19.10.7).

There have been many conflicting reports on the persistence of the agglutinins in bloodstains. Under favorable conditions, the agglutinins can apparently persist for considerable lengths of time. Detectability is bound to be dependent upon the techniques employed; and solubilization as well as optimization of the concentrations of agglutinins and cell suspensions are undoubtedly important considerations. Levine (1932) said that the agglutinins in a four year old O stain had been detected. Matta (1937) found that, in many cases, the agglutinins could no longer be detected in stains 3 months old, although in some samples, they were detectable in 6 month old stains. Balgairies and Christiaens (1937) found that about three-fourths of 3 month old group O bloodstains retained both agglutinins, while some had lost one or the other. About three-fourths of the A stains still had β agglutinin, and the B stains all contained α. Stains aged in sunlight showed somewhat greater losses of agglutinin in all groups. Kayssi and Millar (1937) indicated that the strength of the agglutinins was not attenuated by exposure of 10 day old stains to a dry 64° heat for an hour, nor by exposure to

ultraviolet radiation for the same length of time. A one hour exposure to 100° dry heat, however, resulted in considerable diminution of activity. Faraone (1942) could always detect agglutinins in stains 7-11 months old with his warm extraction procedure, and occasionally in older stains as well. Merkeley (1953), using Harley's (1943) procedure involving long extraction times and long incubation times with test cells, examined 100 stains 12 to 18 months old for agglutinins. Anti-A could be identified in 34 of 43, and anti-B in 17 of 43 group O stains. Anti-A could be found in 12 of 15 group B stains, and anti-B in 13 of 36 A stains. These results indicated that the β agglutinin in O stains is somewhat more labile than the α agglutinin, in accord with what Balgairies and Christiaens (1937) had reported in a smaller sample of more recent stains. Marsters and Schlein (1958) found that, in some cases, agglutinins could not be detected after 5 days, while in others, they were detectable in stains up to three weeks old (when the experiment was terminated). Wiener (1963) got the correct result in 3 A and 3 O stains, which had been kept cold for two years, using the gum acacia technique. Haseeb (1972) said that the agglutinins could still be detected in a B, 2 O and 2 A stains after 12 years at room temperature on the lab bench. Outteridge (1965a) studied the effect of humidity and heat on the disappearance of agglutinins in stains prepared on filter paper. Humidity was the most damaging condition. The agglutinins were more stable cold than either warm or hot, those stains kept at 50° being inactive after 2 weeks in a humid chamber, and after 2 months if kept dry. Stains kept at 4° in a dry container retained activity the longest. At 2 months, and at 9 months, the A and B stains could still be grouped correctly, but the β agglutinin had disappeared in the O stain. The β agglutinin had been weaker than the α in the O stain to begin with.

Many years ago, there was some interest on the part of clinical serologists in storing grouping antisera in the dried state, i.e., simply dried out on slides. A number of experiments were done to see how long such sera, dried on glass slides or cover slips, retained its agglutinating activity. Sanford (1918) said that dried serum remained active for two months kept at room temperature. Kolmer (1919a, 1919b and 1920) found that isoagglutinin activity had already begun to decrease in dried serum within the first to fourth day. If it were kept cold, it could be used up to two weeks. Karsner and Koeckert (1919) said that the isoagglutinins deteriorated within 2-3 weeks in the dried serum, and after 3-5 weeks lost their group specificity. They said that in 7-10 week old dried serum, they always observed non-specific agglutination. These experiments with dried out serum are, of course, only peripherally related to the issue of agglutinin survival in dried out whole blood, but if the results are correct, they would seem to show that the agglutinins survive considerably better in dried out whole blood than in dried out serum.

19.10.3.2 Detection of agglutinogens in bloodstains by absorption technique—absorption inhibition or agglutinin binding.

• Development of the Technique

This procedure derives directly from the absorption techniques that have been used with red cells. It was a number of years after the discovery of the ABO groups before the technique was applied to dried bloodstains. Medico-legal procedures had been focused on the detection of the agglutinins, as noted above.

The absorption precedure has many variations, but in principle is quite simple: A bloodstain containing an agglutinogen placed in contact with an antiserum containing the corresponding agglutinin will reduce the titer of the agglutinin in the antiserum. This technique is often called "absorption-inhibition" in this country, and sometimes too in England. The Germans have usually called it "Agglutininbindung" technique.

It had been known since the early work of Landsteiner and others that red cells containing an agglutinogen would selectively absorb the corresponding agglutinin from an antiserum containing it. There does not appear to be a record in the published literature of anyone having applied this principle to bloodstains prior to 1921. In that year, H. Schütze at the Lister Institute in London recorded a number of relevant experiments. A case had come to his attention in which some blood was alleged to have poured forth supernaturally from a holy picture, and there was interest in determining not only whether this dried blood was of human origin, but also in determining its blood group if possible. Schütze said that the blood was human by the precipitin test, and that the case had prompted him to conduct the experiments reported. Apparently unaware of Lattes' efforts, he indicated that the agglutinins could be detected in dried bloodstains, and the group determined in this way, unless the stain were of group AB, in which case the negative results would be indistinguishable from those from a stain of a different group in which the agglutinins had become inactive. A technique for determining agglutinogens would be quite desirable for this reason, he said. In the experiments, he dried blood in Petri dishes and on cloth substrata, and reconstituted it in saline. Group O serum was added, and, after suitable incubation, tested again to see which agglutinins had been removed. A group B stain removed the anti-B from O serum after the stain had aged 41 days in sunlight, and a 5 month old group A stain removed the anti-A agglutinin.

It is clear that Lattes, then in Messina, and Siracusa in his institute, had carried out a number of experiments on the inhibition technique in the 1922-1923 period, and perhaps earlier (Lattes, 1923; Siracusa, 1923). Outteridge (1965a) mentioned that the inhibition method had been attributed to the Japanese investigator Kwansuke Sera in 1926, but he said that the method probably pre-dated this work, as indeed it did. The reference to Sera's work, which I could not examine, is probably: *Shakai Igaku Zasshi,* number 474, page 375 (1926). Schiff (1926) gave a technique for determination of the agglutinogens by the inhibition method (Der Agglutininbindungsversuch). He did not recommend using O sera, which contains both agglutinins, for the test because of differences in the agglutinin titers. Many other investi-

gators used O sera. He stressed the importance of substratum controls as well, to detect nonspecific agglutinin binding. Siracusa (1923) conducted extensive experiments on the absorption of agglutinins from O sera by bloodstains which had been treated with heat and a variety of chemicals (see in Unit IX, Translations). An O serum was chosen in which the α and β agglutinin titers were about the same. The serum was diluted 1:3 and incubated with appropriate bloodstained material. After a number of hours, the preparation was centrifuged, and an aliquot of the supernatant fluid tested for agglutination with A and B cells. Exposure of dried blood to 100° dry heat did not abolish the ability of the agglutinogens to absorb homologous agglutinin. Exposure of dried blood to a number of denaturing chemicals, including 1N HCl, 0.1N NaOH, ammonia vapors, 5% HgCl, 1% $AgNO_3$, 3 to 6.7% $K_2Cr_2O_7$, 1% OsO_4, $CHCl_3$, acetone and ethyl ether, did not destroy the agglutinogens. Acetic acid and ethanol inhibited the uptake of agglutinins, but did not completely abolish it, while treatment with 2.4% $KMnO_4$ led to nonspecific uptake of agglutinins (or their destruction). Aliyev (1927) described an inhibition technique with which he said there was no problem in grouping bloodstains up to 8 weeks old. Popoff (1929) discussed the use of this and other blood grouping methods for dried stains in Russia. Higuchi (1929) carried out experiments on agglutinin absorption. He recommended the use of A and B sera, rather than O sera, because he said the absorption characteristics were better. Antisera at a titer of about 1:30 were recommended for the test and absorption was carried out for three hours. He said that correct results had been obtained with some stains that were 21 years old.

In 1931, F. J. Holzer in Innsbruck published an important paper on the agglutinin binding method. His procedure, which became the basis for that used in many laboratories for a number of years, was adapted from the one used by Lattes and Siracusa, except that Holzer checked the degree of inhibition by titration of the antiserum after the absorption process. He employed selected O sera with initial titers of between 1:16 and 1:64 (microscopic agglutination after 30 min incubation). A small amount of bloodstained material was incubated with test serum in the cold for 24 hours. The supernatant serum was then titrated against A and B cells in well slides, and the titer compared with that of unabsorbed serum. A number of materials were tested for nonspecific agglutinin binding, and although most were negative, the importance of substratum controls was stressed, In some 387 tests, the group could be correctly diagnosed in 366 cases. Holzer found that A agglutinogen generally gave greater reductions of α titer than did agglutinogen B of β titer. With initial titers of 1:16, the α agglutinin was usually reduced between 3 and 4 dilution steps, while β was reduced between 2 and 3 steps. The reduction was less marked with lower titer serum, and Holzer said that higher titer antiserum should be employed. Note that he was comparing antisera with titers of 16 and 8 in these experiments. One cannot employ antisera at too high a titer for inhibition or no inhibition will be seen. He tested a number of old bloodstained objects in the collection at his Institute, and although there was no way of knowing the correct group in these cases, the combined distribution of groups agreed well with the Innsbruck population distribution for the ABO groups.

From the foregoing, it is clear that there are two general ways of doing the test. One has been called the "all-or-none" method. In it, relatively low titer antisera is incubated with an amount of bloodstained material or extract sufficient to remove all the corresponding agglutinin. From the complete removal of antibody (absence of agglutination upon addition of test cells) is inferred the presence of the corresponding agglutinogen. This method was employed by Siracusa (1923), Higuchi (1929), Schiff (1932), Wiener (1939), Schiff and Boyd (1942) and White (1954). The other alternative is the method of Holzer, in which the antiserum is titrated after absorption to determine the number of doubling dilution steps by which the test serum titer has been reduced by the homologous agglutinogen. Many authors have recommended this technique, or some modification of it (Therkelsen, 1934 and 1936; Boyd and Boyd, 1937; Harley, 1943; Dahr, 1953; and Tan and Wong, 1963). Funk and Towstiak (1965) recommended a technique similar to that of Holzer, except that they added a drop of 11% BSA to the test tubes following the absorption step and incubated an additional 5–10 minutes in a moist chamber before carrying out the titration.

• Sensitivity

One of the problems with the inhibition test is its relative insensitivity. Fairly large amounts of material are needed to carry out the test. Holzer (1931) recommended 10 mg dried blood for each 0.1 mℓ antiserum used. Other authors (e.g. Therkelsen, 1936) have suggested even larger quantities of blood, up to as much as 80 mg. Various modifications have been proposed to increase the sensitivity of the procedure and reduce the amount of material thus required. Ponsold (1934) described a technique in capillaries which could be carried out on 1 mg dried blood. Hausbrandt (1938) gave a modification of the capillary procedure for which only 0.2 mg dried blood was said to be required. Tan and Wong (1963) used a slide technique and titrated in 1½ fold dilution steps. They said the method was applicable to 0.5 to 0.7 mg dried blood. Kishino (1955) used long absorption times, and with incubation at 37°, then room temperature, then cold, and said that the technique was applicable to 0.04 to 0.1 mg dried blood. He also said that in older stains (1–2 years), a 1–2 hour treatment with 0.3–0.5% solutions of trypsin at 37° made the bloodstain much easier to group by the inhibition method. Obviously, the amount of dried blood required for the test is a function of the titer of the test sera. More material is required to inhibit a higher titered serum.

• The Use of O (Anti-A,B) Sera

The issue of using O sera as against separate anti-A and anti-B has been discussed by many workers. The early workers used O serum. Those who have argued against using it have done so principally on two grounds: first, that the α and β agglutinins are often not of equal titer (Schiff, 1926); and

second, that A stains can absorb some cross reacting antibody, capable of reacting with B cells, and B stains can absorb some antibody capable of reacting with A cells, from O sera. On the first point, Schiff (1926), Schiff and Boyd (1942), Dahr (1953) and Moureau (1963) among others have advocated the use of anti-A and anti-B serum. Harley (1943) did not accept difference in agglutination titer as an argument against the use of O serum. Since the titer difference is determined against a control for purposes of interpretation, he did not think it mattered whether the initial titer of anti-A and anti-B were the same or not. The second matter is more serious. This property of some O sera, wherein B cells can appear to absorb α, or A cells β, was discussed in section 19.7.2. Harley (1936) found an O serum which showed this behavior with an A bloodstain. The phenomenon was confirmed with A cells. B stains or cells did not absorb any anti-A from this serum, however. Harley said, therefore, that O sera must be carefully evaluated for this characteristic, and rejected if they have it. But he did not think it was necessary to abandon the use of O serum for the test, and he still recommended it in his book in 1943, provided it had been evaluated. Many workers, though, prefer simply not to use it. The only advantage to it appears to be a saving of one tube, and, of course, twice as much bloodstain is needed if separate anti-A and anti-B absorptions are set up. Some authors have suggested mixing anti-A and anti-B in equal parts (e.g. Tan and Wong, 1963). The principal objection to this practice is that the serum of A and B secretors can contain significant amounts of A and B substances, thus reducing the titers of the anti-A and anti-B unequally (Schiff and Boyd, 1942). Schiff and Boyd recommended that immune anti-A and anti-B be used in the test along with isoantisera. These reagents were mixed together in equal proportions to make an anti-A,B reagent, since the animal serum would not contain any interfering soluble A or B substances.

- Interpretation

Most authorities have indicated that interpretation of these tests be made with caution, especially when only anti-A and anti-B reagents are used. With bloodstains, the test for agglutinins should always be carried out along with the absorption test. The major difficulty is in the diagnosis of weak A receptors and of group O. Negative absorption tests for anti-A and anti-B constitute necessary but insufficient evidence for the diagnosis of a group O stain. A diagnosis can be confirmed only if positive results are obtained for the presence of both the α and β agglutinins, or if a specific anti-H reagent is used (see below). It is more difficult to diagnose A_2 than A_1 in stains because its absorptive power is lower. A_2 in A_2B stains is weaker still. In this connection, Therkelsen (1934 and 1936) recommended that A_2 test cells be used along with A_1 test cells as indicators for A. If an anti-A serum containing both α and α_1 is used with an A_2 stain, the α is absorbed but not the α_1, and the use of A_1 test cells exclusively might cause one to miss an A_2 altogether in an inhibition test. Wiener (1939) and Schiff and Boyd (1942) completely agreed. Schiff and Boyd said that they would not in fact exclude the possibility of a stain which grouped as B being an A_2B when they reported their results. Similarly, stains which gave negative results with absorption tests with anti-A and anti-B, but in which no agglutinin test results were available, would be said to be "probably of group O", but with the possibility left open that they could be of A_2 or a weaker subgroup of A. Benciolini and Cortivo (1977) said that they had not been able to group the A in A_2B stains successfully using the inhibition technique.

- Inhibition with a Doubling Dilution Titration Series of Antisera

In 1932, Hirszfeld and Amzel introduced a somewhat different way of carrying out the inhibition test, which requires more work and needs more material, but which makes the test considerably more sensitive. The procedure was advocated by Kind (1955). Here, a constant amount of antigen (bloodstained material or extract) is incubated with a series of doubling dilutions of antisera, the entire series being tested for remaining agglutinin by the addition of test cells. Kind (1955) discussed the difference between this procedure and the classical (Holzer) technique, as did Outteridge (1965b) in his review. Suppose an antiserum with a titer of 1:256 is incubated with an amount of antigen-containing material sufficient to remove half the agglutinin. If this absorbed serum is titrated, it will show only a "one-tube" reduction, that is, the titer will be reduced to 1:128. Twice that amount of antigen would reduce the titer of the 1:256 antiserum to zero, though. On the other hand, if the same amount of antigenic material (an amount sufficient to remove half the agglutinin from a 1:256 antiserum) were set up by the Hirszfeld and Amzel-Kind technique, the "titer" would be reduced down to "neat" (i.e. 8 steps). This behavior is illustrated in Figure 19.12. In the classical technique, an amount of antigenic material which reduces the agglutinin in the test serum by one-fourth would be missed altogether. Kind (1955) pointed out that the titration procedure in the classical technique does not yield very much more information, for the amount of extra work involved, than does an "all-or-none" test. The all-or-none test is done with relatively low titer antisera (1:8–1:16) to compensate for the antigen content in relatively small amounts of bloodstain. The problem with such a procedure is that if there is significant nonspecific absorption by the substratum, the substratum control will show a reduction along with the bloodstain in the tests, and make the test results impossible to interpret. With higher titer antisera, the nonspecific absorption would generally be much less noticeable. Fiori *et al.* (1963) said that the "all-or-none" procedures should not be used because of this problem. Schleyer (1957) compared the classical technique with the Kind modification, and found that the classical technique was preferable. Alfultis (1965) described a microprocedure for carrying out the Hirszfeld and Amzel-Kind modified procedure.

- The Problem of Non-Specific Absorption and Interference Due to Contamination

The problem of nonspecific absorption by substratum materials has been periodically investigated. Zipp (1931) found

Figure 19.12 Comparison of Results of Inhibition Test by Holzer vs. Hirszfeld and Amzel-Kind Techniques

Doubling Dilution Titration After Absorption (Holzer):

Condition	Reciprocal Dilutions: neat	2	4	8	16	32	64	128	256	Result
No Absorption (Control)	+	+	+	+	+	+	+	+	+	———
¼ Antibody Absorbed	+	+	+	+	+	+	+	+	+	No reduction
½ Antibody Absorbed	+	+	+	+	+	+	+	+	—	One tube reduction
Complete Absorption	—	—	—	—	—	—	—	—	—	Complete reduction

Doubling Dilution Titration Before Absorption (Hirszfeld and Amzel-Kind)

Condition	neat	2	4	8	16	32	64	128	256	Result
No Absorption (Control)	+	+	+	+	+	+	+	+	+	———
¼ Antibody Absorbed	+	+	—	—	—	—	—	—	—	Seven tube reduction
½ Antibody Absorbed	+	—	—	—	—	—	—	—	—	Eight tube reduction
Complete Absorption	—	—	—	—	—	—	—	—	—	Complete reduction

that a number of materials used in textile manufacturing would absorb agglutinins. Werkmeister-Freund (1932) did not encounter problems with nonspecific absorption in bloodstains mixed with clean sand, garden soil or sawdust. Holzer (1931) in the original paper had noted that the mud in a sample of blood-soaked mud showed some absorption of agglutinins, which interfered with the test. Berger (1933) studied 64 dirty bloodstains by the absorption technique and found that 14 of these gave problems due to non-specific absorption of agglutinins. Therkelsen (1936) examined 179 different fabric samples from underwear, regular clothing and outerwear. In 19 cases, absorption was observed, most often of α when tested with A_2 cells. A three tube reduction was seen in some cases. He also examined the absorption properties of papers and the bark and wood of a number of trees. Parchment gave substantial absorption, as did the wood and bark from trees, including chestnut, beech and fir. A number of mineral materials, such as zinc and aluminum powders, and finely pulverized marble, feldspar, kaolin, silica gel and talcum gave no absorption. Guareschi (1937) reported nonspecific absorption by silk, and Granata (1955) observed it with cotton but not with wool. DeRen *et al.* (1970) found that stains on nylon created serious problems in attempting to get an ABO group by the inhibition procedure.

A related problem is that of contamination of the bloodstain with various materials or laundering agents. Siracusa's experiments (1923) on the effects of a variety of chemicals were discussed above. Eisele (1973) said that blood treated with 10% NaOH, 1% acetic acid or 1% $FeCl_3$, and then used to make stains could yield false positive inhibition results upon grouping the stains by the Holzer method. 1% eosin and 1% dextrose treatment had no deleterious effect. Depending upon how the test is carried out, the contaminants may have an effect on the antiserum or on the test cells. Kirk *et al.* (1954) raised this problem in connection with the presence of laundering agents in fabrics. With the detergents that were (and are) available, they noted that much less rinsing was required than was formerly the case with soap, and that there was a stronger possibility of the presence of residual detergent in a fabric. It could be shown that these agents caused lysis of the red cells when present in higher concentrations, and when present in lower concentrations, gave inhibition of antisersa in the agglutinin binding test indistinguishable from that of group specific substances (false positive). They had looked into a procedure for alcohol precipitation fractionation of the agglutinogens in such cases, but said that they had had limited success. Kind (1955) found that the hemolysis observed in the presence of soap or detergent residues in cloth was proportional to the soap solution:AB serum content of the antiserum used. The AB serum apparently served as a protein medium in which to dilute antisera, and protected the cells from hemolysis. However, AB serum can contain A and B substances, which can reduce the titer of the antiserum for which it serves as diluent (Kind, 1956) and it was found that 3% BSA worked just as well for the purpose as the AB serum. Marsters and Schlein (1958) tested the effect of a number of potential contaminants on the inhibition test, and did not find interference, except that the presence of soap or detergent could cause hemolysis of the cells. Simon (1964) tested the effect of two detergents on linen and wool for its effect on grouping by the Holzer technique. The experiment ran for 43 days. Bloodstains alone and detergent residue alone gave the same results with the inhibition test in terms of titer reduction during the entire course of the experiment. Bloodstains on detergent-residue containing cloth, however, were progressively less active with time, i.e., less and less group specific inhibition was observed, and after a few weeks, the group could no longer be determined. Tomita (1967) presented a method for extracting interfering detergent and surfactant materials from bloodstains by chloroform and other solvent extraction procedures. Gramer (1975) investigated the effect of several detergents, a fabric softener and an enzyme-containing pre-treatment product on the determination of the group in bloodstains by the Holzer technique. The enzyme product contained a protease derived from *B. subtilis*. A variety of fabrics were washed or dipped in these materials, and then artificial bloodstains were made on them for the experiments. A number of the fabrics themselves showed nonspecific absorption of agglutinins, and treatment with the cleaning agents in general had a more or less deleterious effect on the test. In a few cases, hemolysis was noted. The importance of comparing a bloodstain test to a proper substratum control test was stressed. While in a number of cases, no results could be obtained, there were no false positives with A_1, A_2, B or O stains. AB stains could be misdiagnosed. Kijewski and Mülmann (1975) found that dried blood mixed with various soils could not be grouped reliably because the concentration of agglutinogen was apparently too low. Clean sand was an exception. With some concentrating procedure, e.g., the filter paper chromatographic technique (section 7.2), the group could be determined.

- Lectins

The unequivocal determination of group O stains was a serious problem with the inhibition method until the development of anti-H reagents. Many authorities felt that a diagnosis of group O could not be made on the basis of negative absorption tests with anti-A and anti-B, unless the presence of both α and β agglutinins could be demonstrated. Before the discovery of useful anti-H lectins, the so-called anti-O reagents—usually animal serum of some kind absorbed with AB cells—were not easy to obtain. Ox serum was a common source of the reagent. Wiener (1939) pointed out the value of such reagents in the inhibition test, and showed that a reagent he had prepared was inhibited by O and by A_2 bloodstains, but not by B or A_1 stains. He said, however, that the reactions were not always clear cut. In 1958, Wiener *et al.* introduced the use of anti-H lectin from *Ulex europaeus* for the test. The reagent used had a titer of about 1:8 to 1:16 against O or A_2 cells. Many investigators began to use anti-H reagents in the test as more sources were discovered and became available (e.g. Funk and Towstiak,

1965). Kissling and Neumann (1972) used an anti-H from *Laburnum alpinum,* and did not get good inhibition results with it at higher titers (1:64 to 1:128). At lower titer values, it worked well. The higher titer material could be used, however, and sensitivity retained, if O test cells, previously sensitized with subagglutinating concentrations of lectin, were used. Potapow (1970a) recommended the use of an anti-B lectin from *Evonymus alata* for the inhibition test. Tryspin treated B test cells were used.

• Testing of the Immunoglobulins in Antisera

It may be noted that Misawa (1968) fractionated the immunoglobulins of anti-A in order to separately test them in the inhibition procedure. IgM was found to be the most easily absorbed antibody (among IgM, IgG and IgA), and test sera containing high concentrations of IgM were recommended for this test.

• Gamma Globulin Deviation Procedure

A rather complicated variation of the inhibition test may be mentioned for the sake of completeness. In 1957, Dell'Erba and Ambrosi proposed to quantitatively assess the amount of antiserum remaining after absorption by means of paper electrophoresis, followed by densitometric quantitation of the gamma globulin fraction. This procedure is the same one as was proposed by Ambrosi for species differentiation (section 16.6.7). Adamo (1957) believed the technique had promise, and recommended it. Laudanna and Segre (1959) criticized the procedure. They said that the extraction of serum protein from the stain and hemoglobin contamination due to hemolysis in the stain interfered with the determination. Dell'Erba and Ambrosi (1960) said that these points were not of practical importance in the method, and did not accept the criticism.

19.10.3.3 Detection of agglutinogen A by inhibition of hemolysis. This technique is mentioned as a matter of historical interest. It was never widely employed in practice, probably because it was never really applicable to group B. Rabbit antisera to group A cells, or alcoholic extracts of them, are also potent sheep red cell hemolysins. Similar sera can be obtained by immunization with sheep red cells. A bloodstain can be tested for its ability to inhibit the subsequent hemolysis of sheep red cells in the presence of complement (e.g. guinea pig serum). From the inhibition of hemolysis is inferred the presence of the A antigen in the bloodstain. The assay is very sensitive. It was put forward by Witebsky in 1927. Hirszfeld and Amzel (1932) confirmed the value of the test for group A stains, and Jadin (1934) thought that the test ought to be used in conjunction with the Lattes test and the agglutinin binding test.

19.10.3.4 Detection of agglutinogens in bloodstains by absorption-elution technique

• Development of the Technique

Absorption-elution is probably the most widely used technique for grouping bloodstains at the present time. The technique is based on the principle that the agglutinogens in dried blood will bind their corresponding agglutinins, the antigen-antibody complex can be dissociated subsequently, usually by raising the temperature, and the agglutinins recovered and detected by agglutination of appropriate, exogenously added test cells. The technique is very sensitive.

The development of the technique grew out of the early studies on agglutinin-red cell reactions, which showed that the antigen-antibody reaction was reversible and that binding was temperature dependent. In 1902, Landsteiner showed that serum agglutinins, absorbed onto red cell receptors at lower temperatures, could be recovered from the cells by raising the temperature to 40–50°. The temperature dependence of agglutinin binding to red cell receptors was studied in detail by Landsteiner and Jagic (1903), and in the same year, Landsteiner indicated that "cold agglutinins" in animal bloods could be recovered from cells by raising the temperature to 20–30° (Landsteiner, 1903). These results were confirmed by Koeckert (1920), Jervell (1921) and many others. Huntoon (1921) eluted bacterial antibodies from their receptors in the same way.

In 1923, Siracusa, working in Lattes' Institute at Modena, first applied the principle to the grouping of bloodstains. He subjected bloodstains to the action of heat, and to a variety of chemicals, and then tested them for their ability to absorb agglutinin (as indicated by the inhibition procedure). He then washed the cellular residue in the cold, added a drop of saline, raised the temperature to 45–50°, centrifuged using a water mantle maintained at 45°, and tested the eluate for agglutination with appropriate test cells. These experiments are discussed in section 19.10.3.2, where the absorption results are given. Absorption was carried out for a number of hours in the cold using O (anti-A,B) serum. In general, those residues which retained their group specific agglutinin binding (inhibition) properties (section 19.10.3.2) would also yield up the bound agglutinins after washing and incubation at 45–50° for 15 min. The paper has been translated, and may be read in Unit IX. In 1927, however, Siracusa called the reliability of the method into question, based on some discordant results that had been obtained with the inhibition and elution procedures. The elution test showed that nonspecific absorption of agglutinins had occurred, which had not been detected by the inhibition test. The problem was not believed to be the result of isoagglutinins present in the stains themselves. Undoubtedly, it had to do with the cross-reacting antibodies in O serum (section 19.7.2), and the fact that the elution test is considerably more sensitive than the inhibition test. Siracusa said that the elution test should be used as a means of confirming the results of the inhibition test. Lattes (1928) mentioned that cold agglutinins in the antiserum might be causing part of the problem of apparent nonspecific absorption in the elution test. Popoff (1929) proposed certain modifications of the elution test, including heating the stained material to 70–80° for a half hour to inactivate the isoagglutinins, to which he attributed the nonspecific reactions. He also recommended washing with, and elution into phosphate buffered saline. Popoff also said that absorption and elution could be carried out a number of times on the same piece of stained material. The elution step could be done several times into the same saline, or else several eluates could be combined

and concentrated, in order to avoid negative results caused by the level of eluted antibodies being too low. Siracusa, in the original work, had tried this device as well. Siracusa (1937) recommended against the use of either O sera, or mixtures of anti-A and anti-B for the elution test. He gave a lengthy procedure, in which the stain was heated to destroy its agglutinins. The inhibition test was then carried out with dilute antisera, followed by reincubation of the stain with strong antisera for the elution test. The stain was then washed with 45° saline, and reheated to 80° to inactivate any agglutinin still present, and the inhibition and elution procedure repeated again with different antisera. In 1941, Faraone working in Siracusa's Institute, investigated the reasons for the nonspecific reactions. He thought that cold agglutinins could be a problem, since absorption was carried out at 4°, and said that cold agglutinin-free serum had to be used for the test. Separate anti-A and anti-B were recommended as well, because of the cross reacting antibodies in some O sera. The phosphate buffered saline recommended by Popoff (1929) was found to inhibit specific agglutination reactions. Schiff (1926) described an absorption-elution technique in his book, calling the procedure "Absprengung des gebundenen Agglutinins". A 2–24 hour absorption in the cold with an equal mixture of anti-A and anti-B were recommended. The problem of false positive results because of nonspecific absorption was discussed, and Schiff regarded the test as confirmatory to the inhibition procedure results. Elution results which did not agree with those of a properly executed and controlled inhibition test which had given good results, he said, should not be taken seriously. Throughout this period, and up until 1960, the technique does not appear to have been widely employed as a means of bloodstain grouping.

Subsequent development of elution techniques took place in England, and the procedure has undergone numerous modifications. It is quite widely used at the moment for the grouping of bloodstains. Kind resurrected the procedure in 1960, and showed that it could be used to group blood crusts on glass slides. Crusts, consisting of 5–10 $\mu\ell$ of blood per cm^2 on slides, were fixed by plunging into 100° McIlvaine's buffer for 30 sec, [a citric-acid-phosphate buffer (McIlvaine, 1921)]. Absorption was for 3 hours at room temperature with occasional agitation and, after rinsing the material with ice cold saline, test cells were added and the temperature raised to 50° for 5 min (Kind, 1960a). Very soon afterward, Kind (1960b) said that the technique, essentially the same one as applied to blood crusts, could be applied to bloodstains on fabrics, a piece about 2 × 5 mm being used for each antiserum being tested. Kind elaborated on the technique in 1962. Among other things, he noted that fabric samples can and do show some nonspecific absorption of agglutinins. The use of thin test cell suspensions was not recommended as a means of increasing the sensitivity, therefore, because one might very well observe agglutination with the cloth control. The fixation in boiling McIlvaine's buffer (pH 7.4) was said to create an impermeable layer of protein on the fabric or surface of bloodstained material, so that nonspecific absorption of agglutinin was inhibited. Evidence that this was so came from showing that a fabric which did absorb anti-A nonspecifically would, after impregnation with group O blood, drying and fixation, fail to do so. Treating the bloodstained material with trypsin solutions inhibited absorption. The fixation process was said to have the additional advantage that it removed soluble soap or detergent material which might hemolyze the test cells, and Kind said that oily contaminants could be washed out of the bloodstain with petroleum ether prior to absorption. Fixation was also said to remove any soluble ABH substances that might be present. Kind said that 68 of 70 samples of dried blood on filter paper, sent by Dr. Dunsford for blind trial, had been correctly grouped. These included 35 A_1, 16 A_2, 2 A_1B, 4 A_2B, 7 B and 6 O stains. Two of the A_2 stains showed no absorption, perhaps because the artificial stains had been made from clotted whole blood specimens, and contained mostly serum. Kind said that a result was not regarded as certain unless the elution results were confirmed by the Lattes test.

Outteridge (1962a) confirmed the value of Kind's procedure, and said that aqueous extracts of stains dried in well slides had worked very well in his hands. A and B were detectable in 10 μg material, and H in 200 μg. Yada (1962) also reported that the technique worked well. He used an alcohol fixation, and did his absorption at 37° for 1 hour. He used 0.1–0.2% cell suspensions, and said that the technique was sensitive down to 0.001 mg dried blood for A and B. In 1961, Schleyer had tried out the technique, but he used tiles rather than cavity slides. He also used an anti-H reagent from *Laburnum*. A_1, A_2 and B stains that were relatively fresh could be correctly grouped, but A_1 and A_2 could not be differentiated (they grouped as A), and problems were encountered with A_1B and O stains. Non-specific anti-B absorption was noted with an A_2 stain, but also with the cloth control, and perhaps could have been accounted for in the bloodstain by insufficient washing. Schleyer recommended that the test be used along with the inhibition test. Outteridge (1962b) noted that Schleyer's difficulties may have arisen from his use of A_2 test cells, the use of *Laburnum* rather than *Ulex* anti-H which perhaps had too low a titer for elution technique, and from the employment of test cell suspensions that were too concentrated. In 1962, Nickolls and Pereira recommended a modified elution technique in which the boiling water fixation step was eliminated, because it was found to reduce the reactivity of the stain considerably. Threads from bloodstained fabrics were employed, rather than larger pieces, because they were easier to wash thoroughly. The test was carried out in cavity slides, with elution at 50° for 10 min into saline containing 1% BSA. Takagi (1968) found the procedure to be completely satisfactory. Fiori *et al.* (1963) proposed an elution procedure carried out in tubes, for which 2 to 3 threads or 0.5–1.0 mg dried blood (pulverized), previously fixed for 15 min in methanol, was used. Absorption was carried out at 4° for 5–6 hours or overnight. The test material was washed 3–4 times with cold saline, appropriate test cells being added

to the last washing, and checked for agglutination to insure that excess antiserum had been washed out. Elution was at 56° for 10 min with gentle shaking into 30–50 $\mu\ell$ saline, which was removed before adding the test cells. Budvari (1963) found this procedure to be completely satisfactory. Göring *et al.* (1969) reported a much higher success rate in grouping stains by Budvari's procedure (actually Fiori *et al.*'s procedure) than with either the Lattes technique or the Holzer inhibition technique. LaCavera and Scafidi (1972) used the technique as well, except that the mixing of the eluate and the test cells could be carried out in a fine bore capillary. After reading, a little methanol could be added, the ends sealed, and the capillary saved. Kind (1963) raised the issue of antiserum selection in connection with the elution test, as well as the inhibition and mixed agglutination (section 19.10.3.5) tests. Goodman (1962) had conducted experiments in which columns of polyurethane support media and formalin treated red cells were prepared, and agglutinin binding studied as a function of temperature. There was significant heterogeneity in the antibody population with respect to the temperature at which the molecules eluted from the column. Kind said in 1963 that these properties of antisera had to be taken carefully into account in the selection and preparation of antisera for the medicolegal tests. Further work on the subject was recommended.

• Further Modifications

A technical modification of the elution test, which is quite useful for routine work, was proposed by Outteridge (1963). Bloodstained threads or fibrils teased out of the fabric were glued into the wells of well slides by one of the ends. Absorption, washing and elution could then be carried out on the same slide. The mountant gave no interference with the test. Outteridge said that single threads could be used, but that he usually used more if there was sufficient material. Howard and Martin (1969) described a very similar technique for single threads on cellulose acetate sheets. Threads from the stained material, about 3 mm long, were affixed to the cellulose acetate using a glue made from cellulose acetate shavings in acetone. Absorption at 4° for 3–24 hours, washing with cold saline or water, and elution at 50° for 15 minutes into added test cells could all be conveniently carried out directly on the threads on the cellulose acetate sheet.

Ueno (1963) proposed a "transfer" technique for carrying out absorption-elution. A thread 6–10 mm long was moistened with distilled water and applied against the bloodstain causing some of the bloodstain material to be transferred to it. The thread could then be cut into three segments, fixed with methanol, and subjected to the elution procedure with anti-A, anti-B and anti-H. With older stains, the transfer could not always be achieved, in which circumstance a thread of the bloodstained material itself could be employed for the test. Ueno said that, in his experience, the fixation step recommended by Kind, in which the fibers were plunged into boiling buffer, did not remove the contaminating soluble group substances present in the stain.

Bashinski and Davis (1973) described a modification of the technique in which the washing step was carried out with the help of a vacuum filtration device. The same sample could be tested repetitively if necessary. Madivale *et al.* (1971) used a Pasteur pipette-shaped "washing tube", drawn so that the threads being tested did not pass through the fine bore of the capillary. Cold saline could then be poured in carefully and allowed to drain out, giving a completely controlled washing step. They determined the optimal washing volumes and flow rates through the washing tubes.

A number of workers have recommended fixation of the stain prior to applying the absorption-elution procedure. Popoff (1929) recommended a 70–80° heat fixation step, which was designed in part to inactivate the isoagglutinins of the stain. Kind (1960a and 1960b) fixed the test material in boiling McIlvane buffer, and Outteridge (1962a) followed this procedure as well. Yada (1962) fixed stains in absolute alcohol before the absorption step. Fiori *et al.* (1963) said that fixation in methanol for 15 min was essential, especially with dried blood clots and fresher stains, before proceeding to the absorption step. Nickolls and Pereira (1962) said that the boiling water (buffer) fixation step decreased the reactivity of the stain.

Extraction techniques have been employed as well. Outteridge (1962a) used an aqueous extraction procedure and carried out the tests on the dried extracts. In 1969, Kind and Cleevely introduced an ammonia extraction procedure. The absorption of agglutinins takes place only at the surface of a bloodstained fiber, they noted, and extraction procedures, followed by fixation of the extract on a glass surface, have the advantage of increasing available antigen receptors. The ammonia was introduced as an extraction medium because of the insolubility of older stains, and the resulting difficulty of extracting them with other aqueous solvents. Bloodstained material about 2 × 2 mm was extracted with 0.2 mℓ 0.880 aqueous ammonia, and the extract divided into six aliquots for duplicate testing with anti-A, anti-B and anti-H. A and B could be detected in about 2 μg dried blood, and H could be detected in 20 μg group O dried blood and 80 μg group A_2 or B dried blood. Absorption was for 90 min at 4°, followed by 30 min of washing with ice cold saline, and elution was carried out at 50–55° for 10–15 min. Chisum (1971) used a modification of this procedure in which very short absorption times (2 min) were employed. Washing was carried out in less than a minute, and the technique was found to be suitable for routine work. Its value, of course, is primarily the amount of time saved. Tröger (1973) found the Chisum procedure to be completely satisfactory, except he said that he used papain-treated O cells for the detection of eluted anti-H. Kind and Lang (1975 and 1976a) reaffirmed the value of the ammonia extraction procedure, in comparison with aqueous extraction, in a series of experiments on various parameters affecting the technique. They noted that they preferred the ammonia extraction to direct determination on threads, as was advocated by Howard and Martin (1969).

• Sensitivity

One of the singular advantages of absorption-elution, in

comparison with absorption-inhibition, is its sensitivity. Most estimates of the minimal amount of dried blood in which the blood group can be determined by inhibition are in the range of 1 to 50 mg, although a few estimates between 0.1 and 1.0 mg have been given. Outteridge (1962a) estimated the sensitivity of the aqueous extraction absorption-elution procedure to be 10 μg dried blood for A and B, and 200 μg for H. Yada (1962) estimated 1 μg dried blood for A and B. Outteridge (1963b) noted that the microtechnique (Outteridge, 1963a) is at least this sensitive, and perhaps more so. Fiori *et al.* (1963) indicated that consistently clear results were obtained with a 10 μg dried blood. Elution is, therefore, at least 100 times more sensitive than inhibition, keeping in mind that any such estimates for either procedure depend very much on the technique employed, the antisera used, and probably on the experience of the person carrying out the test as well. Vitullo (1966) said that the sensitivity of the test could be improved by using bromelin-treated test cells. Seven stains which gave no results with untreated cells were groupable using the enzyme treated ones.

- Interference by Adventitious Substances

The matter of the presence of substances which might interfere with the test has been discussed by a number of authorities. Any discussion of interference with bloodstain grouping tests is related to the fact that ABH-like substances occur in a wide variety of living things, from bacteria to other mammals (see in section 19.7.3). Kind (1962) noted that oily or fatty contaminants could be washed out with petroleum ether prior to carrying out the test, with no deleterious effect on the group substances. Fiori *et al.* (1963) tested bloodstains on wall material, wood and soil, and found that these substrata did not interfere. Sometimes, as with blood in soil, the blood material needs to be reconcentrated for testing, and this may be accomplished by the paper chromatographic method described in section 7.2, and which Fiori *et al.* (1963) also described. The hemoglobin concentrated in the tip of the filter paper strip, and could be recovered for identification and species tests with anti-human Hb antisera. The group substances distributed all along the paper, but were most concentrated in the zone closest to the edge in contact with the solvent (saline). Absorption-elution could be applied for their determination. Fiori *et al.* said that small strips had to be used, so as not to spread out the blood group substances over too large an area of the paper.

It is considered unlikely by some that soluble blood group substances from contaminating body fluids of secretors would present a problem in the absorption-elution procedure. Since they are soluble, the likelihood is that they would be washed out in the washing steps, or in the initial hot water (buffer) fixation step if it is used. Benciolini was said by Fiori *et al.* (1963) to have shown that soluble group substances in stains do not yield up antibodies upon elution. This was attributed to the possible formation of an antigen-antibody bond which was stable at elution temperature, but, as Outteridge (1965a) has noted, it could have been caused by the antigen-antibody complex being washed out in the washing steps. Tha Aye (1978) noted that garments stained with sweat could cause problems in determining the ABO group of a bloodstain on such garments by elution. He tested A and B bloodstains on garments which were contaminated with A and B perspiration. Saneshige *et al.* (1980) recently indicated that determination of the agglutinins (Lattes procedure) could be very helpful in sorting out mixed blood and sweat stains.

Kind and Lang (1976b) conducted a series of experiments on a number of potential contaminants of bloodstains, and their interference with the grouping tests. The studies focused on contaminants which are themselves known to contain ABH receptors. ABH activity has been reported in many animal bloods and tissues, and in numerous plant and bacterial sources. The ammonia extraction technique (Kind and Cleevely, 1969; Kind and Lang, 1976a) was used in the experiments. A number of samples of household dust (from vacuum cleaners), of soil, wood (shavings and sawdust) and microorganisms from the air (allowed to fall on blood-agar and grown) were tested. Twenty-two of 30 dusts reacted, 11 of these with one of the antisera, and another 11 with more than one. Four samples reacted for A, B and H. Both the strength and type of reactivity of the dust samples were greatly affected by the dust concentration. There was some correlation between the group reactivities of the dust samples and the groups of the usual occupants of the locations from which the dust was collected. Three of 24 soil samples, all with higher organic content, gave single or multiple group reactions. Twenty-four of 42 wood samples showed reactivity for B, H or both. No A reactions were seen. Samples were taken from 257 microbial colonies, and 72 showed A, B or H reactions, while 8 others showed AH, BH or AB reactions. Bacterial contamination of a bloodstain, caused mainly by slow drying, can render the stain completely unreliable for ABO grouping. If glassware for the serological tests is washed and re-used, contamination from prior tests could be a problem. The point was raised by Tha Aye (1977) who said that the difficulties are easily overcome by cleaning the glassware with 0.5M periodic acid. Periodic acid, in concentrations of the order of 1 to 5 mM, destroys the blood group receptors A, B, H, M. N, P, Le^a and D (Rh_o) on red cells (Morgan and Watkins, 1951). Fiori *et al.* (1963) noted that, in cases of suspected contamination of bloodstains being tested by the inhibition method by soluble body fluid group substances, a periodic acid treatment of the cloth control might shed some light on the problem, but they did not think this would be necessary when using elution tests. It is of interest to note that Tandon and Naik (1976) found H substance in the saliva of 56 dogs they tested. 92.8% were found to have A subsdtance, and 25% to have B substance in saliva. Contamination of fabrics with dog saliva might thus interfere with grouping of stains on such a fabric.

- Selection and Evaluation of Antisera for Absorption-Elution

Selection of antisera for the elution test was discussed by Kind (1963), who noted that it was important to recognize

that antisera contain heterogeneous populations of antibodies with respect to their thermal dissociation characteristics in antigen-antibody complexes, as shown by Goodman (1962). Everyone is in agreement that relatively high titered antisera are required for absorption-elution. Fiori *et al.* (1963) observed that anti-A and anti-B at 1:16–1:32 give weak, if not negative reactions in the test. Anti-A_1 sera and lectins did not give good results because the titers were too low. *Ulex* anti-H at 1:64 gave good results. Popoff's (1929) "enrichment" method, in which successively absorbed stain material is eluted into the same saline to try and enhance the agglutinin concentration, was tried to see whether it would improve the results with low titer sera or lectins, and it did not. It must be kept in mind that the "titer" of an antiserum is a function of the technique used and the conditions under which it is titrated (see in section 1.3.4.1). Anti-H, for example, will usually give a higher titer with papainized O cells than with untreated ones. Lang (1975a and 1976a) conducted a series of experiments on antisera intended for use in bloodstain grouping. Series of doubling dilutions of antisera were tested using the ammonia extraction elution technique against a series of different amounts of dried blood material. In the original studies on the ammonia extraction technique (Kind and Cleevely, 1969), neat antisera were tested against extracts of dried blood material ranging from 0.002 mg to 1.26 mg. Agglutination with eluted antibody increased up to a point, levelled off, and decreased again in some cases at the highest antigen concentrations (prozone effect). Lang's (1976a) results may be summarized as follows: There was a general positive correlation between antiserum titer and the agglutination score in the elution tests. In some cases, with more dilute antisera, a decrease in agglutination score was seen at high antigen concentrations (prozone). Different antisera with the same group specificity showed different optimal antigen concentrations, as judged by the agglutination score with the eluted antibodies. More A_2 blood solids were required to optimize reactivity with anti-A than A_1 blood solids. For ordinary purposes, it was found to be sufficient to check the antisera in a few dilutions with constant, near optimal concentrations of antigen (one dimensional test). It was noted that the antisera being used had already been quite carefully selected for red cell grouping, and had been tested for spurious irregular antibodies against Rh, MNSs, P, Lu, Le, Kell, Fy and Jk antigens. If such screening had not been done by the supplier, antisera should be tested against a panel of O cells for saline agglutination. Lang also pointed out that the amount of effort to be invested in antiserum screening must, in part, be a function of the size of the stock received at one time. It would not be very productive to invest several days and half the reagent in the screening of a quantity of antisera that was only going to last for a small number of tests.

Among the most carefully controlled quantitative studies on the parameters of the absorption elution procedure are those of Lincoln and Dodd (Lincoln, 1973; Lincoln and Dodd, 1973). The antigen-antibody reaction is reversible, and can be looked at thermodynamically. This matter was presented and discussed in section 1.3.4.1. The uptake of antigen and its elution are affected by the concentrations of antigen and antibody, and by the equilibrium constant for the reaction. The following parameters were studied: Rate of absorption, effect of varying the concentration of reactants during absorption, variations in the type of antibody, effect of varying ionic strength, elution temperature and the effect of cell suspension concentration on the detection of eluted antibodies. The tests were carried out by incubating antisera of known titer with known amounts of bloodstain for specified times, washing, and then titrating the eluates to determine the quantity of antibody recovered. Experiments were conducted with anti-A, anti-D and anti-c. Papain treated cells were used so as to obtain high titer values. The findings may be summarized as follows: (1) Comparison of absorption times of 2 and 16 hours with anti-A showed that 16 times as much antibody was recovered from stains absorbed for 16 hours as from stains absorbed 2 hours at an initial anti-A titer of 1:1000, and 8 times as much antibody at an initial titer of 1:256. Using the 16 hour absorption period, 4 times as much antibody was recovered from the stains incubated with 1:1000 anti-A as from those incubated with 1:256 anti-A. With anti-D, it could be shown not only that the use of *very* high titer antisera (1:1000–1:32,000) for absorption did not increase the quantity recovered from D+ stains, but that D-negative control stains showed nonspecific uptake of antibody. At very low anti-D titer, recovery from D+ stains was low. The nonspecific reactions in the negative control were attributed to failure to remove all the excess antibody in the washing step with the very high titered sera. (2) With anti-A at an initial titer of 1:1000, and varying the quantity of bloodstained material (amounts equivalent to 5, 2.5 and 0.25 $\mu\ell$ packed cells were used), the antibody recovery was 16 times higher in the 2.5 $\mu\ell$ specimen as in the 5 $\mu\ell$ one, and was unchanged by the additional tenfold decrease in going from the 2.5 to the 0.25 $\mu\ell$ specimen. (3) Using cells, it could be shown that the same quantity of red cells can absorb considerably more anti-A than either anti-D or anti-c of similar titer in a 1 hour absorption. This probably results from the very great differences in the number of receptor sites per cell. (4) Using anti-D diluted in 0.145M and 0.03M saline, the eluates from the D+ stain had about twice as much antibody in the lower ionic strength medium, but D-negative control stains showed significant reactivity. (5) Elution temperatures varying from 4° to 70° were tested for both anti-A and anti-D. The optimal temperature for anti-A was 45–65°. 55–65° was effective for anti-D as well, although 70° yielded the greatest amount of this antibody. These differences are attributable in part to heterogeneity in equilibrium constants in the various antibody populations in the test sera. In another experiment done with A cells, the titers of the eluates from cells sensitized with an anti-A were compared in the cases where (a) the eluate was immediately separated from the cells while the temperature was still 56°, and (b) the tube was allowed to sit at room temperature for 5 min before the eluate was separated. Sixteen times as much antibody was

recovered from the eluate which had been separated from the cells immediately (as assessed by both saline and papain titer). In practice, therefore, the eluate should be removed before the samples have a chance to cool. (6) The concentration of cell suspension used to detect the eluted antibody was varied in an experiment using an anti-c serum. Three different dilutions of anti-c were used for absorption. With the more concentrated anti-c, it was clear that detectability of recovered antibody was better with 0.5–0.05% cell suspensions than with 2% ones. At the highest dilution of anti-c, the detectability of eluted antibody increased steadily as cell concentration decreased, 16 times as much antibody being detectable with 0.05% suspensions as with 2% ones. In sum, these experiments indicated the importance of carefully standardizing the test conditions for the antigen being tested. Different antisera behave differently, and must be evaluated. The measurable recovery of anti-A from bloodstains eluted at 22° (saline titer of eluate = 1:4) points up the importance of washing out the excess antibody at 4°. Washing with warmer saline would cause considerable loss of absorbed agglutinin. Additionally, there is an advantage in titrating the eluate, rather than simply adding test cells to it. In those cases where the negative controls showed some reactivity, attributable no doubt to incomplete washing, the titers of the eluates from these were very low in comparison to the antigen-positive material. These differences, however, would not be seen without the titration. The procedures used in these experiments could be productively applied to the evaluation of antisera intended for use in the elution test, and it cannot be assumed that a particular lot of antisera will behave like any other lot. A final point is that the procedures employed must, of necessity, be a function of the nature of the antibodies (IgM, IgG, etc.) in the antiserum being employed. Some antibodies must be detected in colloidal media, others with enzyme treated cells, or by the anti-globulin test (see in section 1.3.4.1).

Lang (1975b and 1976b) showed that considerable enhancement of the reaction of anti-H lectin from *Ulex europaeus* with O and A_2 stains, using the ammonia extraction technique, could be obtained by the addition of BSA. The optimal concentration was found to be 20% BSA with other factors optimized, but this concentration was difficult to work with, and 7.5% BSA concentration was recommended. In some cases, O stains could be detected in this way which were not detected in saline.

- Reliability and Specificity

The age of stains is apparently not a serious drawback to the detection of ABH agglutinogens by elution technique. Yada (1962) said that he had correctly grouped a stain 62 years old, and it is undoubtedly possible to group stains even older, provided that they have not been contaminated. Rees *et al.* (1975) noted that blood preserved for blood alcohol testing could be correctly grouped after more than a year's storage at room temperature in the presence of NaF and oxalate. The elution technique was applied in these studies to stains prepared from the aged blood. Denault *et al.* (1978) recently found that ABH antigens were detectable by elution technique in bloodstains up to 26 weeks old on a number of substrata, including cotton, denim, wool, nylon, and permanent-press fabric, even if the humidity was high.

Most authors have agreed that the results of a properly executed and controlled elution test are entirely specific. With the exceptions noted in foregoing sections (contamination by adventitious substances), the test is ordinarily reliable. It is possible to miss a weaker antigen, however. In an informal quality control test arranged by Fox (1974), one of the laboratories failed to report the A antigen in an AB bloodstain. Benciolini and Cortivo (1977) said that A_2B stains presented problems, only 9 of 11 examples of them being groupable after 5 days aging. Better results were obtained when the stains were methanol fixed than when they were not. False positive results were possible, even in the apparent absence of contaminants. Denault *et al.* (1978) saw occasional false positive A and B reactions in 26 week old bloodstains on denim or on nylon.

- Other Methods of Eluting Antibodies

It may be noted, finally, that antibodies can be eluted from their antigenic receptors in a number of different ways. Heating is probably the only method commonly used in bloodstain grouping. This method of eluting antibodies originated with Landsteiner (1902). Some authors trace the technique of heating to 56° for 5 min to Landsteiner and Miller (1925), and although there is no doubt that they did use this elution technique, it was not the first time that agglutinins had been heat eluted from cells. Of all the different techniques, heating is probably the simplest, and can be applied to the grouping of micro amounts of stain material.

In 1936, Landsteiner and van der Scheer eluted antibodies to haptens on red cell stroma from these "antigens" by brief exposure to 0.1N acetic acid. Kidd (1949) eluted incomplete antibodies from red cells by exposing sensitized cells to 0.1M citric acid-HCl at pH 3.2–3.4. Hughes-Jones *et al.* (1963) gave an acid elution technique for anti-D which yielded up 80% of the bound antibody. Rekvig and Hannestad (1977) applied an acid elution procedure to IgM and IgG antibodies with A, B, D, C, c, E, e, Fy^a and K specificities. Vos and Kelsall (1956) used an ether elution technique, which was modified by Rubin (1963). Weiner (1957) used a cold ethanol procedure to elute immune antibodies to A, B, C, D, E, K and k from cells. Jensen (1959) said that Weiner's method gave better results than heating, but noted that it was quite a bit more involved as well. Chan-Shu and Blair (1979) described still another procedure, using xylene.

19.10.3.5 Detection of agglutinogens in bloodstains by mixed agglutination technique. The use of the term "mixed agglutination" in senses other than the one used here was discussed in section 1.3.4.1. Mixed agglutination, as used in this section, means a technique in which an antigenic receptor is identified in a cell or tissue by using a specific antibody to link the cell or tissue to indicator cells which themselves contain the antigen. The technique was first applied by Coombs and Bedford (1955) to identify A and B receptors on human platelets. In 1956, Coombs *et al.* used the technique to identify A and B on human epidermal cells. It has

been applied to assess the distribution of the A receptor in a variety of human tissue cells (Holborow *et al.*, 1960), the distribution of Forssman antigen in guinea pig tissues (Hawes and Coombs, 1960), and the distribution of ABH antigens in the tissue cells of frogs (Yada *et al.*, 1962). Ogata (1960) reported application of the technique to the grouping of red cells in a thin smear. He called the technique "hemagglutination on a smear" or HOAS, and noted that he had read a paper on it in 1958 at a meeting of the Serological Society of Japan, and published a preliminary report in Japanese in 1959. Medicolegal application of the procedure to the grouping of tissues and secretions is discussed subsequently (section 19.10.5.2). Its application to species determination (mixed antiglobulin technique) has been discussed in a previous section (16.4).

In 1961, Coombs and Dodd applied mixed agglutination to bloodstain grouping. Individual threads about 0.2 mm in length were cut from the stained material, and teased apart. The fibrils were pretreated with dilute acetic acid to fix and clear them. They were then washed in dilute normal rabbit serum (absorbed free of any human A, B or H agglutinins), and incubated with anti-A, anti-B or anti-H for 1-24 hours at room temperature. After washing, indicator cell suspensions were added. A, B and O stains could be grouped reliably. Papain treated O cells were employed for the detection of the bound anti-H lectin. Yada (1961) reported that the procedure was applicable to bloodstains. Fiori (1961) gave a slightly modified technique. He said that some problems were encountered with the preparation of thin microscopic test samples from some substrata, such as wood and paper. Fiori *et al.* (1963) quoted Beniolini as having encountered some false negative results with stains on wood or nylon, and there could be problems with false positive microscopic images with some substrata. Fiori *et al.* thought that the technique was more difficult than elution in its manipulations, but that with most stains it was reliable. Nickolls and Pereira (1962) found that a simplified version of the original Coombs and Dodd technique was quite satisfactory. They omitted the acetic acid step, substituted saline for the normal rabbit serum, and said that the papain treated O cells were not required to obtain satisfactory reactions with *Ulex* anti-H. Good results could not be obtained using *Dolichos* lectin. For most routine work, the elution technique was preferred. Roychowdhury (1963) described a version of the test, which he preferred to call "triple bonded agglutination", or TBA technique. Absorption for 1-2 hrs at room temperature was carried out on 2-2.5 mm long fibrils of stained material, which had been teased apart with a fine needle. After a saline wash, test cells were added and left 1-1½ hours. A gentle wash with saline preceded transfer to a slide, application of a cover slip and reading. Roychowdhury elaborated on this method subsequently (1973, 1974 and 1975), and said that the success rate with it was far better on case samples than with inhibition technique. Mitra and Ganguly (1973) modified the technique, saying that they merely withdrew the excess antiserum by aspiration following incubation with the stained fiber, i.e., the washing step was eliminated. The presence of agglutinates not associated with the fiber in the field due to residual antiserum did not interfere with interpretation in their view. Maresch and Wehrschütz (1963) adopted a technique very similar to that of Nickolls and Pereira (1962) and found it to work quite well. They noted that the positive results could be confirmed by subjecting the mixed agglutinates to a 50° elution step and looking for agglutinates in the field. Certain stained fibers (Nylon) apparently required this confirmation. In 1965, Akaishi described a mixed agglutination procedure in which the dilute acetic acid treatment of the fibers, originally employed by Coombs and Dodd (1961), was used. This step followed methanol fixation of the fibers. The technique gave good results, and it was suggested that it be called the "group specific double combination method" (GSDCM) for reasons mentioned in section 1.3.4.1. Mikami *et al.* (1966) said that catalytic tests for identification of blood (section 6), the fibrin plate method for human species determination (section 17.2) and the GSDCM for the ABO group could be applied consecutively to the same few threads of bloodstained material. In 1967, Halvorsen and Nordhagen described a mixed agglutination technique in tubes. They said that the results did not differ if the stained thread was incubated with antisera at 4° or at 18°, and that the maximal amount of antibody had been absorbed after 12 hours. In 300 samples tested, there was one error in which an A stain was grouped as an O (anti-H was not used). Nordhagen (1967) noted that woolen garments, well worn by A secretors, gave A reactions in mixed agglutination tests even if they had been washed a number of times. Three such A reactions were found in 24 fabric samples tested, and Nordhagen said that this finding emphasized the importance of substratum controls. Göring *et al.* (1969) said that they were considerably more successful in grouping dried bloodstains by the Halvorsen and Nordhagen procedure than with the Lattes test or the Holzer inhibition test. Benciolini and Cortivo (1977) noted that the A reaction in A_2B stains was not detectable by means of mixed agglutination, using the method of Ishiyama and Okada (1975). The latter investigators found that a number of different types of samples could be grouped for ABO using a modified mixed agglutination procedure called the mixed cell agglutination reaction (MCAR). The technique was used by Davidsohn and his collaborators to determine ABO receptors in thin tissue sections (Kovarik *et al.*, 1968). It has developed that this procedure may be applicable to the diagnosis of cancer (Davidsohn, 1972). In effect, the procedure consists of treating the sample with antisera on a microscope slide for 15-30 min at room temperature, washing, and adding test cells. The test cells are allowed to settle onto the specimen for 15 min or so, after which the slide is rapidly inverted and placed on props in a Petri dish filled with buffer or saline, such that the specimen just contacts the liquid. Unagglutinated cells settle out, and the sample can be examined under low power *in situ*. Ishiyama and Okada (1975) modified the procedure slightly for use with samples of medicolegal interest. Ishiyama *et al.* (1977) said that the MCAR mixed

agglutination procedure could be employed to determine the ABO group in latent fingerprints, lifted from surfaces with cellophane tape and transferred to microscope slides. Chatterji (1977) reported good results, equivalent in specificity and sensitivity to absorption-elution, with a mixed agglutination procedure on 2–3 mm long threads affixed to a cellulose acetate sheet. The technique was faster than elution, and saved work.

The mixed agglutination technique is probably equivalent in sensitivity to the elution test. Fiori *et al.* (1963) noted that some limitation is imposed in both methods by the fact that the manipulations can no longer be carried out if the sample is too small. Akaishi (1965) said that stains made from whole blood diluted 1:25,600 could be grouped using mixed agglutination.

19.10.3.6 Detection of ABO agglutinogens in bloodstains by fluorescent and ferritin labelled antibody techniques. In 1962, Hasebe employed fluorescent-labelled anti-A, anti-B and anti-$\mathbf{Rh_0}$ (D) for the detection of the corresponding antigens on red cells (Hasebe, 1962a). Two methods were used, one in tubes and the other on blood smears. In the same year, Hasebe (1962b) extended the application to bloodstains using high titered rabbit immune anti-A and anti-B, and detection of binding with fluorescent anti-rabbit globulin serum prepared in sheep. Both slide and tube methods were used. The latter gave better results, in that there was less non-group specific fluorescent antibody binding. Results with blood crusts were completely acceptable, but Hasebe noted that nonspecific binding of fluorescent antibody to cloth, fiber and wood substrata were still a problem in trying to make the determination on these types of bloodstains. Outteridge (1965a) noted that the method was not as complicated in practice as it seemed upon first looking at it, and that it had promise. Pollet (1969) reported that he had obtained good results with a procedure which he described in full. For ABO and MN antigens, fluorescent-labelled AHG serum was used. Kind and Cleevely (1970) found the procedure to be satisfactory with blood smears, but not with bloodstained fabrics. The fabrics often fluoresced, and an extraction technique was tried to circumvent this problem. Results were not good, and they thought hemoglobin might be absorbing most of the fluoresced light. An agar diffusion procedure was then used to try and separate the blood group active material in the extract from the hemoglobin, but in this case the fluorescent staining became nonspecific. They did not think the procedure looked very promising for bloodstains, but they were able to get good results with secretor saliva stains.

Suzuki (1970) reported that he had been able to group bloodstains by using ferritin-labelled antibodies and then examining the stained material in the electron microscope. A number of artificial bloodstains on fabrics could be correctly determined for ABO in this way.

19.10.3.7 The use of formalin treated red cells. In 1957, Moskowitz and Carb observed that treatment of A red cells with formalin renders them inagglutinable with anti-A reagents. Treatment of the cells with 10% formalin at 25° for 24 hours made the cells completely inagglutinable. Shorter treatment times, or lower formalin concentrations, or a combination of these, reduced the agglutinability of the cells without completely abolishing it. The receptors were apparently not damaged, however, as the formalin-treated cells absorbed the anti-A from an anti-A reagent as effectively as did fresh cells. Gold *et al.* (1958) confirmed the observation, and noted too that formalin treated red cells would, after sensitization with antibody, yield it up at 56° (elution) in the same way as fresh cells. The formalin treated red cells could also be lyophilized, and reconstituted in saline without losing their specific absorptive capability.

Marcinkowski (1970) proposed a procedure for grouping bloodstains based on the use of formalin treated red cells. He did not cite the work discussed above, and apparently thought that the formalin treatment stripped the antigens off the cell. He treated 1 part packed cells (group O preferably, but any group was usable) with 10 parts of 4% formalin in saline (final concentration) for 24 hours at 4°. He then washed the cells, resuspended them in saline and checked them for agglutinability with anti-A and anti-B. He called the inagglutinable cells "E-Li" (from Erythrocytus liber—antigenically free). The cells could be incubated with bloodstain material or its extract, and would take up antigen onto their surfaces. If the cells were washed, and tested with antisera, they agglutinated if they had been in contact with stain material containing the appropriate antigen. Marcinkowski said that 20 stains had been correctly grouped in this way.

19.10.3.8 Reversible agglomeration technique. This procedure is not so much a grouping technique, but a means of getting red cells which are in reasonably good condition from stored blood or cadaveric blood. In 1963, Huggins was looking for simple means to wash the dimethylsulfoxide out of frozen red cell preparations prior to transfusion. These blood units had been cryogenically preserved in glucose solutions containing DMSO. He found that if red cell suspensions were diluted with large volumes (1 vol red cells:10 vols diluent) of isotonic glucose or sucrose, the cells settled out at the bottom of the container quite rapidly (agglomeration). They could be washed with diluent, and allowed to resettle. If an equal volume of plasma were added to the settled cells, they were easily resuspended, and behaved normally (reverse agglomeration). The agglomeration depended on the presence of large quantities of isotonic nonelectrolyte, and on the pH being between 5.2 and 6.1. The mechanism was thought to have to do with the precipitation of γ-globulins which could bind to the red cell surface lipoprotein, and thus carry the cells down as well. In 1977, Michailow said that he had been able to use this procedure to recover red cells in reasonably good condition from putrefying, hemolyzed blood samples for grouping. A substantial number of samples treated in this way could be reliably grouped.

19.10.4 Determination of A subgroups in bloodstains

Efforts to distinguish between A_1 and A_2 in bloodstains are not new. Ponsold (1937) discussed a microprocedure for

carrying out the differentiation by the inhibition technique. Differentiation of A subgroups is mentioned periodically in textbooks prior to the 1960's (e.g. Huber, 1957). A number of authors have said that the inhibition procedure could be used to distinguish between A_1 and A_2 (as well as between A_1B and A_2B) using lectin reagents. Leister and Kirk (1961) tested a commercial *Dolichos* anti-A_1 reagent as well as fractions of *Ulex* extract, separated by continuous electrophoresis, and these fractions were referred to as "anti-A_2" and "anti-O". It was found that A subgroups could be determined in stains. Leister *et al.* (1961) confirmed the usefulness of the lectin reagents in subgrouping stains and red cells, although they appear to have believed that "anti-A_2" and "anti-O" specificities could be separated and used to distinguish between A_2A_2 and A_2O cells. Except for the *Dolichos* reagent, the intensity of the lectin reagent reactions with different cells was shown to be a function of the time of incubation (Sylvia and Kirk, 1961), and the test conditions had to be carefully standardized in order to achieve the desired discrimination. Grünwald and Lackovic (1963) said that anti-H from *Ulex* could not be used to discriminate between genotypes in the ABO system (e.g. AA vs AO). Moureau (1963) reported that there was no difficulty in determining A_1 and A_2 in bloodstains by the inhibition procedure using *Ulex* lectin. DeRen *et al.* (1970) reported, however, that they had been unable to distinguish A_1 from A_2 by an inhibition procedure with H and A_1 lectins.

Poon and Dodd (1964) reported that A_1 and A_2 could be discriminated in bloodstains by mixed agglutination using *Dolichos* anti-A_1 lectin, and testing with papain treated A_1 test cells. Kind's (1960b) studies on the elution procedure indicated that A subgrouping was possible as judged by the *Ulex* anti-H reaction. Outteridge (1962) confirmed Kind's results. Schleyer (1961) had difficulty with A_1–A_2 differentiation by the elution procedure using a *Laburnum* anti-H reagent, but this result could have come about in part because the titer was too low. Morgan and Richards (1967) tested *Dolichos* anti-A_1 in the elution system of Nickolls and Pereira (1962). They found that the intensity of the reaction decreased both with stain age and with the age of the frozen lectin preparation. Six month old reagent did not work, even with 1 day old stains, and 6 month old stains worked only with 1 day old lectin. They tried anti-A_1 serum, obtained by absorption of anti-A with A_2 cells, and did not find it to be very satisfactory. Enzyme treated test cells were used with the serum anti-A_1. Hayward (1969) described an elution procedure for diagnosing A_1 stains with *Dolichos* lectin. The stains were trypsin treated at 37° and washed prior to a 15 hour 4° absorption. Following absorption the preparation was frozen, and washed with ice-cold, partly frozen saline. Elution was at room temperature into trypsin treated A_1 cells. He said, too, that agglutination was optimal at 4°, and ran this part of the test in the cold. A 20 year old A_1 stain was diagnosed by this procedure, while a similarly aged A_2 stain was negative. Hilgermann (1971) described an absorption-elution procedure for differentiating A_1 from A_2 in bloodstains. *Dolichos* lectin and anti-A_1 serum reacted with A_1 and A_1B stains if A_1 test cells were used. A_2 test cells gave negative results. A_2 and A_2B stains reacted with *Ulex* or *Laburnum* lectin if A_2 test cells were used, and A_1 test cells gave negative results. If, however, enzyme treated cells were used (bromelin, papain, ficin, etc.), *Ulex* and *Laburnum* lectins reacted with A_1 and A_1B stains using A_2 test cells, and *Dolichos* lectin reacted with A_2 and A_2B stains using A_1 test cells. Poon and Dodd (1964) had not observed any reaction of *Dolichos* lectin with A_2 stains in mixed agglutination using papain treated A_1 test cells. Tröger (1973) noted that his initial results indicated that *Dolichos* lectin could be used to diagnose A_1 stains by the Chisum (1971) rapid ammonia extraction procedure if papain treated A_1 test cells were used. Pereira (in Culliford, 1971) questioned the reliability of the subgrouping techniques in bloodstains by absorption-elution procedure. Khalap and Divall (1979) reported that bloodstains could be successfully typed for A_1 and A_2 subtypes by elution, using the specific anti-A_1 protectin from the albumin glands of *Helix aspersa*.

19.10.5 ABO grouping in body fluids and tissues

19.10.5.1 Introduction. Medicolegal application of the group substance determination in body fluids could be of two kinds: Determination of secretor status (in saliva) as an additional marker in affiliation cases; and determination of the ABO group from body fluids, stains or human tissues in connection with criminal investigations. Both applications have been practiced for quite some time. There was some hesitation on the part of experts to use the secretor property in affiliation cases for a while, because not everyone was convinced that the distinction between secretor and nonsecretor could always be made with certainty. Holzer (1937) carried out extensive studies, but warned against the introduction of this "new" marker into parentage cases until further experiments had been done. He noted, too, that since most people are secretors, exclusions by the system would be quite rare. In fact, the secretor characteristic can be employed in disputed parentage cases (Boorman *et al.*, 1977). The only type of exclusion that can be obtained, however, is in the case of two nonsecretor parents having a secretor child. Such a result is expected in a very small percentage of cases (see in section 19.10.1.3). Determination of the groups in body fluids, stains or tissues from secretors has long been accepted, provided a clear, unequivocal result was obtained. Fujiwara (1930) reported a case in which he had determined the seminal type as A in a rape-homicide case where the victim was an O. Holzer (1937) mentioned that saliva stains from secretors could be grouped without difficulty.

19.10.5.2 Methods of ABO grouping in body fluids and tissues.

- Inhibition

Up until about 1960, inhibition was the only method used for grouping body fluids. This procedure has been discussed at some length in section 19.8, and most of the considerations that apply to grouping body fluids apply equally to the grouping of body fluid stains. An inhibition technique is often the method of choice with body fluid stains from secretors, because of the high group substance content.

Andersen (1951) conducted an extensive study of the occurrence of H substance in secretor fluids. Using eel serum anti-H, he could always detect H in secretor saliva provided it was concentrated about 4 fold. Nonsecretor saliva had no detectable H. The presence or absence of H in saliva, he said, could be used as a criterion for secretor status, provided the concentration step was carried out. The secretor characteristic, however, should be used as a sole criterion for exclusion of affiliation only with caution, and only when the results are very clear. Seminal stains from secretors could be grouped without difficulty, the distinction between O secretor and nonsecretor being possible using the eel anti-H reagent. The introduction of anti-H reagents simplified and assisted in the interpretation of these tests with regard to O secretors. Jungwirth (1955) used an anti-H serum for this purpose. Wiener *et al.* (1958) introduced the use of anti-H lectin from *Ulex* for grouping blood and saliva. The reagent was equally applicable to seminal typing in rape cases (Helpern and Wiener, 1961). Raszeja and Dziedzic (1971) found that *Ulex* and *Cystisus* lectins could be employed in the differentiation of secretor from nonsecretor saliva in groups A_2, A_2B and O without difficulty. Caution was required in the case of A_1, A_1B and B.

• Mixed Agglutination

In 1961, Yada noted in his paper on the grouping of blood smears by mixed agglutination that the technique was perfectly useful for semen, saliva and vaginal secretion stains as well. Nickolls and Pereira (1962) found that their modification of the original Coombs and Dodd (1961) technique was fully satisfactory in grouping seminal and saliva stains. Dodd and Hunter (1963) compared the mixed agglutination procedure with the classical inhibition method for the grouping of saliva stains. Serial dilutions of saliva in saline were prepared, and used to make stains on cotton fabric. The sensitivity of the mixed agglutination procedure was enormously greater than that of the inhibition technique. In one case, the A antigen could be detected by mixed agglutination in a stain prepared from a 1:256 dilution of saliva, while the corresponding inhibition test detected it only in dilutions out to 1:8. In addition, mixed agglutination required considerably less material. Methanol fixation of the stained threads improved the mixed agglutination results. A, B and H substances could be detected in some saliva stains from nonsecretors as well by mixed agglutination. Maresch and Wehrschütz (1963) carried out mixed agglutination grouping on saliva stains on filter paper. In some laboratories, when saliva needs to be collected for secretor status determination in an affiliation case, it has been found convenient to prepare the sample as a stain on filter paper (see for example Hennig and Rackwitz, 1961). Prokop and his collaborators apparently follow this practice. Maresch and Wehrschütz (1963), noting that groups could sometimes be diagnosed in nonsecretors by mixed agglutination, questioned the ease with which secretor status could be diagnosed, and raised the issue of its validity as a genetic marker. Prokop and Gibb (1965) responded that there was no reason to question the validity of the secretor characteristic as a marker, that inhibition was the method of choice in diagnosing secretor status, and that mixed agglutination on filter paper stains should not be used for the purpose. In 1970, Schulz said that the sensitivity of the mixed agglutination test could be improved by using test cells that had been sensitized with subagglutinating doses of appropriate antisera (the dose used was two doubling dilutions beyond the agglutinating titer). The test was carried out on slides, and found to be applicable to semen, saliva and sweat. Schulz extended his studies in 1974. Secretor and nonsecretor samples (as determined by the inhibition test on saliva) of saliva, semen, vaginal secretions and sweat were tested with anti-A, anti-B and anti-H by mixed agglutination. In vaginal secretion and sweat stains, exceptions were found to the general rule that secretors always secrete H (using the inhibition test). One vaginal secretion stain from an AB secretor failed to react with anti-A, and a sample from another A secretor reacted with anti-A but not with anti-H. Another sample from an A secretor reacted with anti-A but very weakly with anti-H. A sample from a B secretor reacted with anti-H but only very weakly with anti-B (it would have been diagnosed as an O secretor). These were the only four exceptions in 86 samples. By mixed agglutination, the group substances were all diagnosed correctly. Some exceptions were noted in sweat using the inhibition procedure as well. Two A secretor samples did not inhibit anti-H, one O secretor sample did not inhibit anti-H, and one O secretor sample inhibited anti-H and anti-A. Using anti-A and anti-B in the mixed agglutination test, one A secretor sample failed to react with anti-A, but the O secretor which had given the false A positive reaction in the inhibition test did not give an anti-A reaction by mixed agglutination. Palatnik and Carnese (1970) reported good results in grouping saliva stains from secretors by mixed agglutination. Bromelin treated O cells were used to detect the *Ulex* anti-H binding.

• Elution

In 1963, Pereira reported that she had obtained very good grouping results with semen and saliva stains using the absorption-elution method described by Nickolls and Pereira (1962) (Pereira, 1963b). Ueno (1963) said that the elution procedure was not applicable to body fluid grouping, and that the results always came up as group O, regardless of the actual group. Thus, he said that bloodstains could be grouped by elution even in the presence of contaminating soluble group substances. Fiori (1963) rejected both elution and mixed agglutination procedures for body fluid stain grouping. In 1969, Pereira *et al.* conducted a series of experiments on the applicability of absorption-elution and mixed agglutination procedures to group determination in body fluid stains. Both methods were found to be entirely satisfactory. O secretor saliva detection by anti-H with mixed agglutination technique required the use of papain treated O cells. H reactions were occasionally seen in nonsecretor samples as well. With nonsecretors, weak reactions were generally seen with mixed agglutination, while the reactions were generally strong in these samples by absorption-elution. Fixation was not used, but a saline wash step

preceded the addition of antiserum to the threads in order to get some of the soluble group substance out which would dissolve in the antiserum and attenuate its strength. Pereira *et al.* said that, if the threads were not washed, and the fibers teased apart in the antiserum (at the start of the absorption), stronger reactions were seen with nonsecretor samples than with secretor samples. This behavior could be accounted for either by the dissolution of soluble blood group substances in the antiserum in sufficient amounts to partially neutralize it, or by competition of soluble group substances for eluted antibody. In a dilution experiment, in which doubling dilutions of saliva were used to make stains, and these then tested with and without the washing step by absorption-elution, a definite prozone effect could be seen with the anti-A and anti-B reactions in the unwashed samples. It was not seen with anti-H. Thus, at very high concentrations of A or B, negative or very weak reactions are possible. Pereira (1963b) had earlier noted that there appeared to be a kind of different distribution of A and B in a stain placed on cloth, and allowed to spread out, the reactions being weak at the center and periphery, and strongest about midway in between. Pereira *et al.* noted that this effect could be an apparent one, caused by the very high concentrations of A and B substance near the center of the stain (prozone). H reactions, which did not appear to be affected by antigen concentration, were highest at the center and lower proceeding outward toward the periphery, as the concentration of H declined. The relative concentrations of antigen and antiserum are thus found to be very critical in these tests, and this fact was cited as a possible reason for the varying results of different workers, which had led to differing opinions as to the applicability of the technique to body fluid stain grouping. Fiori and Benciolini (1972) compared the inhibition procedure with the elution procedure for body fluid stain grouping. They said that false negative results could be gotten with elution tests, and that, if the procedure were used, only positive results should be interpreted. They again recommended that the last washing liquid in an elution test be checked with test cells to insure that all excess, unwanted antibody had been removed from the sample. Masis *et al.* (1973) reported that semen and saliva could be reliably grouped by the absorption-elution technique. The stains were fixed in boiling water prior to the absorption step.

Tröger *et al.* (1976) reported successful grouping of stain fragments from under the fingernails of dead bodies by a straightforward elution method. Fragments up to 11 months old were determined. Because the nail substances may contain ABH group substances, the victim's blood group must be known in order to interpret the results.

• Other Techniques

In 1962, Yamasawa reported successful grouping of semen and saliva stains on filter paper by a fluorescent-labelled antibody technique. The stains were acetone-fixed, then incubated with fluorescent-labelled rabbit immune anti-A or chicken immune anti-B. The paper was then subjected to electrophoresis to separate the antibody from the antigen-antibody complex, dried and photographed under UV light. While differentiation of positive from negative results was possible, there was a small amount of fluorescence in the negative controls. Kind and Cleevely (1970) also reported good results in grouping secretor saliva stains by fluorescent antibody technique.

In 1974, Okada *et al.* described a rather interesting, if somewhat cumbersome, technique based on prior experiments by Vos and Kirk in 1958. Vos and Kirk were interested in possible ways in which fetal red cells might be protected *in vivo* against maternal antibodies to them which cross the placenta into fetal circulation in incompatible pregnancies. They had noted that adult red cells, sensitized with anti-A or anti-B and agglutinated, then subjected to homogenization procedures which would break up the agglutinates, would reagglutinate upon being centrifuged. If the agglutinates were broken up, however, and the sensitized red cells washed in saline and treated with soluble A or B substances, reagglutination did not occur. Such cells, which had their receptors occupied by antibody, and the other antibody binding sites apparently occupied by soluble group antigen, were referred to as "protected" cells. Okada *et al.* (1974) took advantage of this behavior. A series of doubling dilutions of the body fluid to be tested was prepared, and "test cells" were added. These "test cells" were prepared by breaking up the agglutinates of red cells sensitized and agglutinated by homologous antibody. The incubations were carried out at 45° for 30 min, and the tubes read after 30 min. In the presence of soluble group substances in the body fluid being tested, agglutination (actually reagglutination) was inhibited at the higher body fluid concentrations, whereas in the absence of soluble group substances, reagglutination was seen in all dilutions. The technique was called "reagglutination inhibition".

19.10.5.3 ABO grouping of different body fluids and tissues. Of the body fluids likely to be encountered, semen and saliva are probably the most common. As a general rule, these fluids and their stains are not difficult to group by the inhibition procedure if they are from secretors. As noted above, nonsecretors do have a small amount of group substance in their secretions, and it is sometimes possible to get a group on such a sample using one of the sensitive techniques (mixed agglutination or elution). Mixtures of body fluids from different individuals lead to problems in the interpretation of these tests. The most common example is probably encountered in samples collected from victims of sexual assault. The contamination of the semen with vaginal or other secretions was discussed at some length in section 10.3.5 in connection with semen identification. The same considerations apply to grouping tests. It is essential that the victim's group and secretor status be known before the results of grouping tests on a sample from a victim can be interpreted. Hayashi (1974) claimed to have devised an acrylamide gel disc electrophoretic method whereby the seminal and vaginal fluid protein could be separated, eluted from the gel, and then separately grouped.

Various methods have been used to determine blood

groups in a variety of body fluids, secretions and tissues. A number of the older studies on this subject were discussed in section 19.8. The early studies on mixed agglutination were done on tissues, and the procedure could obviously have medico-legal value. Swinburne (1962) applied mixed agglutination to the grouping of skin fragments, such as scrapings from live persons or dead bodies, and to dandruff particles. A brief pretreatment with mild alkali, and collection of the test material by centrifugation was recommended to get rid of grease, oil and other contaminants. 39 of 40 samples could be grouped, regardless of secretor status. Poon and Dodd (1964) likewise used mixed agglutination for the grouping of epidermal cells. Slavík and Meluzín (1972) reported successful grouping of histological sections of spleen, heart, kidney, lung and liver tissue after alcohol fixation by the absorption-elution technique. Brain tissue was negative. Suyama and Imai (1975) said that ABO grouping could be done on the pulp or dentine of teeth by absorption-elution. Schaidt (1958) noted that ABO groups could be determined by the inhibition test from samples of urine or feces provided these were concentrated. He used the paper chromatographic procedure described in section 7.2 to concentrate the group substances into a zone on the filter paper. There were a number of reports in the older literature that secretor urine could be grouped by inhibition if it were concentrated. It has been known for decades that the group substance concentration in this fluid is low. Gibb and Vogt (1965) said that urine stains could not be reliably grouped by mixed agglutination. Schulz (1974), however, said that he had correctly grouped 26 urine stains from A and B secretors by mixed agglutination. Urine stains from 5 nonsecretors gave negative results. In 1958, Hayashi grouped the earwax (cerumen) of secretors by an inhibition technique. He said that the amount of ABH substance was small in this material, and that he could identify small amounts of Le^a in the earwax of nonsecretors. Yada *et al.* (1966h) could group "flakes" of earwax by absorption-elution after methanol fixation, and the group was obtained regardless of secretor status. Dillon (1971) grouped dried saline extracts of earwax by absorption-elution. Bromelin treated O cells were used to detect the eluted anti-H. 49 secretors could be correctly grouped, while 51 nonsecretors gave no reactions with any of the antisera. Trela and Turowska (1975) employed an inhibition method for the grouping of inner ear fluid (perilymph) from secretors. Saneshige *et al.* (1980) found that the amount of A substance in A secretor sweat was far less than that in A secretor saliva. Identical results were obtained with the same fluids from B secretor individuals. An inhibition test was used for these determinations.

In some circumstances, determination of the blood group of dead bodies may be desirable or necessary. When the blood has undergone hemolysis and/or putrefactive changes so as to make it untypable, one can consider attempting to determine the group from one of the tissues. Holzer examined this problem in 1937. Group substances could be found in bile, duodenal lining and in other tissues of secretors. But the richest source was the stomach. A piece of stomach wall lining about 1 cm^2 yielded group substance to 0.5 $m\ell$ saline upon overnight steeping sufficient to give inhibition of appropriate antiserum in dilutions up to 1:16. Holzer also found that the stomach contents contain enormous quantities of group substances, apparently leeched out of the walls. Inhibition testing of stomach contents yielded inhibition in some cases out to dilutions of 1:2000. Holzer rinsed out a stomach excised at autopsy, washed it, and filled it with saline. Over the course of time, samples were taken and tested, and the inhibition titer increased from 1:16–1:32 at 6 hours to 1:64–1:128 at 70 hours. A case reported by Moureau *et al.* (1963) illustrates the potential usefulness of this sort of approach. In a body which had been in the water for three weeks, and which had undergone putrefaction, the hemolyzed blood sample gave inhibition with both anti-A and anti-B. This was the body of a child whose mother was of group O, and the child could not therefore have been an AB. A culture of the hemolyzed blood samples revealed microorganisms which gave strong B reactions. A piece of the gastric mucosa, well washed, steeped in saline and tested, showed inhibition only with anti-A. Group A was thus confirmed, the B having been acquired. Moureau *et al.* said that they had tested gastric mucosa from many corpses, and were always able to diagnose the blood group. Masis (1971) said that the secretor status of bodies could be reliably determined in bile, and somewhat less reliably in urine, using an inhibition procedure. An anti-H lectin derived from *Sambucus nigra* was used for group O diagnosis.

Pereira (1973) applied the absorption-elution technique to the problem of grouping muscle tissue from decomposed bodies. The decomposition process apparently gave rise to the presence of every kind of ABH receptor, unrelated to the genetically determined blood group substance. False reactions due to A, B and H were observed, and Pereira said that this approach was utterly unreliable.

19.10.5.4 Problems in the ABO grouping of body fluids. In any ABO blood group determination on other than fresh material, the possible presence of contaminants which have A, B or H receptors must be kept in mind. This issue was discussed in sections 19.10.3.2 and 19.10.3.4 in connection with the examination of bloodstains. The receptors in adventitious substances which react with the grouping sera to give false positives are sometimes referred to as "acquired". The matter was discussed in the foregoing section in connection with the determination of blood groups in dead bodies.

It is known that the red cells of patients suffering from carcinoma of the rectum or colon, who are of blood group A, can begin to show B reactions. This phenomenon is very rare, occurs *in vivo*, and was termed "acquired B" (section 19.7.3). Not surprisingly, the culprits in these cases were bacteria, their B-like substances being able under certain circumstances to coat the red cells. Many microorganisms have surface receptors which are recognized by anti-A, anti-B or anti-H, as is clear from the experiments of Illchmann-Christ and Nagel (1954), Springer (1956) and a number of others. Jenkins *et al.* (1972) looked into the problem of "acquired B" from the standpoint of forensic serology. Their curiosity

had been raised by two cases which had come to their attention, both involving dead bodies taken from the water. In one, a body had been dismembered and the parts put into the river. The first part recovered yielded enough intact red cells to allow for grouping as an O Rh+. Another part, recovered later, yielded no red cells, but the muscle tissue grouping gave group B. Thus, although the pathologist's examination made it virtually certain that the parts were from the same body, the blood grouping results did not support the conclusion. Further study showed that *E. coli* could be isolated from the deep muscle layers, and culture filtrates could induce B receptors on red cells lacking them. The B was, therefore, acquired. In the other case, samples were received from 10 victims of an airplane disaster at sea. The bodies had been in the water for several weeks. Eight of the ten reacted as B, a most improbable result with ten persons of Western European origin.

A number of anti-B sera were examined for their reactions with ordinary B cells and with cells from two patients having *in vivo* acquired B. The acquired B cells showed very low titers with the antisera in comparison with the normal B cells. The titers of the eluates from the acquired B cells, and from stains made from the acquired B blood, were significant. The eluates from ordinary B cells were of much higher titer than those from acquired B cells, and similarly with the stains, but the eluates from the acquired B material were fully detectable with B cells. In the usual absorption-elution test, these would have been called positive. Ten seminal stains were made, and inoculated with *E. coli* O_{86} and incubated for 24–48 hours in a moist environment at 37°. Prior to inoculation, all the specimens except for one from an AB secretor, were negative in the absorption-elution test with anti-B. The tests were carried out with and without papain-treated B cells. After 24 hours incubation, 2 samples showed definite B reactions and 4 others showed weak reactions with ordinary test cells. Eight of the samples showed strong reactions with papain treated cells (not counting the AB sample). In the control (not inoculated) stains, however, some B reactions could be seen with the papain cells. While the controls were not inoculated, neither were they sterile, and the B reactions which developed were probably the result of naturally occurring bacteria in the samples. The B activity of the samples was somewhat more marked at 48 hrs of incubation. In the course of these experiments, none of the stains acquired A, but in some cases, H activity decreased or was lost after inoculation. Four samples of fresh muscle tissue showing A and H, but not B activity were placed in a river for a week, and all of them acquired B activity, which increased after another week in the water.

A possible solution to the problem would be to prepare an anti-B which reacted with ordinary B bloodstains but not with the acquired B. Jenkins *et al.* tried four approaches: (1) Absorption of anti-B with red cells having an *in vivo* acquired B; (2) Absorption of anti-B with rabbit cells—rabbits have a B antigen related to, but not identical with, human B, and closely related to the *E. coli* O_{86} B-like receptor; (3) Absorption of anti-B with human group B cells; and (4) Serial dilutions of anti-B. The various antisera were tested with group A and B bloodstains, a bloodstain made from the *in vivo* acquired B blood, a seminal stain from an A secretor inoculated with *E. coli* O_{86} and incubated, and a sample of muscle tissue which had acquired B reactivity in the river. Results of the elution experiments are shown in Table 19.9.

It was found to be more difficult to induce acquired B in a bloodstain than in a seminal stain. Acquired B can be detected by the inhibition test. The use of the considerably more sensitive elution test will detect it all the more readily. Acquired B seems most frequently to be a problem in seminal (and probably in vaginal secretion) stains, and in muscle tissue from bodies under certain circumstances. Jenkins *et al.* noted that not every anti-B would stand all the procedures carried out to try and render the antiserum specific, and that the simplest procedure was probably absorption with the rabbit red cells. There is no reason to believe, however, that acquired B antigens will behave identically in all cases, and an anti-B absorbed so as to be inactive with one specimen could still be active with a different one.

Pereira and Martin (1975 and 1976) discussed a number of problems involved in body fluid grouping. They pointed out that pseudo-A and pseudo-B reactions (i.e., acquired) were not uncommon in saliva samples. They had been found to be more common in saliva and vaginal secretion samples than in uncontaminated semen samples. Changes in the ABH specificity of saliva can be brought about by incubating the specimen at 37° for 24 hours, thus implicating bacteria in the transformation. The changes could be caused by receptors on the bacteria themselves, or by blood group enzymes (those which transfer terminal sugars involved in the immunodeterminant oligosaccharides—section 19.9.3) elaborated by the bacteria. The concentrations of pseudo-A and pseudo-B receptors are very much lower than those of the genetically determined substances in secretors, and discrimination can be achieved in some cases by using short absorption times or by diluting the sample 1:1 with saline. If corresponding blood samples are available, these simple procedures were said to be adequate in most cases, but not in cases of saliva stains or samples of unknown origin. It was noted too that AB secretors do not always secrete A and B substances to the same extent, and that occasional individuals may be encountered who secrete in one fluid but not in another. It was recommended that inhibition and elution tests be carried out in parallel, and on a series of dilutions of antigen-containing material. With whole saliva, tests were done on a series of 10-fold dilutions. True group substance reactions from secretors will be detected by inhibition, and confirmed by elution. Further, the sensitive elution test may show a prozone effect at very high antigen concentrations which can be of diagnostic value. This prozone effect was discussed in section 19.10.5.2 in connection with the experiments of Pereira *et al.* (1969). Nonsecretor material will be negative in the inhibition test, but the group may sometimes be detected by elution. It was considered unwise to rely on an elution result for diagnosis of the group in nonsecretor

Table 19.9 Reactions of Several Acquired B Samples with Anti-B Antisera, Variously Treated to Remove the Acquired B Reactions (after Jenkins et al., 1972)

	Reaction of B Cells With Eluate From:				
Antiserum	B Bloodstain	A Bloodstain	Acquired B Bloodstain	Acquired B Semen Stain	Acquired B Muscle Tissue
Neat	+++	–	+++	+++	++
1:16	+++	–	+	++	++
1:64	+++	–	–	–	+
Absorbed with acquired B cells	+++	–	(+)	nt	nt
Partially absorbed with B cells	++	–	–	–	–
Absorbed with rabbit cells	++	–	–	–	–

+++ macroscopic agglutination; ++ strong microscopic agglutination; + less strong microscopic agglutination; (+) weak agglutination; nt not tested

material, unless the nonsecretor status could be confirmed by Lewis typing (secretions from nonsecretors group as Le(a+b−), except in cases of Le(a−b−) individuals). A spurious reaction can be observed as weak in the inhibition test, and will disappear in the elution test as the dilutions become greater, while a true group substance reaction from a secretor gives strong inhibition reactions and persists in the elution test throughout the dilution series. With saliva stains, aqueous extracts were used. The neat sample was used for the inhibition test, and a series of dilutions (neat, 1:5, 1:10, 1:20 and 1:40) were subjected to the elution test. Lewis typing was carried out on 2 × 2 mm stains by an inhibition test, using a substratum control and control saliva stains from Le(a+b−), Le(a−b+) and Le(a−b−) persons. Elution had not been successful for Lewis typing. Pereira and Martin concluded by noting that the results of the two procedures, inhibition and elution, had to agree with one another before they would report the results. Pereira and Martin (1977) published a full paper on Lewis grouping in body fluid stains and in saliva by inhibition technique. Results were very good, and the procedure was found to be quite useful in certain cases in the determination and confirmation of secretor status.

19.10.5.5 Fractionation of soluble ABH substances from secretor fluids (and red cells). In 1969, Fiori *et al.* reported that the soluble substances in human saliva could be fractionated on molecular sieve media (Sephadex, Sepharose). Three fractions could be obtained: Fraction 1 was of high MW, and fractions 2 and 3 were considerably smaller. Not every secretor saliva had the same combination of fractions. All secretors had fraction 1, but, depending on the person, it could be present alone, in combination with fraction 2, in combination with fraction 3, or in combination with both. There were thus distinguished four categories or "types" of secretors: namely, I, having fraction 1; II, having fractions 1 + 2; III, having fractions 1 + 3; and IV, having all 3 fractions. These characteristics were found in A, B and O secretors, the fractions in each group having the respective blood group activity. A series of papers followed in the *Journal of Chromatography* in which the studies were expanded (Fiori *et al.*, 1971a and 1971b). Nonsecretors (in the usual sense) were studied, and it was found that they always lacked fraction 1. They could, however, possess either of fractions 2 and 3, or both of them, or neither of them, giving four "types" of nonsecretors, which were called V (fraction 2), VI (fraction 3), VII (fractions 2 + 3) and VIII (neither fraction 2 nor 3). The type VIII would be a "true nonsecretor". A person could be any one of the eight types for A, B or H, depending upon which substances were secreted. A nomenclature was suggested to indicate the combination present. Group O is the simplest, since only H is secreted. An O person found to have H fractions 1, 2 and 3 would be designated O(H-1,2,3), while an O person having only fraction 1 would be called O(H-1), and so on. An A person secreting A fractions 1 and 2 and H fractions 1, 2 and 3 would be A(A-1,2/H-1,2,3), and an AB person secreting all three A fractions, B fraction 1 and H fractions 1 and 3 would be AB(A-1,2,3/B-1/H-1,3), and so forth. Human red cell stroma were solubilized with Triton-X-100 and subjected to the fractionation procedure. All three fractions were found in all subjects, corresponding to the phenotype of the donor, i.e., all three H fractions in O cells, all three H and A fractions in A cells, etc. Fiori *et al.* (1972) reported that population and family studies had been carried out to see whether they supported the notion of genetic control of these phenomena. Detailed data were not given, but it was said that the best genetic model was one in which two structural genes controlled the synthesis of fractions 2 and 3, with two additional gene pairs regulating the expression of these structural genes in secretions. There was said to be agreement with this hypothesis in the results of the analysis of mating combinations. It was also reported that two more low MW fractions had been isolated from red cell stroma (fractions 4 and 5) which inhibited anti-A, anti-B and anti-H equally. A full paper on fractions 4 and 5 followed (Fiori *et al.*, 1973), in which it was said that fractions 4 and 5, or both, or neither, could be found in saliva in association with fraction 3. This meant that type III, IV, VI and VII people could each be subdivided into four subtypes on the basis of fractions 4 and 5. The subtypes were called α(fraction 3 alone), β(fractions 3 + 4), γ(fractions 3 + 5) and δ(fractions 3 + 4 + 5)

Quite clearly, confirmation of these observations would have far reaching implications for the present understanding of the ABH substances on red cells and in secretions, and of the secretor system itself. Rutter and Whitehead (1975 and 1976) have reported that they could not confirm the observations, and were able to find only the higher MW fraction with blood group activity in the Sephadex fractions. Saliva from 12 nonsecretors and 16 secretors was studied. In the meantime, Panari *et al.* (1976) reported that the molecular heterogeneity seen in saliva is also present in the group substances in semen. Fiori *et al.* (1978) found the heterogeneity in group substances of urine as well. They said that there were four "types", and that this system was independent of the one in saliva. Fiori (1976) replied to the Rutter and Whitehead findings by stressing the importance of using the standardized technique which had been carefully devised by trial and error, not only for the fractionation, but also for the hemagglutination inhibition tests used to locate the activity. The smaller fractions were not all that easy to detect, he said. Ueda (1974) was said to have confirmed the saliva ABH fractions. Ueda's paper is written in Japanese, but it contains a table showing the various ABH fractions apart from which I could not read it. The salivary fractions as reported by Fiori and collaborators have also been confirmed by Hamilton and Kimberling (1973), Hamilton *et al.* (1974) and by Kimberling (1979). Hamilton and Kimberling (1973) had not yet found all eight of the phenotypes, but had essentially confirmed Fiori's findings. By 1974, Hamilton *et al.* reported that they had observed all the phenotypes, and that family studies suggested the involvement of more than one genetic locus in this polymorphism. Kimberling (1979) gave a genetic explanation for all the

findings and the family studies based upon three separate genetic loci, called Sec_1, Sec_2 and Sec_3. The Sec_1 locus was the "traditional" secretor locus (*Se/se*), he said, and controlled the high MW component. The other loci were distinct, but closely linked, and were said to control the low MW components of the polymorphism. These interesting developments prompted the Editor of the *Journal of the Forensic Science Society* of England (Editorial, 1976) to call for some other laboratories to take an interest in this question, and attempt to decide upon an explanation for the disparate observations. Kind *et al.* (1979a) and Lang *et al.* (1979a) from the Central Research Establishment group in England have recently noted that they find only a single fraction of blood group active substance upon fractionation of nonsecretor salivas, and that they could not, therefore, agree that "fraction 1" was absent in these samples.

The most recent series of papers from the C.R.E. group (Kind *et al.*, 1979b; Lang *et al.*, 1979b; Rothwell, 1979) gives the details of the efforts which have been made to repeat the observations reported by Prof. Fiori's group. It has not been possible to confirm the results, the papers say in effect. Apparently, Prof. Fiori visited the C.R.E. laboratories and demonstrated the procedures on four occasions. On two of them, there seems to be agreement that they worked, while on the other two occasions, the procedures did not work. These efforts are discussed by Kind *et al.* (1979b), who cautioned that they did not think these procedures were at all ready for use in forensic cases until the discrepancies could be worked out.

Perhaps other workers will become interested in the problem so that some understanding can be reached. For the moment, the whole matter remains something of a puzzle.

19.10.6 Factors affecting the success of medico-legal blood group determinations and unusual samples

Experienced forensic serologists all recognize the obvious fact that success or failure in group determination depends to a large extent on the condition of the evidence submitted or collected. Bhatnagar *et al.* (1974) gave an analysis of their success in grouping 1295 stains from 166 cases in the Delhi region in India, and tried to correlate it with the condition of the evidence. Almost 2/3 of the stains were on textile material, and about 3/4 of them could be grouped. Most of the remainder were badly contaminated, and in a few cases, the substratum control reacted. Only 23% of putrefied liquid blood samples collected at scenes (either as liquid, or on swabs), or samples mixed with soils, could be grouped. 41% of samples on weapons could be grouped; the remainder were contaminated. Only 8% of putrefied postmortem bloods could be grouped. Bhatnagar *et al.* gave some recommendations for the proper preservation of biological evidence.

Occasionally, it could be necessary to try and determine the blood group of a badly burned or charred body. Nagano *et al.* (1975a, 1975b and 1976) attempted to get some information about this situation by studying the thermal stability of the red cell blood group active glycolipids. Partially purified A, B, AB and H active glycolipids from red cells were heated, and tested for agglutinin binding activity. Heating to 120–130° for an hour lessened all the activities, the AB material being slightly more stable. A, B and H activities were abolished by 185° heat for 5 min, and AB activity by 200° heat for 5 min. Thermostability was higher if heating was carried out in the presence of serum. If tooth material (dental pulp) is used as a source of material for ABO grouping in burned bodies, the results of Korszun *et al.* (1978) are relevant, and indicate that the tooth enamel and dentine do not adequately insulate the ABH substances in pulp when the external temperature rises above 200° (see in section 19.10.7.2).

Finally, a few reports in the literature have dealt with samples having weak receptors or unusual antibodies. These matters have been discussed in various parts of section 19. Morgan (1964) had a case in which both whole blood and stains were submitted. The stains grouped as A, the cells as A_2, but the Lattes test on the stain and the agglutination tests on the serum showed that both A_1 and B cells were agglutinated. A_2 cells, however, were not agglutinated by stain extract or by serum, showing that the sample was of group A_2, with an unusual anti-A_1 in serum (see in section 19.3.1.1). Ishigu (1968a and 1968b) encountered a blood of group B_m (section 19.3.2). The cells did not agglutinate with anti-A or with any example of natural or immune anti-B, even if bromelin treated, or by the Coombs test. The B receptor was demonstrated by absorption and elution. Serum contained an anti-A. A bloodstain made from the blood grouped as O by inhibition, elution and mixed agglutination tests, and as B by the Lattes test. Saliva contained B substance, which could be detected in a saliva stain by inhibition or mixed agglutination. Masis and Ol'khovik (1970) had a case of an AB blood with a very weak B receptor, and in which the serum contained an anti-B. B-substance was present in the person's secretions.

19.10.7 ABO grouping of hair, nails and teeth

Determination of ABO groups in hair, fingernails or toenails, and various tissues of the teeth is a matter of medico-legal interest. Hair is relatively common physical evidence, and the hair shaft has few if any individualizing characteristics apart from its morphology. While there are reports in the literature on methods for the ABO grouping of hair that are claimed to be reliable, there are also many indications that the techniques have not been found to be reproducible in other laboratories. Determination of the ABO blood group from nails or from teeth can be of some help in the identification of persons under various circumstances, the primary value being in demonstrating non-identity.

19.10.7.1 ABO grouping of hair. In 1953, Krefft investigated the possibility of determining the group substances in hair. An inhibition procedure employing 0.1 g of pulverized hair was used. The hair from 35 people was tested and the correct results obtained in each case. High titered anti-A and anti-B was reduced about 6 to 7 dilutions in the presence of the respective antigen. "Anti-O" of titer 1:32 was re-

duced to 1:8 by an O sample, but there was no effect on anti-A or anti-B. Tesař (1954) in Prague carried out similar experiments. The hair was extensively washed in ether, and then pulverized into powder, sometimes using glass powder. 0.2 g of this material was then extracted with 5 mℓ saline in a 100° water bath for an hour, and the extract was used for the test. A three dilution reduction in antiserum titer using high titer anti-A and anti-B was taken as the minimal criterion for a positive result. Correct results were obtained with all samples. Disruption of the hair structure is apparently necessary to expose the group specific substances, since Vogt *et al.* (1965) got no inhibition of anti-A or anti-B in the absence of it. McWright (1961) applied a sonication procedure, which Thoma (1954) had developed for fingernails (see in section 19.10.7.2), to the grouping of hair. Sonication was carried out on about 7 mg hair material in the presence of anti-A or anti-B. Correct results could be obtained by the inhibition technique with donors from all four groups. Sonication time was critical.

The mixed agglutination technique for hair grouping was investigated by Lincoln and Dodd (1968a). Hairs were washed in ethanol, ether, acetone, and finally in saline containing 0.3% albumin. About 10 small lengths of hair were used with each antiserum. Absorption was for 2 hours, followed by at least four washes with saline, and a last wash in saline-albumin. Papain treated test cells in 1% suspension were used for detection. 17 examples of shavings were grouped correctly, but only 19 of 45 samples of head hair corresponded to the blood group of the donor. It was said that the blood group activity of the hair might reside in the sebaceous layer, and that too much preliminary washing with solvent might remove this layer, leading to false negatives, while too little washing might leave behind adventitious substances, leading to false positives.

Most studies have employed absorption-elution procedures. Beginning around 1966, Yada and his collaborators in Japan published an extensive series of papers on hair grouping by elution. Hairs were washed in ether, and crushed with a hammer and anvil. Two 1 cm lengths were incubated each with anti-A and anti-B. After two hours at room temperature, the samples were washed once in a large volume of cold saline, and elution carried out at 55° for 10 min. Test cell suspensions of 0.2% were employed. Forty-four samples were correctly grouped in this way, and secretor status appeared to have no effect on the presence of the blood group substances in hair (Yada *et al.*, 1966a). These studies were extended to include formalin-fixed hairs, some of them decades old (Yada *et al.*, 1966c) axillary, pubic and crural hairs (Yada *et al.*, 1966b), eyebrows, eyelashes and vibrissae (Yada *et al.*, 1966f), and an 88 year old hair rope (Yada *et al.*, 1968d). In all cases, correct results were obtained in all the samples. Hairs from Caucasian donors gave similarly correct results (Yada *et al.*, 1968c). A detailed description of the technique was published (Yada *et al.*, 1968b), and a double roller device was found to be very suitable for crushing the hair (Yada *et al.*, 1968e). A blind trial study was carried out in eight laboratories (Yada *et al.*, 1968d) in four of which the examiners had been trained in the procedure. Four errors were made in 250 hair samples by the experienced personnel, while in the four laboratories in which the examiners were previously unfamiliar with the technique, there were 33 incorrect groupings in 240 samples. Yada *et al.* (1966g) reported that rabbit hairs, subjected to the procedure, give a B result in most cases, with an occasional A. Yada *et al.* (1968a) said that scalp hair from persons with very weak forms of red cell B (B_m and AB_m cells) gave correct grouping results with the elution procedure.

In 1971, Wynbrandt and Chisum reported successful results with an elution procedure. Hairs were washed in shampoo and in methanol, and then crushed by subjection to 12,000 psi in a press for 30 sec between steel plates. The hair was then boiled in McIlvaine's buffer, pH 7.4, and dried. Absorption was for 24 hrs at 4°, after which the hair sample was washed with 7 to 11 mℓ ice cold saline and blotted dry. Elution was carried out for 10 min at 55° into an 0.25% test cell suspension, and the results read every 10 min for an hour. 50 samples could be correctly grouped in a blind trial, with the test on two of them having had to be repeated because of technical errors in handling. Anti-H reacted with all the samples, regardless of secretor status.

Gramer and Tausch (1973) gave a detailed elution procedure with which 42 of 43 samples were correctly grouped. Hairs were ether-washed and physically disrupted, and then incubated with antisera overnight in the cold. They were then washed with ice cold saline by means of a vacuum suction device, like the one used by Wynbrandt and Chisum (1971). Elution was allowed to proceed at room temperature for an hour into 1% test cell suspensions that had been bromelin treated. Technique was critical, and not all commercially obtained bromelin preparations gave satisfactory results. Cegla and Popielski (1976) said that they had used a microelution procedure with trypsin treated test cells to test 120 hair samples from living and dead persons. The group obtained corresponded with the blood group in 90% of the cases, was uncertain in 6.6%, and did not agree in 3.4%.

Boettcher and Kay (1973) reported success in hair grouping using ^{125}I-labelled antibody and autoradiography. The positives gave distinguishably darker images than the controls, and *Ulex* anti-H was not found to be satisfactory in the test. The presence of group specific substances in hair was independent of the secretor status of the donor.

Oepen and Noever (1980) carried out a study on 346 hair samples from 195 donors using a modification of the elution procedure. The group determined from the hair failed to match the red cell type in a substantial fraction of the cases (about 20%). A good review of the literature was given in this paper.

Kishi and Iseki (1977) recently reported isolating a glycolipid from hair which had A activity. The material gave all the reactions to be expected from an isolated A substance. H and B substances were isolated by these same workers in 1978.

19.10.7.2 ABO grouping in nails and teeth. In 1954,

Thoma reported on ABO group determination in fingernails and toenails by an inhibition technique. Sonic oscillations were found to expose the group substances in some way. From 25 to 50 mg nail substance was sonicated in saline for 30 min at 1000 KHz. The nail material was then dried, and overlaid with antiserum for 24 hrs at 5°. Anti-A,B (O serum) was used for the test. Reliable results were obtained with samples from all four groups, which were collected only from secretors. Sonication time was found to be very critical. McWright (1961) applied the procedure to 15 mg amounts of nail material, except that sonication was for 10 min and in the presence of the antisera. Reliable results were obtained, the sonication step being required for any inhibition of the antisera.

Outteridge (1963c) reported successful results in the grouping of 105 samples from 28 donors belonging to all four groups. The amount of antigen was variable, and was varied accordingly from 10 to 30 mg. Longer (36 hr) absorption times were used, and the anti-H reaction showed peculiar variations. Yada *et al.* (1966d) reported successful grouping of fingernail samples of about 1 mg from 62 persons of all four groups and including nonsecretors by their elution procedure (see in Section 19.10.7.1). The nail material was first scraped, and then crushed prior to absorption.

Schiebe *et al.* (1961) noted that the soft tissue of teeth, the dentine and pulp, could be grouped for ABO if the material came from secretors. An inhibition test was used. Takata (1973) showed that the elution technique could be applied to crushed tooth enamel for determination of ABO, secretor status being immaterial. The test needed 6–10 mg of material. In 1974, Takata said that the test was equally applicable to teeth which had been in the sand or in water for 2 years, or in air for 3 years, and from teeth that had been exposed to high heat (100° for 4 hrs, 150° for 2 hrs, or 200° for 15 min). Funatsu (1975) said that dental calculus could be used in an elution test for group determination. Mukai *et al.* (1975) tried a number of tooth tissues using elution, and could not get results from enamel, but they always got reliable results from pulp. Dentine and cementum were variable. Anti-H lectin and eel serum anti-H were used, with papain treated O indicator cells, for H antigen. Korszun *et al.* (1978) studied the thermostability of the ABH antigens in pulp, and estimated the protection afforded by enamel and dentine against heat by mathematical methods. At temperatures of 200° or greater, the hard tissue could not effectively insulate the pulp antigens from denaturation. Typing of pulp was reliable by elution technique, but typing of the hard tissues was not. Mukherjee and Chattopadhyay (1976) were successful in grouping the deeper hard tissue of teeth by elution. Yada *et al.* (1966e) noted that their elution procedure (Section 19.10.7.1) was applicable to bone fragments, if these were soaked in water for a couple of days, etherwashed, and then crushed prior to absorption.

19.11 Distribution of ABO Groups and Secretors in U.S. Populations

The data are shown in Tables 19.10 and 19.11. The criteria used in the selection of the data shown in these all subsequent population distribution tables were given in the Preface of this book.

Table 19.10 Frequency of ABO Groups in U.S. Populations

Population	Total	O	A		B	AB		Reference
			A_1	A_2		A_1B	A_2B	
			Frequency of Occurrence — Number (Percent)					
CAUCASIAN								
Iowa City, IA	49,979	22,392 (44.8)	21,144 (42.31)		4,695 (9.39)	1,748 (3.5)		Buckwalter & Knowler, 1958
Iowa City and Des Moines, IA	6,313	(45.8)	(41.6)		(9.0)	(3.6)		Buckwalter et al., 1958
Brooklyn, NY								
Leukemia patients								
Jewish	665	226 (34)	261 (39.2)		120 (18.0)	58 (8.7)		Macmahon and Folusiak, 1958
Non-Jewish	639	291 (45.5)	235 (36.8)		90 (14.1)	23 (3.6)		
Blood donors								
Jewish	375	142 (37.9)	165 (44.0)		46 (12.3)	22 (5.9)		
Non-Jewish	548	281 (51.3)	184 (33.6)		62 (11.3)	21 (3.8)		
Seattle, WA								
University students	5,657	2,399 (42.4)	2,458 (43.4)		599 (10.6)	201 (3.6)		Van Arsdel and Motulsky, 1959
St. Louis, MO								
Veteran's Hospital	359	169 (47.1)	149 (41.5)		29 (8.1)	12 (3.3)		Sievers, 1959
Control Donors	32,945	14,918 (45.3)	13,611 (41.3)		3,248 (9.9)	1,168 (3.5)		
New Haven, CT								
Yale students	1,000	431 (43.1)	422 (42.2)		110 (11.0)	37 (3.7)		Niederman et al., 1962
New York, NY								
Memorial Hospital								
Donors	4,738	2,029 (42.82)	1,828 (38.58)		636 (13.42)	245 (5.17)		Osborne and DeGeorge, 1962
Transfused patients	2,332	976 (41.85)	932 (39.97)		291 (12.48)	133 (5.7)		
Tumor patients	525	192 (36.57)	233 (44.38)		70 (13.33)	30 (5.72)		
Southeast Georgia	300	172 (57.3)	97 (32.3)	39 (13.0)	20 (6.7)	4 (1.3)	1 (0.3)	Cooper et al., 1963
Boston, MA								
Rheumatoid	608	276 (45.4)	183 (30.1)	63 (10.4)	55 (9.0)	25 (4.1)	6 (1.0)	Dublin et al., 1964
Non-rheumatoid	605	281 (46.4)	173 (28.6)	52 (8.6)	76 (12.6)	16 (2.6)	7 (1.2)	

Table 19.10 Cont'd.

Population	Total	O	A		B	AB		Reference
			A_1	A_2		A_1B	A_2B	
Pittsburgh, PA								
School population	1,578	609 (38.6)	630 (39.9)		250 (15.8)	89 (5.6)		
County Fair sample	3,871	1,586 (41.0)	1,595 (41.2)		489 (12.6)	201 (5.2)		Kaplan et al., 1964
Male blood donors	1,959	840 (42.9)	742 (37.9)		253 (12.9)	124 (6.3)		
Salt Lake City, UT								
College age population	247	111 (45.0)	108 (43.7)		23 (9.3)	5 (2.0)		Mayeda, 1966
Oakland - Eastern San Francisco Bay area, CA								
Mothers	4,928	(44.91)	(40.93)		(10.33)	(3.84)		Reed, 1967
Newborns		(45.43)	(39.77)		(11.24)	(3.55)		
San Francisco Bay area, CA								
"Caucasians"	8,962 ★	4,067 (45.4)	2,856 (31.9)	783 (8.7)	958 (10.7)	228 (2.5)	70 (0.8)	Reed, 1968
"Caucasians of Western European origin"	5,056	2,361 (46.7)	1,557 (30.8)	476 (9.4)	507 (10.0)	118 (2.3)	37 (0.7)	
New York, NY	500 ☆	229 (45.8)	134 (26.8)	37 (7.4)	62 (12.4)	29 (5.8)	5 (1.0)	Wiener, 1969
Denver, CO	3,648	1,717 (47.1)	1,473 (40.4)		324 (8.9)	134 (3.7)		Charney, 1969
West Virginia - central and southern area	1,412 ✪	(45.9)	(41.3)		(9.1)	(3.7)		Juberg, 1970
Tecumseh, MI	8,965	3,916 (43.68)	3.048 (34.0)	859 (9.5)	811 (9.05)	234 (2.61)	97 (1.08)	Schreffler et al., 1971
Brooklyn, NY								
Kings County Hospital								
Adults	599	265 (44.24)	220 (36.73)		76 (12.69)	38 (6.35)		
Newborns	253	114 (45.05)	102 (40.31)		29 (11.46)	8 (3.16)		Robinson et al., 1971
Patients	68	32 (47.06)	28 (41.18)		8 (11.76)	0		
Los Angeles, CA	205	79 (38.5)	63 (30.7)	25 (12.2)	31 (15.1)	3 (1.5)	4 (2.0)	Sturgeon et al., 1973
California and Hawaii	6,004	(48.2)	(35.8)		(12.1)	(4.0)		Grunbaum et al., 1978
Bexar County, TX	200	(54.0)	(34.0)		(8.0)	(4.0)		Ganaway and Lux, 1978
Detroit, MI	507	(41.2)	(33.5)	(1.4)	(17.0)	(4.1)	(2.8)	Shaler, 1978 ■
Baltimore, MD								
Postmortum samples	1,095	(44.1)	(39.7)		(11.7)	(4.5)		Profili and Hurley, 1979
Miami/Dade Co., FL	366	189 (51.6)	111 (30.3)		44 (12.0)	22 (6.0)		Stuver, 1979 and see Shaler, 1978
Los Angeles, CA								
Case material	419	190 (45.3)	169 (40.3)		52 (12.4)	8 (1.9)		Siglar, 1979 and see Shaler, 1978

Table 19.10 Cont'd.

Population	Total	O	A		B	AB		Reference
			A_1	A_2		A_1B	A_2B	
NEGRO								
New York, NY	200	(50.0)	(15.0)	(7.0)	(23.5)	(2.5)	(2.0)	Miller et al., 1951
Miami, FL	502	244 (48.6)	122 (24.3)		117 (23.3)	19 (3.8)		Butts, 1955
Washington, D.C.								
Howard University	1,188	(53.03)	(27.2)		(17.25)	(2.52)		Moore, 1955
Students	937	(49.3)	(20.7)	(6.5)	(20.0)	(2.9)	(0.6)	
Iowa City and Des Moines, IA	6,722	(49.1)	(26.5)		(20.1)	(4.3)		Buckwalter et al., 1958
St. Louis, MO								
Veterans Hospital	99	53 (53.5)	24 (24.2)		19 (19.2)	3 (3.0)		Sievers, 1959
Control Donors	1,395	713 (51.1)	353 (25.3)		269 (19.3)	60 (4.3)		
Iowa City, IA								
Controls	1,261	563 (44.65)	424 (33.6)	88 (6.98)	144 (11.42)	31 (2.46)	11 (0.87)	Newman et al., 1961
Southeast Georgia	333	162 (48.6)	50 (15.0)	24 (7.2)	58 (17.4)	1 (0.3)	5 (1.5)	Cooper et al., 1963
Oakland - Eastern San Francisco Bay area, CA								
Mothers	1,453	(49.28)	(26.84)		(19.13)	(4.75)		Reed, 1967
Newborns		(46.59)	(27.24)		(21.40)	(4.27)		
San Francisco Bay area, CA	3,146	1,540 (49.0)	603 (19.2)	267 (8.5)	605 (19.2)	84 (2.7)	47 (1.5)	Reed, 1968
Birmingham, AL								
School children	610	302 (49.5)	163 (26.7)		122 (20.0)	23 (3.8)		Casey et al., 1968
New York, NY	500 ☆	242 (48.4)	100 (20.0)	23 (4.6)	110 (22.0)	10 (2.0)	8 (1.6)	Wiener, 1969
West Virginia - central and southern area	133 ✪	(55.0)	(27.0)		(16.0)	(2.0)		Juberg, 1970
Brooklyn, NY								
Kings County Hospital								
Adults	1,150	580 (50.43)	271 (23.57)		246 (21.4)	53 (4.61)		Robinson, et al., 1971
Newborns	2,933	1,441 (49.13)	790 (26.93)		592 (20.18)	110 (3.75)		
Patients	628	298 (47.45)	171 (27.23)		137 (21.82)	22 (3.5)		
California and Hawaii	1,025	(47.7)	(25.9)		(21.5)	(5.0)		Grunbaum et al., 1978
Bexar County, TX	200	(55.0)	(21.0)		(19.0)	(5.0)		Ganaway and Lux, 1978
Detroit, MI	507	(53.1)	(16.0)	(0.2)	(26.2)	(2.8)	(1.8)	Shaler, 1978 ■
Baltimore, MD								
Postmortum samples	1,289	(46.6)	(27.5)		(22.3)	(3.6)		Profili and Hurley, 1979
Miami/Dade Co., FL	346	180 (52.0)	75 (21.7)		76 (21.9)	15 (4.3)		Stuver, 1979 and see Shaler, 1978
Los Angeles, CA								
Case material	185	87 (47.0)	56 (30.3)		35 (18.9)	7 (3.8)		Siglar, 1979 and see Shaler, 1978

Table 19.10 Cont'd.

Population	Total	O	A		B	AB		Reference
			A_1	A_2		A_1B	A_2B	
HISPANIC								
Southern Texas Mexican Surname	1,597	962 (60.2)	452 (28.3)		144 (9.0)	39 (2.5)		King et al., 1955
San Francisco Bay area, CA "Mexicans"	335	186 (55.5)	74 (22.1)	17 (5.1)	44 (13.1)	13 (3.9)	1 (0.3)	Reed, 1968
Brooklyn, NY — Kings County Hospital Puerto Rican								
Adults	288	161 (55.9)	90 (31.25)		27 (9.38)	10 (3.47)		
Newborns	928	533 (57.44)	275 (29.63)		96 (10.34)	24 (2.59)		Robinson et al., 1971
Patients	248	131 (52.82)	78 (31.45)		29 (11.69)	10 (4.03)		
California and Hawaii ◇	1,596	(56.5)	(30.9)		(10.8)	(1.9)		Grunbaum et al., 1978
Bexar County, TX	200	(61.0)	(26.0)		(11.0)	(2.0)		Ganaway and Lux, 1978
Miami/Dade Co., FL	357	204 (57.1)	117 (32.8)		29 (8.1)	7 (2.0)		Stuver, 1979 and see Shaler, 1978
Los Angeles, CA Case material □	215	133 (61.9)	60 (27.9)		19 (8.8)	3 (1.4)		Sigler, 1979 and see Shaler, 1978
CHINESE								
New York, NY Blood donors	103	(45.6)	(27.2)	(0)	(22.3)	(4.9)	(0)	Miller et al., 1951
New York, NY	400	172 (43.0)	108 (26.0)	0	101 (25.25)	19 (4.75)	0	Wiener, 1969
New York, NY	946 *	395 (41.75)	253 (26.74)		240 (25.37)	58 (6.13)		Wiener, 1974
ASIAN								
California and Hawaii	3,053	(32.5)	(37.8)		(21.0)	(8.8)		Grunbaum et al., 1978

★ Includes the 5,056 "Caucasians of Western European origin" in the next row.

☆ 2 A_1 and 2 A_1B were found in Caucasians; 4 A_1, 2 A_1B and 1 A_2 were found in Negroes.

✪ In 485 Caucasian A types, 76% were A_1 and 24% were A_2; in 34 Negro A types, 82% were A_1 and 18% were A_2; in 36 Caucasian AB types, 72% were A_1B and 28% were A_2B; in 3 Negro AB types, 1 was A_1B and 2 were A_2B.

* Includes the 400 previously reported in the row above (Wiener, 1969)

◇ "Chicano/Amerindian"

□ Primarily Mexican

■ Data of Stolorow and collaborators

Table 19.11 Frequency of Secretors in U.S. Populations (Caucasian)

		Frequency — Number (Percent)		
Population	Total	Secretor	Nonsecretor	Reference
Iowa City, IA	1,261	971 (77.0)	290 (23.0)	Newman et al., 1961
New Haven, CT				
Yale Students	1,000	773 (77.3)	227 (22.7)	Niederman et al., 1962
Boston, MA	1,194	857 (71.8)	337 (28.2)	Dublin et al., 1964
Tecumseh, MI	8,664	6,461 (74.57)	2,203 (25.43)	Schreffler et al., 1971
Los Angeles, CA	205	141	64	Sturgeon et al., 1973

SECTION 20. THE LEWIS SYSTEM

20.1 Introduction

The Lewis system is rather complex, related in a number of ways to the ABO and Secretor systems, and not yet completely understood. Some discussion of the Lewis groups appeared in parts of Section 19, and the nature of biosynthesis of Le^a and Le^b was covered in Sections 19.9.2 and 19.9.3. The Lewis groups may be thought of as a body fluid group system, the Lewis substances being absorbed onto the red cells under certain conditions.

20.2 Discovery and Development

The antigen Le^a was identified in nonsecretor saliva in 1939 by several Japanese workers. It was called "T", and the chicken immune precipitating serum which detected it was called "anti-T". This work was overlooked in the West until recently. It is discussed by Race and Sanger (1975) and by Prokop and Uhlenbruck (1969). There is another antigen at present which is called "T" (the Thomsen-Friedenreich T—see in section 21.8.2), and these two should not be confused. Wiener *et al.* (1964a) mention a number of examples of Lewis antibodies which were found, but were not named, prior to 1946.

In 1946, Mourant described a new agglutinin in a serum from a woman whose baby was suspected of having hemolytic disease, and which agglutinated about 25% of group O adult red cells from English people. The woman, Mrs. Lewis, and her husband gave Dr. Mourant permission to call the new antigen "Lewis". In 1947, Andresen found an antibody with similar characteristics in a woman called Anna Erikson. The antigen being detected was called "L", and about 21% of adults were L+. The percentage was much higher in children, depending upon their age. It was noted that L− parents could have L+ children, and Andresen supposed two alleles, *L* and *l*, where *LL* and *Ll* children were L+, but where only *LL* adults were L+. Somehow, the *L* gene was expressed in adults only in the homozygous state. In 1948, Andresen noted that Anna Erikson serum contained the antibody which Mourant had called "anti-Lewis", and which he now called anti-L_1. The serum of an A, MN man had been found to contain another agglutinin, called anti-L_2. L_2 behaved almost, but not quite, as if it were the product of the allele of the L_1 gene (e.g. 21.4% of adults were L_1-L_2-). There was a relationship between A_1 and L_2, but not between A_1 and L_1, when the L-system phenotypes were looked at within the ABO blood groups. Mourant's Lewis and Andresen's L_1 are now called Le^a, and L_2 is called Le^b.

In 1948, Grubb made the important observation that persons whose red cells were Le (a+) were nonsecretors of ABH, while the majority of people with Le(a−) cells were secretors. He found too that the saliva of Le(a+) people inhibited anti-Le^a. Brendemoen (1949) found that the serum of all Le(a+) people he tested, and most of the salivas, inhibited anti-Le^a. In 1949, Grubb and Morgan noted that Le^a and Le^b were present in the secretions of persons of appropriate genotype, and that these substances were very similar in their properties to the ABH substances. Some human ovarian cyst fluids were found to be rich sources of these substances. Their serological properties were destroyed by incubation with filtrates from cultures of *Clostridium welchii*. Brendemoen (1950) noted that Le^a was secreted in the saliva of people whose red cells were Le(a+b−), and in some people whose cells were Le(a−b+) as well, but to a variable extent. People whose cells were Le(a−b−) did not secrete Le^a in saliva. In 1950, Grubb said that he did not think of the Lewis system primarily as a blood group system, but as a serological system of water soluble mucoproteins. Consideration of Lewis as a blood group system, he said, was perhaps due to the ease with which red cells could be tested.

As a general rule, then, Le(a+b−) red cells came from ABH nonsecretors, Le(a−b+) red cells came from ABH secretors; and Le(a−b−) cells may be from ABH secretors or nonsecretors, the proportion of each being the same as in the rest of the population. There are exceptions. Andresen (1948) described a few people whose cells were "L_1 L_2" with a weak "L_1", i.e., Le(a+b+) where the Le^a was weak. Cutbush (1956) described other examples of these cells in adults and in infants. In populations other than Europeans, secretors of ABH may be found whose cells are Le(a+). Lewis *et al.* (1957) found this circumstance in two Japanese, and Boettcher and Kenny (1971) found that 10–15% of Australian aborigines have Le(a+) red cells and are ABH secretors.

In 1951, Grubb set forth an explanation for the Lewis system which, with minor modifications, still forms the basis of the way the system is looked at. Essentially, the presence of Le^a in saliva was regarded as being independent of the secretion of ABH substances in saliva. Le^a is found in the saliva of about the same proportion of secretors as of nonsecretors. The Secretor and Lewis genes were thought of as being independently segregating, but as interacting in some of their phenotypic expressions. Ceppellini's studies have tended to confirm Grubb's ideas, and helped to form the basis for the general framework. Ceppellini and Siniscalco (1955) analyzed data from family material which had been collected for another purpose, with the idea of understanding the relationship between Lewis and Secretor. ABH secretor status was determined, as was the presence or absence of Le^a in saliva and on red cells. Some of the results had been presented elsewhere, and details are given in the paper. The data indicated that two genes, called *Le* and *le*

comprised an allelic pair which controlled the synthesis of Le^a (and thus its appearance on red cells and in body fluids). Persons of genotypes *LeLe* and *Lele* had Le^a; persons who were *lele* lacked it. The *Le/le* genes were inherited independently of the genes controlling the ABH secretion characteristic (*Se/se*). The *Se* gene, however, exerts an effect on the phenotypic expression of the *Le* gene, in that *Le* expression is suppressed in saliva and nullified in red cells in the presence of *Se*. Thus, *LeLe* and *Lele* people are Le(a−) on cells and have some Le^a in saliva if they are *SeSe* or *Sese* as well, but are Le(a+) on cells and have more Le^a in saliva if they are *sese* (see Table 19.8). In this view, Le(a+) parents can produce Le(a−) children (*Lele sese* × *Lele sese* → 1/4 *lele sese*), and such families have been found. Le^b is now regarded as the product of the interaction of *Le* and *H*, as shown by the biochemical studies of Morgan and Watkins (section 19.9.3).

20.3 Lewis Antigens on the Red Cell

In 1955, Sneath and Sneath made the important observation that Le(a+b−) cells transfused into an Le(a−b+) recipient, the transfused cells and "native" cells later being separated by differential Rh agglutination, had become Le(a+b+) (Sneath and Sneath, 1955a). Thus, Le(a+b−) cells had acquired Le^b *in vivo* in an Le(b+) person. The transformation could be brought about *in vitro* as well: Le(a+b−) cells + Le(a−b+) plasma → Le(a+b+) cells, and Le(a−b+) cells + Le(a+b−) plasma → Le(a+b+) cells. Mäkelä and Mäkelä (1956a) confirmed this observation for Le^b, and said that plasma Le^b did not inhibit the agglutination reaction, but could transform Le(b−) red cells into Le(b+). The work was extended (Mäkelä *et al.*, 1967), and the plasma substances found to be stable to 100° heating for 2 min, and nondialyzable. Plasma substances could transform the red cells, but salivary ones could not, and there was no evidence for enzymatic involvement in the transformation. Nakajima *et al.* (1968) noted that there were no differences between the abilities of fetal and adult red cells to take up plasma Lewis substances, and uptake did not appear to depend on the amount of the substance present in the plasma.

20.4 Lewis Substances in Saliva and Other Body Fluids

It is now accepted that the symbol "Les" stands for the presence of Le^a in saliva, and the symbol "nL" for the absence of Le^a in saliva (Race and Sanger, 1975). From what has been said, the general scheme predicts that nL × nL matings will not produce any Les children, and no exceptions to this rule have been found in family studies (Race and Sanger, 1975).

Brandemoen (1949) showed that the serum of Le(a+) people contains Le^a, but that the serum of Le(a−) people does not. This was found independently by Grubb and Morgan (1949) and has been confirmed.

Brown *et al.* (1959) found that the Le^a antigen in the saliva of Le(a+b−) people is immunologically different from that in the saliva of Le(a−b+) people. The former is precipitated by an anti-Le^a precipitin serum prepared in rabbits. The latter is not, even though it may very strongly inhibit an anti-Le^a agglutinin serum tested with Le(a+) cells. The same observation was made by Baer *et al.* (1959) using antisera prepared in chickens by injection of certain ovarian cyst fluid or nonsecretor saliva substances. Some Le^a substance in the saliva of Le(a−b−) nonsecretors has been reported (Sturgeon and Arcilla, 1970; Andresen, 1972; Gunson and Latham, 1972). This perplexing observation could be related to the kind of anti-Le^a used for the tests, or to Le^c (Race and Sanger, 1975).

In 1964, Kaklamanis *et al.* proposed that it should be possible to distinguish homozygous from heterozygous secretors (*SeSe* from *Sese*) on the basis of the Le^a/H ratio in saliva. Based on prior studies (Brown *et al.*, 1959), they had the view that Le^a and H specificities reside on the same macromolecule, and that phenotypic expression of the genes gives rise to different numbers of the different determinants on the molecular surface. Kelso (1968) agreed that H and Le^a expression in saliva was competitive, but said that it was not strictly reciprocal. He denied that the genotype at the secretor locus could be determined. The amount of Le^a in saliva in various ABO, secretor and Lewis phenotypes has been studied by a number of investigators, and is variable (Kaklamanis *et al.*, 1964; Kelso, 1968; Sturgeon and Arcilla, 1970; Boettcher and Kenny, 1971; Randeria and Bhatia, 1971; Arcilla and Sturgeon, 1973).

There is evidence that the Lewis substances in plasma are glycosphingolipids, whereas those in the saliva and ovarian cyst fluids are glycoproteins (Marcus, 1970). The plasma Lewis antigens are found in both high and low density lipoprotein fractions, isolated from plasma. These preparations, but not the residual plasma, can transform red cells with respect to Lewis antigens. It was noted that all Le^b glycoprotein preparations have H activity, whereas Le^b glycosphingolipids do not. Concentrated solutions of HLe^b glycoprotein preparations had a weak transforming effect on Lewis negative red cells. These data help to explain some of the prior observations on red cell Lewis transformation.

Grubb (1951) found no Le^a in five seminal samples. Lodge and Usher (1962), however, found Lewis substances in semen in concentrations parallel to what is found in plasma. McConnell (1961) said that Lewis substances were present in semen at lower concentrations than in saliva, and that occasionally a man may have Lewis substances in saliva but not in semen. Lewis substances are present, however, in fairly high concentrations in both gastric juice and in urine.

20.5 Some Complexities of the Lewis System

20.5.1 Antigens of Le(a−b−) red cells

In 1957, Iseki *et al.* described an immune antiserum which contained an incomplete, cold antibody reacting with Le(a−b−) cells from secretors as well as from nonsecretors. The antiserum was obtained by immunizing rabbits with saliva from a secretor whose red cells were Le(a−b−)

and then absorbing with Le(a−b+) and Le(a+b−) red cells. The antibody was called anti-Le^c, and the antigen it was detecting was Le^c. 1.86% of Japanese people tested reacted as Le(a−b−c+), and a three allele scheme, Le^a, Le^b and Le^c, where Le^c was dominant over Le^b and Le^a, and Le^b dominant over Le^a in red cell expression, was needed to explain the family studies. Race and Sanger (1975) have noted that this antibody, in view of the fact that it reacts with Le(a−b−) cells from *both* secretors and non-secretors, may be a mixture of the anti-Le^d of Potapov (1970b) and the anti-Le^c predicted by Potapov (1970b) and described by Gunson and Latham (1972) (see below). Lodge *et al.* (1965) did a rather involved analysis of five Lewis antisera, which were said to be anti-Le^{ab} (equivalent to anti-Le^x - see below), and one of which reacted with Le(a−b−) and O_hLe(a−b−) cells. Andersen (1958) found an antibody in the serum of a 12 year old male cancer patient who was A_2Le(a−b+) and had no history of transfusions. This "Magard" serum reacted with A_1Le(a−b−) cells from secretors in preference to A_2Le(a−b−) cells from secretors, and Andersen said that the *Se* gene modified the A substance on the cells, in the absence of *Le*, so as to make them reactive with the Magard serum. Such an interpretation would not be compatible with the overall scheme of gene action indicated in Fig. 19.9 (Race and Sanger, 1975).

In 1970, Potapov prepared an antiserum by injecting goats with saliva from an OLe(a−b−) secretor, and absorbing the serum with trypsin treated Le(a+b−) cells (Potapov, 1970b). The antiserum contained an anti-Le^b, and an incomplete, cold antibody which did not react with Le(a+b−) or Le(a−b−) cells from nonsecretors, but did react with Le(a−b−) cells from secretors. The new agglutinin was called anti-Le^d, and its antigen Le^d. The designation "Le^c" was reserved for the predicted antigen that would characterize Le(a−b−) red cells of nonsecretors. Gunson and Latham (1972) found anti-Le^c in a woman who had been transfused and who had had four pregnancies. It reacted with the cells of Le(a−b−) nonsecretor people, and the reaction was inhibited by saliva from Le(a−b−) nonsecretors, less strongly by saliva from Le(a−b−) secretors, and very little by saliva from Le(a−b+) secretors. It was also inhibited by purified glycoprotein from ovarian cyst fluids of Le(a−b−) nonsecretors, and by 3-fucosyl-lactose. These tests were done by Dr. Tippett and Dr. Sanger in London (Race and Sanger, 1975), who also confirmed that a goat anti-Le^c prepared by Dr. Potapov in 1973 had the same specificity as Gunson and Latham's human one. Dr. Winifred Watkins commented in the discussion section of Gunson and Latham's paper that the anti-Le^c reaction inhibition by 3-fucosyl-lactose suggested that Le^c had a terminal chain configuration corresponding to "X" in Fig. 19.11. It could be expected, too, that Le^d would be equivalent to "Y" in Fig. 19.11, since in secretors, the second α-fucosyl residue would be transferred to the galactosyl residue. It is not necessary to postulate any new alleles to understand these new Lewis antigens in this way, since Le^a and Le^b are derived from type 1 chains, while Le^c and Le^d (X and Y) would be derived from type 2 chains (Figs. 19.10 and 19.11). Table 20.1 shows some of the relationships that were given in Table 19.8, extended to include the Le^c and Le^d factors. Hirsch and Graham (1980) have recently shown that Le^c and Le^d, like Le^a and Le^b, are adsorbed onto the red blood cells from plasma.

20.5.2 Le^x

In 1949, Andresen and Jordal described an antibody, which they termed "anti-X", and which later became known as anti-Le^x. Some have considered anti-Le^x to be anti-Le^a + anti-Le^b, or anti-Le^{ab}, but the discoverers do not so consider it. Andresen (1961) said that anti-Le^x is a specific agglutinin, detecting a specific red cell receptor Le^x, closely related to Le^a. Sturgeon and Arcilla (1970) studied families having members with red cell phenotype Le(a+b+x+), and the data suggested to them that the condition could be explained by a variant *Se* gene, in which Le^a is not completely converted to Le^b. In their view, and in Andresen's, Le^x is considered a product of the *Le* gene, but independent of secretor status. Sturgeon and Arcilla (1970) said that the *Le* gene might have cistrons, coding for the presence of Le^a and Le^x. Andresen (1972) and Arcilla and Sturgeon (1973) studied subjects with Le(a−b−x−) cells, and small quantities of Le^x and of Le^a could be found in the saliva of nonsecretors. These findings are not readily understandable at the present time.

20.5.3 Anti-A_1Le^b

Seaman *et al.* (1968) described an antibody in a serum "Siedler" from an A_1Le(a−b−) subject which reacted only with A_1Le(b+) cells, but not separately with A_1 or Le(b+) cells. A few additional examples of this antibody have been reported.

20.6 Lewis Antisera

20.6.1 Anti-Le^a

Many examples of human anti-Le^a have been found since Mourant found the antibody in the serum of Mrs. Lewis, most of them very weak, and, according to Race and Sanger (1975), frequently hemolytic. They are nearly always found in Le(a−b−) subjects, and may contain some anti-Le^b. Potent anti-Le^a is rare. The use of papain treated cells is often helpful.

Anti-Le^a may be prepared in animals by injecting a variety of Le^a containing fluids or red cells. A number of examples have been mentioned in section 20. Potapov (1976) prepared a variety of Lewis antisera in goats.

20.6.2 Anti-Le^b

Some of the confusion in the literature is undoubtedly because of the fact that there seem to be two types of anti-Le^b. One of these, the anti-Le^{bH} of Ceppellini *et al.* (1959) or anti-$Le_1{}^b$ of Sneath and Sneath (1955b), is inhibited by all secretor saliva, and behaves very much like anti-H in an inhibition test. The red cell Le^b detected by this antiserum shows considerable suppression in the presence of A_1. The other, anti-Le^{bL} (Ceppellini *et al.*, 1959) or anti-$Le_2{}^b$

Table 20.1 Saliva and Red Cell Relationships for the Four Lewis Factors

Saliva		Red Cell Reactions With			
ABH	Le^a	Anti-Le^a	Anti-Le^b	Anti-Le^c	Anti-Le^d
Secretor	Les	−	+	−	−
Nonsecretor	Les	+	−	−	−
Secretor	nL	−	−	−	+
Nonsecretor	nL	−	−	+	−

(Sneath and Sneath, 1955b) is not inhibited by saliva from Le(a−b−) secretors, but only by saliva which contains (inhibits) anti-Le^a. The red cell Le^b detected by this antiserum does not show epistatic suppression by A_1. Thus, many examples of anti-Le^b react with O and A_2 cells. Bird (1959) has suggested that "anti-H" may be regarded as anti-H + anti-H_1, where anti-H_1 would be equivalent to anti-Le^b, presumably to anti-Le^{bH}. Wiener *et al.* (1964a) shared this view of anti-Le^b.

20.6.3 Other Lewis Antisera

Lewis antisera other than anti-Le^a and anti-Le^b have been discussed in section 20.5.

20.7 Theories About the Lewis System

The ideas of Grubb and of Ceppellini about the Lewis system were developed in section 20.2. As will be evident, there are things about the Lewis system and its interaction with other genes that are not very clear. Some other ideas about the Lewis system will be mentioned here. Andresen and Henningsen (1951) and Andresen *et al.* (1950) developed explanations to take care of the reactions of anti-Le^x. These ideas have been extended (Andresen, 1961) to include the two types of anti-Le^b and the "Magard" antibody. Andresen's paper (1961) must be read. It is much too complicated to summarize easily.

Pettenkofer (1953a and 1953b) put forward a three allele hypothesis which included the genes Le^a, Le^b and Le^c. Le^c was dominant over Le^b and Le^a, and Le^b was dominant over Le^a. The theory explained the anti-Le^a, anti-Le^b and anti-Le^x reactions, and their relations to the *Se/se* locus. Wiener *et al.* (1964a) are for the most purposes in agreement with Grubb and Ceppellini, except that Le^b is regarded as a form of H, as suggested by Bird (1959). Wiener *et al.* suggest that if Le^b is really a variant of H, it should be ignored in the Lewis system notation. A review of the ABO, Secretor and Lewis systems is given by Andersen (1969), and Hossaini (1977) reviewed a number of aspects of the Lewis system.

20.8 Biochemical Studies

Most of this material was presented in Section 19.9. Studies on fractionated Le^a-active ovarian cyst fluid glycoprotein were done by Bhaskar and Creeth (1974). Hanfland *et al.* (1978) isolated and purified a number of Le^a-active and some related glycolipids from the serum of ALe^a people. Prohaska *et al.* (1978) demonstrated the *in vitro* enzymatic synthesis of Le^a and Le^b from the glycolipid Lewis precursor found in the sera of Le(a−b−) people. They showed further that the precursor is present on the red cells of Le(a−b−) people. Staal *et al.* (1977) carried out studies on a family with a rare congenital disease called fucosidosis, in which an α-L-fucosidase specific for α1→2 linkages is congenitally absent, causing Le^b to accumulate in tissues. The condition is inherited as an autosomal recessive trait.

20.9 Medico-legal Applications

The Lewis system is not used in disputed affiliation cases because its mode of inheritance does not allow conclusions to be drawn. Good Lewis antisera are relatively uncommon, and the system has only one major medico-legal application, that of confirmation of secretor status in body fluids and body fluid stains. Pereira and Martin (1975, 1976 and 1977) have discussed this application (see in Section 19.10.5.4), which was carried out using an inhibition technique. They said they had not had success with the elution method. Piner and Sänger (1980) recently reported successful results in grouping body fluid stains for Le^a and Le^b by inhibition technique. Paired saliva stain-vaginal secretion stain samples from women and paired saliva stain-semen stain samples from men were tested and shown to give identical Lewis results. The Lewis substances in body fluid stains could be detected for a matter of weeks in these experiments. It was said that the finding of Le(a+b−) results in such a stain could be used to verify ABH nonsecretor results. The finding of Le^b in mixtures of semen and vaginal secretions might be useful in confirming the presence of secretor semen in vaginal material which was Le(b−) as well.

In 1974, Yudina in Moscow reported that Le^a and Le^b could be determined in bloodstains up to a year old using an absorption-elution procedure modified from that of Nickolls and Pereira (1962). Anti-Le^a and anti-Le^b from goats was used, test cells were trypsin-treated, and the detection reaction was carried out in 3% serum albumin (human or bovine). The antisera had a titer of 1:32–1:64, and elution was carried out at 52–54° for 30 min. Davie (1979) described a procedure for Lewis grouping on microtiter plates, which was applicable to blood or body fluids, and had the advantage of conserving material.

20.10 Distribution of Lewis Phenotypes in U.S. Populations

These data are presented in Table 20.2.

Table 20.2 Distribution of Lewis Plenotypes in U.S. Populations

		Frequency — Number (Percent)					
Population	Total	Le(a+b−)	Le(a−b+)	Le(a−b−)	Le(a+)	Le(a−)	Reference
CAUCASIAN							
New York, NY	460	(22.8)	(71.5)	(5.7)			Miller et al., 1951
Boston, MA	1,194	307	666	221			Dublin et al., 1964
South Central West Virginia	1,412				(25.9)	(74.1)	Juberg, 1970
Tecumseh, MI	7,775				7,296 (93.84) saliva	479 (6.16)	Schreffler et al., 1971
Los Angeles, CA	205				189 saliva	16	Sturgeon et al., 1973
NEGRO							
New York, NY	211	(23.2)	(54.5)	(22.3) ☆			Miller et al., 1954
New York, NY	397	44 (16.95)	138 (58.5)	54 (24.5) ★			Ceppellini et al., 1959
South Central West Virginia	133				(20)	(80)	Juberg, 1970
CHINESE							
New York, NY	85	(23.5)	(70.6)	(5.9)			Miller et al., 1951

☆ Of 23 Le(a−b−), 17 were ABH Secretors and 6 were ABH Nonsecretors

★ Of 54 Le(a−b−), 40 were ABH Secretors and 14 were ABH Nonsecretors

SECTION 21. THE MNSs SYSTEM

21.1 Discovery and Mode of Inheritance of the MN Blood Groups

In 1927, when ABO was the only blood group system known, Landsteiner and Levine were looking for other antigenic differences in human red cells. Cells of different people were injected into rabbits, and the rabbit antisera absorbed with human cells of various types. Antibodies were being looked for which would discriminate cells from different individuals on the basis of other than ABO distinctions. An antibody was found which agglutinated the cells of about 83% of Caucasians and about 69% of Blacks, independent of their ABO groups (Landsteiner and Levine, 1927a). The antigen being detected was designated "M". Soon afterward, two additional factors, called N and P, were found (Landsteiner and Levine, 1927b), and it was established that M was an inherited characteristic. P will be discussed in Section 24.1. In the papers which followed (Landsteiner and Levine, 1928a and 1968b), further studies on the population distribution of the characteristics were done, along with studies on 161 families. N was clearly inherited as well, and both M and N behaved as dominants, since parents who both possessed a factor could have children who lacked it, but parents who both lacked a factor could not have children who possessed it. If the M and N characteristics were inherited independently, it would have been expected that some M-N- people would have been found in the number of people grouped, and none were. Assuming that there was not some peculiar irregularity (e.g. M-N- being lethal, etc.), the easiest explanation of the observations was a two allele hypothesis, in which the homozygotes were M or N, and the heterozygotes were MN. This hypothesis was very soon confirmed by Schiff (1930) in 42 families, and by Wiener and Vaisberg (1931) in 131 families. Thus, M and N are inherited as a Mendelian codominant pair of alleles *M* and *N*, where the genotypes *MM*, *MN* and *NN* give rise to the phenotypes M, MN and N, respectively. This mode of inheritance has been confirmed by studies on thousands of families, and is widely accepted. There have arisen a number of complexities with MN over the years, and these will be described in a subsequent section.

21.2 The S and s factors

In 1947, Walsh and Montgomery found an antibody in a puerperal patient which reacted with about 48% of randomly selected cells, and whose reactions appeared to be independent of ABO, Rh, P, Lutheran, Kell and Lewis systems. A sample of the serum was sent to Sanger and Race in London, who tested it and agreed that it was detecting an antigen independent of the systems noted above, but it was not independent of MN (Sanger and Race, 1947). The possibility that the antigen, called "S", was an allele of MN was considered, along with the possibility that it was governed by a separate locus. In the latter case, a corresponding allele for *S*, namely "*s*", would be predicted. It became clear that *S* was not an allele of *M* and *N*, and it appeared to be related to MN as a linked locus. A joint report by all four investigators appeared in 1948 (Sanger *et al.*, 1948). It was predicted that an anti-s would eventually be found. In 1951, Levine *et al.* did find the anti-s in the serum of a Mrs. Guth, who had had a child that suffered from hemolytic disease (Levine *et al.*, 1951b). They conducted a number of studies on it, and sent some of it to Sanger and Race in London, who tested it in connection with their ongoing MN studies. These studies (Sanger and Race, 1951) indicated that the Ss locus was separate from, but closely linked to the MN locus, and the MNSs system genotypes, phenotypes and reactions using anti-M, anti-N, anti-S and anti-s are indicated in Table 21.1.

21.3 Recombination and Mutation in the MNSs System

The genetic evidence clearly shows that *S* and *s* are not alleles of *M* and *N*, but the linkage is apparently very close. If this were not so, recombination would be relatively common, for crossover frequency is directly proportional to the distance between loci. When recombination is rare, it can be difficult to distinguish between recombination and mutation. A most interesting family study was reported by Gedde-Dahl *et al.* (1967). The family was pretty large (12 sibs in the second generation), and data for most members of four generations was available. The family was being studied because a rare, inherited disease called Epidermolysis bullosa simplex was segregating in it. In a male of the second generation was found the phenotype MNS, and his genotype could be diagnosed through his descendants as *MS/NS*. The genotypes of his parents, established by the informative sibs in his generation, were the same: *MS/Ns*. The possibility of his being illegitimate was considered, but it is virtually ruled out by the other blood groups, and by the fact that he, like his father, had the rare variant form of Epidermolysis bullosa. Affected male relatives of the propositus were excluded as possible fathers by other blood groups. The choice as to how this observation should be interpreted came down to one between recombination and mutation, and the investigators favored the former. In a careful survey of the literature, three other possible recombinations were discussed. Details are given in Race and Sanger (1975). Although mutation cannot be ruled out, it is known that mutation in all organisms is exceedingly rare. There is only one example of an apparent mutation in the

Table 21.1 MNSs Types Using All Four Antisera

Reaction With					
Anti-M	Anti-N	Anti-S	Anti-s	Phenotype	Genotype(s)
+	+	+	+	MNSs	MS/Ns; Ms/NS
+	+	+	−	MNS	MS/NS
+	−	+	−	MS	MS/MS
+	−	+	+	MSs	MS/Ms
+	−	−	+	Ms	Ms/Ms
+	+	−	+	MNs	Ms/Ns
−	+	+	−	NS	NS/NS
−	+	+	+	NSs	NS/Ns
−	+	−	+	Ns	Ns/Ns

blood groups, this in a case studied by Henningsen and Jacobsen (1954), in which an M mother had an N child. This child was born at home, and maternity was not in question. The mother reacted as *MM* but the child's cells reacted as having a single dose of N. A special anti-N antiserum from a normal healthy MN person, which reacted only with NN cells, but not with MN cells (Metaxis-Bühler *et al.*, 1961), was used to test the child's cells. The explanation did not appear to be any known rare variant of the MN system (see below), and it remains something of a puzzle.

21.4 The Variant S^u—The Problem of U

In 1953, Wiener *et al.* described a fatal hemolytic transfusion reaction in a 35 year old Black female, who had had three pregnancies, and was being treated for a bleeding ulcer (Wiener *et al.*, 1953d). Her serum turned out to contain a strong, saline agglutinating antibody against all cells from Caucasians and almost all cells from Negroes. The factor being detected was called "U", and 4 out of 425 Black people tested turned out to be U-. In further studies (Wiener *et al.*, 1954), the cells of 1100 Whites reacted with anti-U, and 12 out of 989 Blacks were found to be U-. Cells from U- people were injected into rabbits to see if an "anti-u" could be made, but without success. There was a suspicion that U was MNSs related in some way.

A second example of anti-U was found in another Black woman, and studied by Greenwalt *et al.* (1954). In the course of the study, it was noted that the two U- bloods available failed to react with *either* anti-S or anti-s. Anti-U could thus be thought of as anti-S + anti-s, and *U* could be interpreted as an allele of *S* and *s*, which was called S^u. This tidy explanation did not quite do, however, since it was soon found that not all S-s- samples are U-. Race and Sanger (1962) recorded this result in samples sent from various places. Further studies by Allen *et al.* (1963) and by Francis and Hatcher (1966) indicated that about 15% of S-s- samples were U+. Allen *et al.* (1963) observed too that MS-s- cells, whether U+or U−, do not contain the small amount of N that is characteristic of all other M cells. Thus, anti-U is not anti-Ss. The S-s- phenotype may be due to an allele S^u, or to an inhibitor gene operating on the Ss locus. Race and Sanger (1975) said that they would continue to use the designation S^u for the S-s- phenotype. Apparently, anti-U reactions with S-s- cells are not always that clear cut, leading to problems of interpretation. There have been suggestions that there may be two different anti-U. The U-phenotype has been found thus far only in non-white populations, mostly in Blacks. However, S^u or something like it, has been observed rarely in Europeans. An interesting example is given by Austin and Riches (1978).

21.5 Complexities of the MN System

21.5.1 Variants of M and N

The major outlines of the MNSs system, in terms of reactions with the four antisera, and in terms of inheritance, are not especially complicated. The problem of U has been discussed. For most practical purposes, the peculiarities of the system are not an issue, since the majority are rare. In terms of understanding the system, however, there will eventually have to be an explanation for the unusual observations that have built up in fair quantity. There are variants of both M and N, and a number of antigens that have their own names, but which are in some way associated with the MNSs system. Ultimately, all these pieces must be put together in a way that is consistent with the underlying biochemical basis for the system. The extensions of the MNSs system are mentioned briefly here, but are not discussed in detail.

In the older literature are found descriptions of weak M and N receptors. In 1935, Crome described an apparent M mother with an N child, and the grouping results were confirmed by a number of laboratories. Thomsen and Landsteiner apparently agreed that the mother could have a very weak N receptor which had been inherited and was fully expressed in the child. Friedenreich (1936c) had an exactly similar situation, except that the mother did react weakly with selected examples of potent, immune anti-N. The mother had two sisters in this case who shared the characteristic. Friedenreich called the normal N antigen "N_1", and this weak variety "N_2". Moskow (1935) reported the subdivision of both M and N into M_1/M_2 and N_1/N_2. Pietrusky (1937) reported studies on the N_2 receptor, as did Krah (1949a and 1949b). It is hard to know what to make of these studies today in terms of the present versatility of the system, and the wider range of antisera that are available.

The antigen M^g was discovered by Allen *et al.* (1958b), and it appears to be of very low frequency. Oddly enough, however, anti-M^g is relatively common. The M^g antigen does not react with anti-M or anti-N. M^g is a little more frequent in Switzerland. M^g is governed by a gene M^g, an allele of M and N. People who are M^gM^g are known. Brocteur (1968) mentioned the possible problem of missing M^g in paternity testing. Ikin (1966) made good anti-M^g in rabbits by injection of cells from one of the rare homozygotes.

In 1964, Metaxas and Metaxas-Bühler described an apparently silent allele of M and N, called M^k. From the data on this and subsequent families that have been studied, it is clear that M^k gives no M or N antigen, and no S or s antigen. It may not be a true allele, but some sort of operator gene variant which can affect MN or Ss. M^k is quite rare.

In 1960, Jack *et al.* found that some human anti-M reagents contain an "anti-M_1", which divides the cells of group M people in a qualitative way into M and M_1. M_1 is fairly infrequent in Caucasians (about 3% of M genes), and more frequent among Black people. In the Bantu of Africa, half the *M* genes are M_1 (Le Roux and Shapiro, 1969). Most antisera to M_1 are anti-M + anti-M_1, which gives rise to complications. Occasionally, an almost "pure" anti-M_1 can be found (Le Roux and Shapiro, 1969). Anti-M_1 is occasionally found in the serum of an individual with MN red cells (Molthan, 1980).

In 1966, Gershowitz and Fried reported an antigen called M^v. M^v is dominant and may be associated with S or s. It is quite rare. It is peculiar in that it behaves in certain ways like M, and in others like N.

Some other variants: M^c, M^r, M^z, and M^a and N^a. Coverage may be found in the classic references (Race and Sanger, 1975; Prokop and Uhlenbruck, 1969).

21.5.2 Other associated antigens

An antigen called Hu (for Hunter) occurs in some 7% of American Black people. Another antigen called He (Henshaw) occurs in about 3% of African Black people, and not in Caucasians. M. Shapiro (1956) has studied He, and MacDonald *et al.* (1967) found an anti-He in the serum of a pregnant White woman which reacted in saline at 12° and in albumin at 37°. H. A. Shapiro (1956) commented on the medicolegal significance of He. Anti-He sera can be produced in animals (Wiener *et al.*, 1964b). The Miltenberger series of antigens has an intricate history involving the antigens Vw, Gr and Mi^a. Cleghorn (1966) brought much order to the confusion, there being apparently 5 classes of antigens in the series. Some other antigens: Vr, Sul, Far, Mt^a, Cl^a, Ny^a, Ri^a and St^a. A reference to "Sul" is Konugres and Winter (1967), and for some background on Ny^a, see Schimmack *et al.* (1971). Information on Tm and Sj may be found in Issitt *et al.* (1968). Judd *et al.* (1979b) described an apparently new MN-related antigen, defined by a serum called "Can" from a 57 year old white male with cancer. All M+ cells tested had the Can antigen, although some had to be treated with neuraminidase before they would react. Some N cells reacted as well. The antiserum also reacted with a higher percentage of cells from Black donors than from White donors.

21.6 Antisera to MNSs Antigens

Anti-M and anti-N occur in human serum. Wolff and Jonsson (1933) found an anti-M in a human serum. There are many examples of these sera. Prokop and Uhlenbruck (1969) give a thorough discussion of this subject. Kao *et al.* (1978) have suggested that anti-M formation in M-people may come about as the result of bacterial infection. Antisera to M and N are often made in rabbits, and have been made in other animals as well. Kerde (1965) had no luck in trying to make the reagents in sheep. Occasionally, antibodies which appear to react with part of M, or part of N, are disclosed. Konugres *et al.* (1966) found an anti-M in an MN person which was not autoagglutinating. It had to be assumed that the antibody was detecting something of M that the possessor lacked, from whence we get the antibody being called anti-M^A, and the person's cells M^aN. A rather similar kind of anti-N, called anti-N^A, was found in Melanesians (Booth, 1971). Metaxas-Bühler *et al.* (1961) found a very interesting anti-N, called "AP", in an MN donor. Exhibiting a sort of ultimate dosage effect, this serum reacted with NN cells, but not at all with MN cells. It has been very useful in the study of peculiar MN antigens.

Smith and Beck (1979) studied 50 human sera containing saline agglutinating anti-M. 78% of these were IgG while the remainder were IgM. Ordinarily, IgG antibodies are not expected to be saline agglutinins, but anti-M is an apparent exception to this rule.

Anti-M and anti-N lectins have been found. The seeds of *Vicia gramineae* have a rather specific anti-N (Ottensooer and Silberschmidt, 1953), while those of *Iberis amara* have an anti-M (Jack *et al.*, 1960). Several species of *Bauhinia* seeds contain anti-N, but it is not usually saline reacting (Mäkelä and Mäkelä, 1956b; Mäkelä, 1957). The specificities detected by these lectins are not precisely identical, and the lectins have been used as probes to get information about the structure of the receptors.

Anti-S and anti-s are found in sera on occasion. Anti-s can be made in rabbits. Human anti-s is not as common as anti-S. Antisera to other factors in the system have been mentioned briefly in the foregoing sections.

21.7 Heterozygous Advantage and MN

There has been considerable discussion in the literature about the fact that, if all the data are looked at, there is an excess of MN offspring produced by MN × MN matings, i.e., it is significantly greater than the 50% predicted (One expects from MN × MN matings a 1:2:1::M:MN:N ratio). Wiener (1962) believed that this effect was an apparent one, caused by grouping errors made by using underabsorbed anti-N. Rabbit anti-M and anti-N reagents are made by injecting group OM and ON cells into the animal, and then absorbing with human cells of the opposite specificity. It has been known for a long time that anti-N sera often react with M cells. Overabsorption with M cells renders the sera weak and nonspecific, while underabsorption leaves them cross-reactive, i.e., MM may be grouped as MN. The matter is discussed below. Wiener (1962 and 1963) presented a compelling argument for his position, and showed that in his own studies representing a large number of families, the production of MN offspring by MN × MN matings was very close to 50%. Race and Sanger (1975) and Prokop and Uhlenbruck (1969) agreed with Wiener on this point. Prokop and Uhlenbruck (1969) pointed out the fact that this shows that accurate MN grouping can be less trivial than is perhaps widely appreciated.

21.8 Biochemical Studies on the MN System

21.8.1 Introduction

The nature of the M and N determinants has been studied intensively in a number of laboratories, and there is now substantial information on the immunodeterminants. The picture is, however, less clear than in the case of ABO, and there are a number of complications in the data which will eventually require explanation. Although the various plant agglutinins have aided in the research, their reactions are not all exactly identical, and this situation has complicated the data somewhat.

Like ABH determinants, MN determinants are not restricted to red cells. Springer (1965) regards both classes of determinants as rather generally distributed cell surface structures, which have been named "blood group substances" essentially on the basis of their having been first discovered on red blood cells. They are not specific to blood, nor even to animals.

Uhlenbruck (1965) brought up some useful biochemical distinctions, which it is well to keep in mind in discussions of the chemical nature and biosynthesis of the blood group substances. The molecules are glycoproteins, a general class of substances which contain protein and carbohydrate. Further information about glycoproteins may be found in Gottschalk (1972). Uhlenbruck (1965) distinguished the following categories of these molecules: *mucins*, consisting of a protein chain with many low MW carbohydrate units and with hexosamine-N-Ac-neuraminic acid disaccharides; *mucoids*, which are quite similar, but contain higher MW carbohydrate moieties, which are branched, and in which the same molecule may contain a number of different kinds; *serum glycoproteins*, containing both of the above type of prosthetic carbohydrate groups; and the *γ-globulin type*, in which the molecule has one or two high MW carbohydrate chains. The term "mucopolysaccharide" is equivalent to "mucoid", British and American authors tending to favor the former. Mucopolysaccharides and mucoproteins are not quite identical. Further discussion of this richly complex subject is given by Prokop and Uhlenbruck (1969). The MN substances (and the ABH and Lewis ones) are mucoids. Further information on the nature of many kinds of mucus secretions may be found in *Ann. N.Y. Acad. Sci.* **106**: 157–809 (1963).

21.8.2 The Thomsen phenomenon

This could very well be considered a separate subject, but it is brought up and discussed briefly here, since references to it arise in connection with the nature of M and N. Simply put, the Thomsen phenomenon is polyagglutinability. It was first observed in blood samples a few days old. The cells were agglutinated by the serum from blood of any ABO group, and this agglutination took place better at temperatures less than 37°. Hübener (1926) and Schiff and Halberstaedter (1926) both described this behavior. Oluf Thomsen (1927) observed the same phenomenon and studied it in more detail. He showed that the agent bringing about the change was of bacterial origin, and that exposure of cells brought about a change in them such that a previously hidden antigen was exposed. He called it "L" (for "latent"). His student, Friedenreich, studied the phenomenon in quite a bit of detail (Friedenreich, 1928a and 1928b), and wrote his doctoral dissertation on it (Friedenreich, 1930). It was found that certain bacteria produced the "transforming principle", which led to the development of the previously hidden receptor. Friedenreich re-named the receptor "T", and the serum agglutinin for it was called "anti-T". The terminology has persisted, and one must not confuse Friedenreich's T with the salivary antigen first described by the Japanese workers in 1939, but which we now call Le^a (section 20.2). Friedenreich showed that the transforming principle was an enzyme. Anti-T could be found in the serum of all adults in variable amount. Prokop and Uhlenbruck (1969) discussed the subsequent development of the T/anti-T system and panagglutinability generally. The subject has become quite complicated. The enzyme causing the transformation is now known to be neuraminidase, and the transformation of cells, such that they are anti-T agglutinable, was called the Hübener-Thomsen-Friedenreich Phenomenon by Prokop and Uhlenbruck (1969). Springer *et al.* (1979) have conducted a series of studies, the results of which suggest that T and/or related antigens may be associated with certain types of human cancer cells.

It is now clear that T is a member of a general category of receptor which Uhlenbruck (1965) has called "cryptantigens". The red cell (and other cell) surfaces are now known to contain a number of structures whose immunodeterminant specificity resides in various carbohydrate chain end groups. Removal of the end residue changes the immunospecificity, sometimes generating an antigen which was not there before. In the particular case of cryptantigens revealed by the removal of neuraminyl groups (like T), Uhlenbruck uses the term "Friedenreich antigens", and there are now a number of these known (see Kim *et al.*, 1970). They are not limited to human cells nor to red cells. Studies on the distribution of the T antigen in cells and membranes from various sources were carried out by Newman and Uhlenbruck (1977). The "true" Thomsen-Friedenreich antigen, now called T_F, has the terminal disaccharide β-D-Gal(1→3)NAc-D-GalNH$_2$ as its immunodeterminant structure. This is recognized by serum anti-T, and by a number of lectins. It is known that an agglutinin from the peanut is anti-T specific, and Skutelsky *et al.* (1977) took advantage of this property to study the distribution of T receptors and agglutinin binding to a number of cells using ferritin-conjugated lectin, and examination of the reacted material in the electron microscope.

21.8.3 The nature of the MN receptors

In 1954, Hohorst used a phenol extraction procedure to obtain an MN-active preparation from red cells, which was partially characterized and contained polysaccharide. Around 1958, Georg Springer and his collaborators took up their extensive studies of MN substances. Certain influenza viruses were found to inactivate M and N, but not other known blood group receptors. Identical effects could be produced by neuraminidase (which was called RDE, receptor destroying enzyme) from a bacterial source. The virus was known to code for neuraminidase (Springer and Ansell, 1958). These observations were independently made by Mäkelä and Cantell (1958) in Finland. In 1959, Baranowski *et al.* in Poland isolated and partially purified M and N substances from red cells and determined their carbohydrate and amino acid composition. In 1960, Lisowska showed that several proteolytic enzymes reduce, but do not abolish M and N activity. No sialic acid was released upon treatment with these enzymes (pepsin, papain, α-chymotrypsin and trypsin). Klenk and Uhlenbruck (1960) isolated NANA-containing mucoids, and could show that papain did release sialic acid from these preparations, concomitantly destroying M and N reactivity, without, however, destroying the influenza virus receptor. It is to be noted that neuraminidase

treatment of cells always generated the T agglutinogen (section 21.8.2) in cells and purified preparations. Stalder and Springer (1960) could show that preparations from red cells and from human kidneys inhibited anti-M and *Vicia gramineae* anti-N reactions. Springer and his collaborators have isolated M and N substances from red cells and other sources, and purified some of the preparations to a high degree. Isolation and partial characterization of M and N substances from red cells and N substances from meconium were reported by Hotta and Springer (1965). In 1966, Springer *et al.* purified the N substance from red cells and that from meconium (which was called Me-Vg antigen, because it reacted with *Vicia graminea* lectin) extensively. The preparations were potent myxovirus receptors, and induced specific anti-N formation in rabbits. The peptide content of the red cell preparations was 44%, and of the Me-Vg antigen, 13%. The MW of the red cell preparation was 595,000, and that of the Me-Vg was 520,000. There was evidence that the carbohydrate residues were linked to the peptide by way of serine or threonine residues.

Other information has been gathered from studies on rare variants of the MN system, and from differences in the reactivities with lectins and other agglutinins of nonhuman origin. Springer and Stalder (1961) had an opportunity to examine MM^g and NM^g cells (see in section 21.5.1). The M^g was not destroyed by influenza virus nor by neuraminidase. Trypsin and papain did inactivate M^g. The evidence on M^g had suggested that it was an allele of M and N, but these studies clearly indicated that, at minimum, the arrangement of sialic acid residues in the M^g receptor differed from what it is in M and N receptors.

It has been known for a long time that M cells will react with anti-N (Landsteiner and Levine, 1928b). Rabbit anti-N has to be absorbed with M cells in order to obtain specificity, but overabsorption can remove too much anti-N and make the serum useless. Many investigators have confirmed this finding. The same behavior characterizes the *Vicia graminea* anti-N reagent. Levine *et al.* (1955a) found that, although the *Vicia* reagent does not agglutinate M cells too well—although it does to some extent, and especially if they are centrifuged—it is absorbed onto M cells and can be eluted from them. On this basis, and on the basis of the finding that mucoid preparations from M cells inhibited anti-N reactions, Uhlenbruck (1960) suggested that N, or something like N, served as the precursor substance in the biosynthesis of the MN substances. Allen *et al.* (1960) interpreted this behavior to mean that the *M* gene produces a small amount of N substance. There are, however, some M cells that do not react with anti-N reagents, including *Vicia* lectin. Allen *et al.* (1960) showed that an example of MU cells did not react with anti-N, and they tested an example of MU- cells and found the same behavior. These cells, which were S-s-, did not, therefore, appear to make the N substance found in most other M cells. Uhlenbruck and Krupe (1965) reported, however, that MuMu cells do in fact have the receptor for *Vicia* anti-N, but as a cryptantigen, and that neuraminidase treatment is required in order to reveal the reaction. Romanowska (1964) showed that the reactions of several lectins were a little bit different. Neuraminidase treatment of M and N substances inhibited but did not abolish their ability to inhibit the reactions of *Iberus amata* anti-M and *Bauhinia variegata* anti-N. Exposure of M and N substances to receptor destroying enzyme from *T. fetus* abolished their reactions with *Vicia* and *Iberus* reagents, but not the *Bauhinia* reagent. Springer *et al.* (1972) have shown that isolated M antigens inhibit most anti-N reagents tested (rabbit, human, and *Vicia*). Mild acid hydrolysis of M substance releases sialic acid, and leads to an increase in anti-N inhibition, such that in the course of hydrolysis, M substance becomes indistinguishable from N substance. Springer and Huprikar (1972) noted that the virus receptor and the isoantigen were thought to be controlled by a single gene, leading to a branched terminal structure possessing β-galactose and N-Ac-neuraminic acid, both of which are required for N activity. β-galactosidase destroys N activity without affecting M. Cohen *et al.* (1972) studied the reactions of an agglutinin from the horseshoe crab (*Limulus polyphemus*). Absorption of the agglutinin onto MN cells is inhibited by prior sensitization with anti-M or anti-N, and MN mucoid substance inhibits *Limulus* reagent agglutination of cells. This evidence suggested that *Limulus* reagent was reacting with a receptor common to M and N determinants, but not determining the difference between them.

As noted previously, the increased number of plant and animal agglutinins and the sometimes subtle differences in their reactivity has complicated the picture to some degree. At the same time, any general theory of the biochemical genetics must be able to explain the reactions. Uhlenbruck has proposed to try and put some order to the dozens of "reactivities" with various "receptors" of sera and of lectins and protectins. The effort needs to be appreciated in the broad terms of looking at the red cell (and other cell) surface as a structurally complex mosaic of antigenic determinants. Uhlenbruck accepts the concept of Wiener that many of the antigens may be complex, i.e., that they may have a number of determinants (which Wiener calls blood factors). The nomenclature is fairly involved, but it is covered in Prokop and Uhlenbruck (1969). It is descriptive, and general, in that it allows for expansion as new reactions are found, and does not carry with it any mechanistic or genetic implications. There is much to recommend a standardized set of nomenclature, but, as Prokop and Uhlenbruck (1969) themselves noted, such an effort ". . . . occasionally leads to strained relationships between serologists!" Two examples will illustrate the idea: $A^{hum}(A^{pig})$ indicates the A antigen of *human* red cells, which contains the A factor of *pig* red cells that is detected with *human* anti-A. It also contains the so-called sheep cell Forssman antigen, which can be detected with rabbit serum. Thus, A^{she}_{ra}. And the full designation of human red cell A would be $A(A^{pig}_{hu}, A^{she}_{ra})$. Cryptantigens are denoted by square brackets. Thus human red cell M substance is $M(M_{ra}, M_{Ia}, M_{Vg}, [N_{Vg}])$, where "ra" indicates

rabbit, "Ia" indicates *Iberus amata* and "Vg" indicates *Vicia graminea*.

As to the biochemical genetics of MN, it is not yet as clear as in the case of ABO, as pointed out at the beginning of the discussion. Uhlenbruck's original suggestion that N, or something like it, was a precursor of M has been modified to take into account the reactivity with the various phytagglutinins, and the studies on the rarer kinds of MN variants (Uhlenbruck, 1969). The model is quite involved, but invokes a precursor substance which has the *Vicia*, *Bauhinia* and *Iberus* reagent receptors. The precursor is the molecule to which sialic acid is added, thus generating the M and N receptors. The U/u genes are postulated to act next in the sequence, and the S/s genes last. Springer and Huprikar (1972) and Springer *et al.* (1972) took the view that N gene product is the immediate precursor of M gene product, and that the difference between M and N resides in the carbohydrate chain. In this view, M and N are not alleles, and the allele of *M* is an amorph. More recently, Springer and Desai (1974 and 1975) have said that the difference in M and N structures is one NANA residue attached to a β-galactopyranosyl residue, as indicated in Fig. 21.1, which also indicates the proposed relationship of M and N with T and Tn. The results of Sadler *et al.* (1979) and of Judd *et al.* (1977a) with M and N reactivity as functions of sialic acid content are not in agreement with the view that sialic acid is the integral determinant of the antigen structure.

There is now considerable biochemical evidence that the substantial difference between M and N resides in the structures of the N-terminal peptides of the glycoprotein molecules, and does not depend on carbohydrate. At the same time, there is no doubt that carbohydrate, especially N-acetyl-neuraminic acid, plays an important role in defining the structure of the antigens, some anti-M and anti-N reagents being more capable of detecting NANA-dependent differences than others (Sadler *et al.*, 1979; Judd *et al.*, 1979a; Judd *et al.*, 1979b; Springer and Yang, 1978). In 1967, Lisowska and Morawieki showed that blocking the free amino groups of M and N substances (mostly ϵ-NH_2 groups of lysine were involved) interfered with their reactivity toward rabbit antisera. Activity toward *Vicia* reagent was, however, not affected. They suggested that the peptide moieties were important features in determination of the basic differences between the structures. Dahr *et al.* (1975a

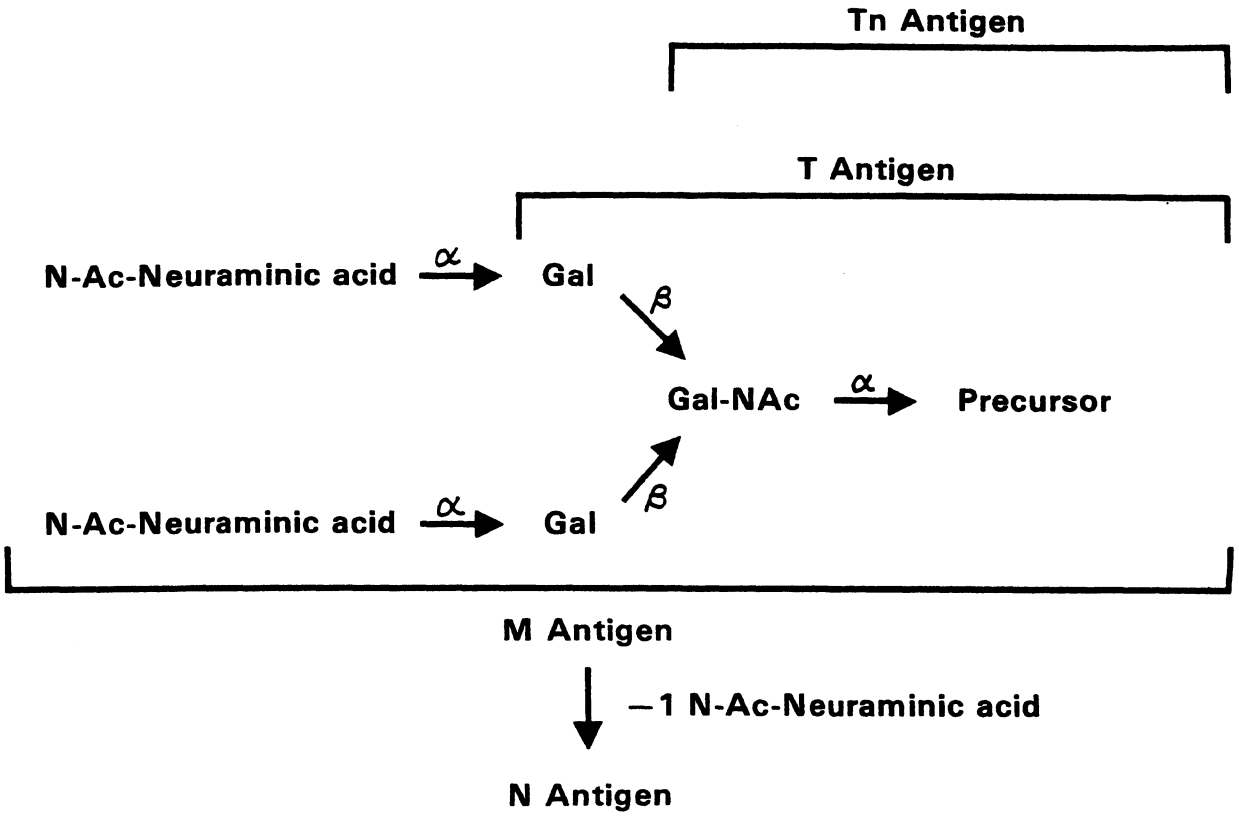


Figure 21.1 Terminal Oligosaccharide Structure of M, N, T and Tn Immunodominant Groups (according to Springer and Desai, 1974)

and 1975b) did a number of similar experiments and reached similar conclusions. Lisowska and Duk (1975) expanded the previous studies, and suggested that the peptide moieties in the molecule conferred certain steric configurations on the end groups which explained the available data. The *Vicia* receptor was said to be more internal, accessible in N cells, but not in M cells. Dahr *et al.* (1975a) agreed with this view. Waśniowska *et al.* (1977) have now shown that the N terminal amino acid of the peptide chain in M substance is Ser, whereas in N substance it is Leu. Dahr *et al.* (1977) reported identical results, and additionally that MN cells yield both amino acids. Waśniowska *et al.* found another amino acid substitution—probably at position 5—where M has Gly and N has Glu. These results have been confirmed in a number of different laboratories (Blumenfeld and Adamany, 1978; Dahr and Uhlenbruck, 1978; Furthmayr, 1978). Lisowska and Kordowicz (1977) have shown that antisera can be prepared in rabbits against desialyzed (sialic acid removed) M and N glycoproteins. These sera agglutinate desialyzed red cells, and precipitate desialyzed glycoprotein, but do not react with native cells or glycoprotein. After appropriate absorption of the anti-desialo-M and N, the reagents can be rendered specific, and this is taken to mean that sialic acid is not an essential feature in determining the difference between M and N structures. Springer *et al.* (1977) have said that only serine is found as the N-terminal amino acid of the peptide in gently isolated M and N glycoproteins. A partially purified glycopeptide fraction from M cells showed N-terminal serine predominantly, but sometimes leucine, while the reverse was true in the case of the fraction derived from N cells.

Walker *et al.* (1977) studied the reactions of normal M and N cells, MU cells and of the cells of an M/- patient and his family quantitatively, and they also determined the electrophoretic mobility of the cells. The data did not support the idea that M and N differ only in sialic acid residue content (Fig. 21.1). They did not support the idea of an N precursor relationship to M either, because if this were the case, MU cells—which do not cross react with rabbit anti-N—would be expected to have more M receptors, and they did not. The previously reported "generation" of rabbit anti-N receptor by neuraminidase treatment is explained on the basis of the presence in rabbit anti-N of an antibody which reacts better with desialized red cells. A theory was proposed to account for these findings and to take into account the evidence suggesting that the peptide moieties from M and N specific structures were different. It was called the "acceptor site" theory. The *M* and *N* genes, in this view, determine only the peptide sequences. The M and N specificities are produced by transferases which put together the oligosaccharides, but which are coded for by genes unrelated to *M* and *N*. It was suggested that the terminal oligosaccharide structure may be identical in M and in N, the conditions for antigenic specificity being brought about by configurational differences dictated by the peptide, which by its primary structure, determines the location of the attachment sites for the first sugar, N-Ac-Gal-NH_2. At least two such attachment sites would be specified by the primary structures in each MN protein, and possibly many more. Springer's data, suggesting that M specificity needs two closely juxtaposed sialic acid residues, could be explained by supposing that the *M* gene yields a peptide in which acceptor sites (i.e., amino acids) are close by, or next to one another. The difference in M and N would reside, therefore, in the distance between the amino acids to which the terminal oligosaccharide chain became attached. The *M* and *N* genes are thought to dictate the synthesis of distinct and independent glycoprotein, thus explaining the M^k and M/- hemizygous conditions. MU and Mu phenotypes are thought to represent alterations in the sequence.

The structural studies on the glycoproteins of the red cell membrane in a number of laboratories appear to have revealed that the difference in M and N lies in two amino acids in the N-terminal glycopeptide of the glycoprotein. Marchesi (1979) has reviewed the studies on the red cell membrane glycoproteins. This work is closely related to the structural studies discussed in section 21.9.4. The glycoproteins which carry MNSs specificities have been called "glycophorins" by Marchesi, Furthmayr and their collaborators at Yale. There are now known to be three separate glycophorins, designated A, B and C. Glycophorin A has been purified, and the complete amino acid sequence of its peptide moiety determined (Tomita *et al.*, 1978). It is in amino acids 1 and 5 (counting from the N-terminus) that the glycophorin A molecules from MM and NN cells differ (Waśniowska *et al.*, 1977; Dahr *et al.*, 1977; Dahr and Uhlenbruck, 1978; Furthmayr, 1978; Blumenfeld and Adamany, 1978; Tomita *et al.*, 1978). The data of Blumenfeld and Puglia (1979) fully support the structure for glycophorin A set forth by Tomita *et al.* (1978) (see Fig. 21.2). Furthmayr (1978) has shown that the N-terminal sequence of glycophorin B is identical to that of the glycophorin A from NN cells in at least the first 23 residues. This accounts for the fact that anti-N reacts with MM cells. Glycophorin B also carries the Ss antigens. Investigations on S-s- cell glycoproteins have shown that the glycopeptide carrying these specificities is very much reduced (Tanner *et al.*, 1977) or perhaps absent altogether (Dahr *et al.*, 1978). Red cells of type M^g have the same amount of glycophorins A and B as normal cells, but MM^k cells have only 50% of the normal complement (Furthmayr, 1978). Anstee and Tanner (1978) and Anstee *et al.* (1979) showed that cells from M^g and Miltenberger types have altered sialoglycoproteins.

21.9 Medico-legal Applications

21.9.1 Introduction—disputed parentage applications

As noted, M and N factors were first found in 1927. Within a short while, it was established that these were consistent serological characteristics of the human population, and the mode of inheritance was clear (Landsteiner and Levine, 1928; Schiff, 1930; Wiener and Vaisberg, 1931). The potential for use of the system in disputed affiliation cases was immediately recognized (Wiener and Vaisberg, 1931; Schiff, 1932b and 1933a). According to Schiff and

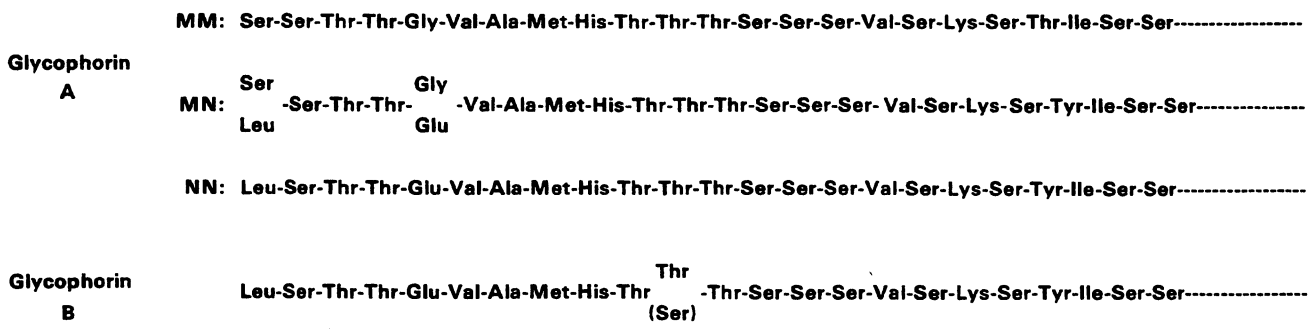


Figure 21.2 Amino Terminal Sequences of the First 23 Amino Acid Residues in the Peptide Moieties of Glycophorin A from MM, MN and NN Red Cells and of Glycophorin B from Red Cells, regardless of MN Type (after Furthmayr, 1978 and Tomita et al., 1978).

Boyd (1942), the first legal case in which MN was employed was in Potsdam in 1931. Wiener (1935) reported an exclusion in a case in this country involving a mother, three children, and two different men, based on MN grouping, but he did not say when it was done, nor whether it went into court. The system is well established as a marker in disputed affiliation cases, and S and s are regularly included as well by many laboratories (see, for example, the case of Silveira, 1967). Application of the system to the grouping of bloodstains developed more slowly.

If anti-M and anti-N are used in paternity tests, the probability of excluding a falsely accused father is close to 18% (AABB, 1978). Should anti-S and anti-s be employed in the tests as well, the probability value is increased to about 31% for Whites and 32% for Blacks (Chakraborty *et al.*, 1974; AABB, 1978). MN is the most powerful two allele system for paternity exclusion, and exclusion probabilities if all four antisera are used are higher than for any other red cell antigen system. It is important to be aware of the rare variants discussed in section 21.5.1 when carrying out these tests. On the one hand, it is not expected that these variants would be encountered very often, if ever; on the other, the presence of one of them (e.g. M^k or M^g) in a sample being tested could cause one to misinterpret the results of the in vestigation. If it could be shown that a rare allele were passed from a putative father to a child, this fact would greatly increase the probability of his being the true father. The peculiarities of MNSs must be appreciated and considered in interpreting exclusions obtained with the system (Morel, in AABB, 1978). Problems of interpretation may be encountered if S^u types are present, and Black populations are more likely to show them than White populations (Moores *et al.*, 1980).

21.9.2 Detection of MN in bloodstains

Landsteiner and Levine (1928a) noted in the original paper that blood dried on a glass surface would absorb appropriate anti-M or anti-N and reduce its titer, and they noted that bloodstains might, therefore, be amenable to MN typing. In 1933, Lauer presented an inhibition technique for MN grouping in dried blood. Quite a bit of blood was needed for the tests (about 10 mg dried blood was used), and care had to be exercised in preparing and selecting antisera. Proper controls had to be run as well, because of dosage effects with some sera—homozygous bloods giving stronger reactions than heterozygous samples—and the problem of cross-reactivity. It has been known since Landsteiner and Levine's work that M cells can absorb some anti-N. M grouping was more reliable than N grouping. Lauer presented his results in two criminal cases. In 1933, Clausen carried out studies on MN grouping in stains, and gave a slightly modified inhibition technique. His results did not look terribly promising. he said that he could find no trace of MN factors in saliva, semen, serum or urine. Schiff (1932b) mentioned that MN determination in bloodstains would be very uncommon, because in most cases, the rather large amount of material required for the test would be unavailable. Therkelsen (1934) reported a modified inhibition technique for M. He used a large amount of material (50 mg dried blood) in order, he said, to get a large quantity of specific binding with M containing stains, and minimize the role of nonspecific binding. Results with N were not very good, and its determination was not recommended. M was determined in a 14 month old stain. The subject was covered again in Therkelsen's lengthy paper in 1936, the conclusions being the same. Moureau (1935a) reported results quite similar to Therkelsen's in the grouping of MN in bloodstains by inhibition technique. In 1936, Holzer tested animal material, including blood and tissues, for absorption of anti-M and anti-N, and found it to be minimal where it occurred at all. He did not think that this was an important consideration in MN grouping in bloodstains. Wiener (1939) was in agreement with Therkelsen and Moureau that M could be determined reliably, but that N could not be. Schiff and Boyd (1942) briefly mentioned Therkelsen's (1934 and 1936) procedure for M in bloodstains. Harley (1943) said

that he had been unsuccessful in devising a technique for MN grouping in "smaller stains". Mueller (1953) noted that Balgairies and Christiaens had had only 20–30% successful results in their studies on bloodstains up to 4 days old in 1938, and he said that the success rate with MN in his own laboratory was probably still less.

21.9.3 More recent developments in MN grouping in bloodstains

In 1961, Sylvia and Kirk investigated the problem of MN grouping in stains, using an anti-M serum, and the anti-N lectin derived from *Vicia graminea*. An inhibition procedure was used for stains. The antiserum and lectin preparations were selected and calibrated for use in grouping on the basis of the time dependence of agglutination of different cells, which was itself a function of the titer of the antiserum or lectin. M cells could be agglutinated by *Vicia* reagent, but much more slowly than MN cells. In 100 bloodstains, M and N could be correctly grouped in all the samples.

In 1963, Pereira reported success in the determination of MN in bloodstains by the elution technique (Pereira, 1963a). Carefully selected rabbit antisera were used in 2 hr absorptions in a moist chamber at room temperature. Elution was at 56° for 15 min following three washes with saline. Cloth controls gave no reactions. N stains did not react with anti-M. M stains often reacted weakly with anti-N. A full series of controls, and caution in interpretation of results was recommended. Pereira said that if all the reactions were weak, no conclusions should be drawn. Stains up to 3 months old could be grouped, but the reactions tended to be weak in 9 month old stains. Fiori *et al.* (1963) also reported successful results in grouping MN in bloodstains by elution. Absorption was at 4°, as for ABO, and high titer ($\geq$1:64) antisera were required. Budvari (1963) confirmed these results. In 1969, Howard and Martin reported successful results with M and N by elution, using the technique in which the threads are affixed to a cellulose acetate backing, and the entire procedure carried out on the backing sheet (see in section 19.10.3.4). Katsura *et al.* (1971) proposed a solution to the problem of antiserum specificity. Rabbit antisera, raised against OM or ON cells, were absorbed onto boiled red cells of appropriate type. The material was washed, and the absorbed antibodies then eluted, the eluate consisting of "specific" antiserum. The eluates had titers of about 1:32 for anti-M and 1:16 for anti-N in saline. The titers were improved by the use of an antiglobulin test. These preparations could be successfully employed for MN grouping of blood-stained threads by elution technique (Sagisaki *et al.*, 1971). Elution of the antibody and addition of appropriate test cells was followed by the addition of anti-rabbit globulin serum raised in goats to effect agglutination. Stains a year old, and sometimes up to twice that, could be determined reliably. Yudina (1972) reported an absorption-elution method for grouping M and N in bloodstains.

Mixed agglutination procedures have been found to be satisfactory as well. Nickolls and Pereira (1962) employed a mixed agglutination procedure, which was a modified version of that of Coombs and Dodd (1961), for MN determination. Fiori *et al.* (1963) agreed that mixed agglutination was a suitable procedure for MN grouping, although somewhat more delicate and involved than elution. Akaishi (1965) got good mixed agglutination (GSDCM) results for M and N as well. Maresch and Wehrschütz (1963) confirmed the successful results obtained by Nickolls and Pereira (1962) using mixed agglutination. They followed the mixed agglutination determination with a 50° elution step which gave agglutinates in the field no longer adhering to the fibril. They regarded this step as giving additional certainty to the determination, and with certain kinds of materials (e.g., Nylon), it was essential to obtain a good result. Driesen and Keller (1973) followed a procedure rather similar to that of Maresch and Wehrschütz with good results.

Schwerd (1978) examined several procedures for MN grouping in stains. Elution techniques similar to those of Kind (1960b) and of Lincoln and Dodd (1973) were employed, along with a mixed agglutination procedure similar to that of Coombs and Dodd (1961), as modified by Kobiela *et al.* (1972). Methanol fixation of the threads was always done. It was found to be important to carefully arrange the quantities of antigen and antibody, as Lincoln and Dodd had shown, and to do the washing steps at 4°. The use of rabbit anti-globulin did not improve the results. Selection of antisera was stressed as the most important consideration. Useful anti-N were the most difficult to find. With carefully standardized test conditions and antiserum selection, Schwerd said that false N reactions should not ordinarily be observed. Failure to detect N (false negative) could be avoided by the use of several anti-N sera. The use of *Vicia graminea* lectin was found to be useful as well, and rabbit antisera worked better on the whole than did human derived antisera.

Denault *et al.* (1978) found that M and N were detectable for the most part in stains on a number of substrata for up to 26 weeks by the elution procedure. There was a hint that N was slightly affected by high humidity when the bloodstain was on wool. Both M and N could be missed, and there were occasional false positive M and N reactions.

21.9.4 The problem of anti-N cross reactivity

All investigators familiar with the MN system are aware of the reaction of anti-N with M cells. The reasons for this "cross-reactivity" are not simple, nor yet fully understood. It has as its basis the fundamental immunochemical structures of the M and N determinants, and their arrangement on the surface of the cell membrane. These matters were discussed in section 21.8. The problem must be dealt with in practice by carefully screening antisera for specificity, and in bloodstain grouping, control bloodstains of all three types must be used. In spite of precautions, errors in MN grouping are possible. N antigen can be missed, and MM stains can be misgrouped as MN because of their cross reactivity with anti-N. A recent blind trial study in this country, in which many laboratories participated, indicated that the

MN types in bloodstains were not correctly determined in a significant number of samples. Although the subject has not been widely discussed in the published literature, a number of laboratories that we know about have taken MN testing out of their routine bloodstain examination procedures.

Shaler *et al.* (1978) have recently employed a different approach to this problem, based on what is known about the biochemical composition of the MN-active glycoproteins. Some additional background material is introduced here as a basis for understanding these studies. These studies closely relate to the material discussed in section 21.8.3. In 1971, Fairbanks *et al.* fractionated the major polypeptides of the human erythrocyte membrane by electrophoresis in polyacrylamide gels containing the detergent sodium dodecylsulfate (SDS). Coomassie Blue staining of the gels located six fractions containing protein. If the gels were stained with so-called periodic acid-Schiff reagent, four bands could be seen, one of which was regarded as being a (glyco)lipid fraction. Periodic acid is known to oxidize structures containing neighboring C-OH groups to the level of aldehydes, and these compounds can form colored products with Schiff reagent. For details of the reaction schemes, see Pigman (1957), Lillie (1977) and Clark (1973). Periodic acid-Schiff staining can thus be used for histochemical location of glycoproteins. The major glycoproteins located on the polyacrylamide gel by Fairbanks *et al.* (1971) were called PAS-1 through PAS-3. The arrangement of these proteins and glycoproteins on the membrane was studied in a subsequent paper (Steck *et al.*, 1971) using proteolytic enzymes to partially hydrolyze the molecules on membrane vesicles—both "right side out" and "inside out"—from erythrocytes. Hamaguchi and Cleve (1972) looked at the serological specificities of the glycoprotein fractions and said that PAS-1, with MW about 58,000, was probably the MN glycoprotein. PAS-3 contained most of the S activity. Interestingly, rabbit anti-N inhibition was seen with PAS-3 as well as with PAS-2 on occasion. Dahr *et al.* (1975a) showed that trypsin and α-chymotrypsin had differential effects on the MNSs activities as assessed with different antisera. Rabbit anti-N reactivity with M cells was abolished by chymotrypsin, its reactivity with N cells being unaffected. Anti-S or anti-s reactions were greatly inhibited or abolished by chymotrypsin treatment, while trypsin had no effect or slightly enhanced them. This, and other data, suggested that the "cross-reacting N" receptor of M cells is present on the same glycoprotein as the Ss activity (PAS-3), but not on the MN glycoprotein (PAS-1). Dahr *et al.* (1975c) looked at the glycoproteins from M or N S-s-U- cells, and PAS-3 was absent. S-s-U+ cells showed the same profile. These observations constituted compelling evidence for the assignment of MN activity to PAS-1, and "cross reacting N" and Ss activity to PAS-3. It is now clear that PAS-1 and PAS-2 comprise glycophorin-A, and that PAS-3 comprises glycophorin B (Furthmayr, 1978). It may also be noted that Tanner *et al.* (1977) find *some* PAS-3 in S-s- cells. Furthmayr (1978) has pointed out that there are great technical difficulties in these SDS-polyacrylamide gel fractionations which could account for differences in results. These studies help to explain some of the observations in the literature on the serological effects of proteolytic enzyme treatment of different types of red cells (Hirsch *et al.*, 1957; Morton, 1962). Judson and Anstee (1977) have examined the effects of enzyme treatment on various red cell types, and have correlated these with alterations seen in the glycoprotein profile. Shaler *et al.* (1978) took advantage of the fact that cross-reacting N activity, which resides on a different glycoprotein than does MN, is differentially sensitive to treatment by α-chymotrypsin. M cells, previously determined to possess N cross reacting activity, were used to make bloodstain, along with control M and N cells. These stains, treated with appropriate concentrations of chymotrypsin (4 mg/mℓ) for up to 15 min at 37° showed selective destruction of cross reacting N in the subsequent elution test, without affecting normal M and N activity. Incubation time and chymotrypsin concentration were both critical variables. Shaler *et al.* emphasized that this delicate procedure, while showing promise and illustrating the value of fundamental biochemical approaches to practical problems, was not being recommended as a routine procedure in casework for the time being.

21.9.5 The detection of the Ss and other antigens in bloodstains

In 1968, Lincoln and Dodd first reported the detectability of S in bloodstains by elution technique (Lincoln and Dodd, 1968b). Four anti-S, containing complete antibodies active at room temperature, were tested in the elution procedure, all operations being carried out at 20°. None of these were satisfactory. One serum was found, however, that contained incomplete, AHG detectable antibodies which were most active at 37°. Absorption at 37°, elution, and detection of the antibody with test cells at 37° with this serum yielded excellent results using the anti-human globulin technique. S+ and S− stains up to 6 months old could be grouped reliably. Kobiela *et al.* (1972) confirmed that S could be reliably grouped in stains up to 4 months old, the details of their procedure being beyond our ability to decipher the Polish text. Lincoln and Dodd (1975a and 1975b) recently added s to the list of antigens that can be grouped reliably in bloodstains. As in the case of S, the anti-s contained incomplete antibodies, and 37° absorption and reaction temperatures were employed, detection being by means of anti-human globulin technique. Washing steps were carried out at 4°. The two useful anti-s reagents had AHG titers of 1:8 against cells. 1 cm long bloodstained threads could be grouped for s in samples up to 7 months old, SS stains giving uniformly negative reactions. McDowall *et al.* (1978b) compared an auto-analyzer procedure with a manual one for the detection of anti-S eluted from bloodstains. Autoanalyzer procedures are briefly discussed in Section 22.8. The manual detection method was found to be superior to the automated one.

Denault *et al.* (1978) found that S survived aging in bloodstains less well than did s. The effect did not seem to be specifically attributable to humidity differences, nor to any particular substratum. It also appeared that s could be missed in

stains more easily than could S. A single false positive S reaction was seen with denim.

Some time ago, Ducos *et al.* (1969a) reported that the MNSs related antigen Vw could be detected in stains up to 6 months old in most cases. An inhibition technique was used, and a human anti-Vw was available for the experiments. Vw is a low incidence antigen, classified by Cleghorn (1966) as part of Class I of the Miltenberger series of antigens (see section 21.5.2 and Race and Sanger, 1975).

21.10 The Frequency of MNSs Phenotypes in U.S. Populations

Frequences of MN blood groups in some U. S. populations using anti-M and anti-N are shown in Table 21.2, and MNSs groups determined using anti-M, anti-N and anti-S and/or anti-s are shown in Table 21.3.

Table 21.2 Frequencies of MN Groups in U.S. Population

Population	Total	Frequency — Number (Percent) M	MN	N	Reference
CAUCASIAN					
New York, NY	3,263	1,037 (31.73)	1,621 (49.6)	603 (18.6)	Wiener and Gordon, 1951
New York, NY	954	287 (30.1)	481 (50.4)	186 (19.5)	Wiener et al., 1953
Western Alaska	784	(36.61)	(45.03)	(18.37)	Pauls et al., 1953
University of Iowa "Controls"	2,186	587 (26.85)	1,208 (55.26)	391 (17.89)	Buckwalter et al., 1962
Boston, MA					
Rheumatic	606	200 (33)	293 (48.4)	113 (18.6)	Dublin et al., 1964
Non-rheumatic	600	182 (30.3)	305 (50.9)	113 (18.8)	
Eastern San Francisco, CA Bay Area					
Mothers	4,928	(31.29)	(48.86)	(19.85)	Reed, 1967
Babies		(28.37)	(52.42)	(19.22)	
New York, NY	500	158 (31.6)	249 (49.8)	93 (18.6)	Wiener, 1969
Miami/Dade Co., FL	366	108 (29.5)	181 (49.5)	77 (21.0)	Stuver, 1979 and see Shaler, 1978
NEGRO					
Baltimore, MD	580	136 (23.45)	280 (48.25)	164 (28.3)	Glass and Li, 1953
Washington, D.C. Howard University Students	937	(39.7)	(36.0)	(24.3)	Moore, 1955
Eastern San Francisco CA Bay Area					
Mothers	1,453	(24.36)	(50.79)	(24.85)	Reed, 1967
Babies		(23.19)	(51.34)	(25.47)	
Birmingham, AL	610	154 (25.2)	298 (48.9)	158 (25.9)	Casey et al., 1968
New York, NY	500	119 (23.8)	242 (48.4)	139 (27.8)	Wiener, 1969
Miami/Dade Co., FL	345	81 (23.5)	176 (51.0)	88 (25.5)	Stuver, 1979 and see Shaler, 1978
CHINESE					
New York, NY	400★	141 (35.3)	192 (48.0)	58 (14.5)	Wiener, 1969
New York, NY	946☆	321 (33.93)	460 (48.62)	148 (15.64)	Wiener, 1974
HISPANIC					
Miami/Dade Co., FL	359	116 (32.3)	167 (46.5)	76 (21.2)	Stuver, 1979 and see Shaler, 1978

★ Includes 9 MN_2 types ☆ Includes the 400 people reported by Wiener, 1969, and there were 17 (1.79) MN_2 types

Table 21.3 Frequencies of MNSs Groups in U.S. Populations

Population	Total	Frequency — Number (Percent) MS	MSs	Ms	MNS	MNSs	MNs	NS	NSs	Ns	Reference
CAUCASIAN											
New York, NY	394★	88 (22.1)		37 (9.6)	106 (26.9)		87 (22.0)	27 (7.0)		49 (12.4)	Wiener et al., 1953
Southeastern GA	333	13 (3.9)	39 (11.7)	34 (10.2)	11 (3.3)	76 (22.8)	79 (23.7)	1 (0.3)	21 (6.3)	59 (17.7)	Cooper et al., 1963
New York, NY	332	12 (3.6)	44 (13.3)	39 (11.7)	7 (2.1)	75 (22.6)	77 (23.2)	4 (1.2)	26 (7.8)	48 (14.5)	Issitt et al., 1965
New York, NY	900	50 (5.6)	121 (13.4)	109 (12.1)	29 (3.2)	194 (21.6)	206 (22.9)	11 (1.2)	53 (5.9)	127 (14.1)	Issitt et al., 1968
San Francisco, CA Bay Area	8,962	575 (6.4)	1,327 (14.8)	853 (9.5)	331 (3.7)	1,992 (22.2)	2,088 (23.3)	41 (0.5)	457 (5.1)	1,298 (14.5)	Reed, 1968
South Central West Virginia	1,051	(6.4)	(13.8)	(9.6)	(3.6)	(24.1)	(24.1)	(0.6)	(4.5)	(13.4)	Juberg, 1970
Tecumseh, MI	8,447	587 (6.95)	1,262 (14.94)	697 (8.25)	278 (3.29)	1,959 (23.19)	1,954 (23.13)	29 (0.35)	423 (5.01)	1,258 (14.89)	Schreffler et al., 1971
Detroit, MI	461	(6.3)	(17.6)	(13.4)	(2.4)	(24.3)	(22.3)	(1.7)	(3.0)	(10.0)	Shaler, 1978 ◇
Miami/Dade Co., FL	370	28 (7.6)	50 (13.5)	30 (8.1)	19 (5.1)	82 (22.2)	80 (21.6)	3 (0.8)	19 (5.1)	59 (15.9)	Stuver, 1979 and see Shaler, 1978
NEGRO											
New York, NY	580★	(6.9)		(17.2)	(16.6)		(33.1)	(26)		(20.2)	Miller et al., 1951
Ann Arbor, MI	96★	10 (10.4)		18 (18.8)	12 (12.5)		31 (32.3)	8 (8.3)		17 (17.7)	Neel and Hanig, 1951
Southeastern GA	304☆	6 (2.0)	22 (7.2)	42 (13.8)	9 (3.0)	31 (10.2)	113 (37.2)	4 (1.3)	15 (4.9)	58 (19.1)	Cooper et al., 1963
New York, NY	204●	10 (4.9)	16 (7.8)	28 (13.7)	4 (2.0)	33 (16.2)	64 (31.4)	5 (2.5)	10 (4.9)	34 (16.7)	Issitt et al., 1965
Houston, TX	263○	–	12 (4.6)	48 (18.3)	4 (1.5)	49 (18.6)	79 (30.0)	–	15 (5.7)	56 (21.3)	Francis and Hatcher, 1966
New York, NY	493□	10 (2.0)	32 (6.5)	78 (15.8)	8 (1.6)	71 (14.4)	163 (33.1)	9 (1.8)	23 (4.7)	99 (20.1)	Issitt et al., 1968
San Francisco CA Bay Area	3,146	57 (1.8)	201 (6.4)	482 (15.3)	98 (3.1)	349 (11.1)	1,132 (36.0)	33 (1.0)	167 (5.3)	627 (19.9)	Reed, 1968
South Central West Virginia	106	(2.0)	(6.0)	(16.0)	(4.0)	(10.0)	(34.0)	(1.0)	(4.0)	(24.0)	Juberg, 1970
Detroit, MI	485	(3.1)	(6.2)	(13.4)	(2.7)	(11.0)	(38.1)	(2.3)	(4.0)	(19.4)	Shaler, 1978 ◇
Miami/Dade Co., FL	337	7 (2.1)	23 (6.8)	49 (14.5)	11 (3.3)	40 (11.9)	122 (36.2)	6 (1.8)	11 (3.3)	68 (20.2)	Stuver, 1979 and see Shaler, 1978
CHINESE											
New York, NY	103 ★	(3.9)		(35.0)	(5.8)		(37.9)	●	(1.0)	(16.5)	Miller et al., 1951
HISPANIC											
San Francisco CA Bay Area "Mexican"	335	23 (6.9)	62 (18.5)	41 (12.2)	18 (5.4)	77 (23.0)	78 (23.3)	1 (0.3)	12 (3.6)	23 (6.9)	Reed, 1968
Miami/Dade Co., FL	363	24 (6.6)	57 (15.7)	36 (9.9)	12 (3.3)	77 (21.2)	81 (22.3)	2 (0.6)	12 (3.3)	62 (17.1)	Stuver, 1979 and see Shaler, 1978

★ Tests with anti-M, anti-N, and anti-S only

☆ 4 people were S – s –

● 1 person was MS – s – U – and another was MNS – s – U –

○ Anti-M and anti-M, were used. M and M, combined in the table.

□ Two people were Mu, one was MNu and four were Nu

◇ Data of Stolorow and collaborators

SECTION 22. THE Rh SYSTEM

21.1 Introduction

The Rh System is one of the most complicated cell or serum group polymorphisms in human beings, a distinction which it now shares with the Gm and HLA systems. In its practical essentials, Rh is not too complex, but there are many pecularities and intricacies of Rh if all the information is taken into account. There are two systems of nomenclature (three, if the numerical system is counted) for Rh in common use, which reflect real differences in the opinions of the people who proposed them about the genetic basis of the system. Efforts to standardize Rh nomenclature have never been successful. Very much is known about Rh serology, but very little illuminating work has been done on the biochemistry and biochemical genetics of Rh. It is clear that the Rh antigenic determinants are not carbohydrate in nature, and it has not been possible to use the very enlightening studies on the ABH, Lewis (and to a lesser extent, MNSs) as models.

22.2 Discovery and Development of the Rh System

In 1940, Landsteiner and Wiener reported that a new human red cell agglutinogen was recognizable using an immune serum prepared by the immunization of rabbits with the red cells of Rhesus monkeys. The factor appeared unrelated to the known blood factors (ABO, MN and P), and was called "Rh". Wiener (1952) traced the beginning of this work to 1937. In 1941, 60 families with 237 children were investigated by Landsteiner and Wiener, and the "Rh factor" behaved as a simple Mendelian dominant characteristic, there being no Rh+ children from Rh− × Rh− matings.

In 1939, Levine and Stetson had found an agglutinin in the serum of a mother who had given birth to a macerated, stillborn fetus as the result of her second pregnancy. The fetus could be shown to have the antibody. Fetal death was the result of a hemolytic reaction, and the mother had serious hemolytic reactions following transfusions with ABO compatible blood. The agglutinin in her serum agglutinated about 80% of random red cells, and Levine and Stetson thought that this antibody was the result of frank immunization of the mother by the cells of her fetus in response to an antigen which had been inherited from the father. This work represented one of the first major steps in the understanding of hemolytic disease of the newborn. In 1940, Wiener and Peters reported on three cases of hemolytic transfusion reactions in cases of transfusion of apparently compatible (for ABO) blood. The best explanation for the incidents was the formation in the patients, who were Rh−, of antibodies in response to transfusion by Rh+ blood. The antibody in the patients' sera closely resembled the anti-Rh serum prepared in rabbits by immunization with Rhesus monkey red cells. It was, therefore, clear by 1940 at least that the new Rh factor could be immunogenic in human beings (Wiener and Peters, 1940; Landsteiner and Wiener, 1941; Wiener, 1942). In 1941, Levine and his collaborators put forward the notion that the Rh immunization of Rh− mothers by Rh+ fetuses was the principal etiologic factor in the pathogenesis of a number of cases of erythroblastosis fetalis (Levine *et al.*, 1941a and 1941b). This substantially correct explanation of certain types of hemolytic disease associated with pregnancy could not, at the time, account for the fact that the incidence of erythroblastosis fetalis was considerably lower than the number of Rh+ infants delivered from Rh− mothers, nor for the fact that hemolytic disease of the newborn was sometimes seen in cases where the mothers were Rh+. Recognition of a number of Rh antigens in the system, of individual variation in isoimmunization phenomena, and of the fact that agglutinogens in other than the Rh system can be responsible for hemolytic disease associated with pregnancy, has made the explanations much clearer (see Wiener, 1946).

In 1943, Wiener and Landsteiner had looked at the sera of a number of mothers of infants born with hemolytic disease, and had found some antibodies detecting further variants of Rh, which, at the time, were called anti-Rh_1 and anti-Rh′, these in addition to the original anti-Rh (or human sera which behaved just like it). People who were "Rh positive" could be "Rh_1" and react with all the antisera, "Rh_2" and react with anti-Rh and anti-Rh′, or "Rh′" and react with anti-Rh_1 and anti-Rh′. "Rh negative" people reacted with none of the antisera. Wiener and Sonn (1943) reported a newly found anti-Rh antiserum, and the nomenclature in this paper changed a little bit from that just described. Wiener (1943b) described six allelic genes, called rh, Rh_1, Rh_2, Rh′, Rh″ and Rh on the basis of what was then known. Not all combinations had been found, and some different genotypic combinations gave identical phenotypic reactions. There were only three known agglutinins in Wiener's view, but there were some sera (which had names) that contained more than one of them.

In England, Race and his collaborators were uncovering additional Rh antisera as well. At first, the sera were designated by descriptive combinations of letters derived from the donors' names. A serum reacting with "Rh negative" cells was described by Race and Taylor (1943). Two further examples of sera with an additional Rh specificity were described by Race *et al.* (1943). By 1944, there were four antisera defining seven alleles (Race *et al.*, 1944a). In 1944, Prof. R.A. Fisher (see in Race, 1944) formulated a genetic hypothesis based on the results with the four antisera then known. Two of the sera defined antithetical reactions, and

Fisher postulated that the genes responsible for the synthesis of the two antigens were alleles. The antigens and the genes were called C and c. The remaining sera were said to be defining antigens D and E. Fisher supposed that the genes D and E would have alleles, d and e, and that, ultimately, antisera would be found to them as well. At the time, the antisera were given Greek letter designations by Fisher, Γ being "anti-C", γ "anti-c", Δ "anti-D", H "anti-E", and the predicted "anti-d" and "anti-e" were, respectively, δ and η. Fisher supposed that the three gene loci, if separable at all, must be very closely linked, for no crossing over had been observed (Race *et al.*, 1944b). The possible Rh gene complexes or assemblages were, therefore, CDe, cDE, cde, cDe, Cde, cdE, CDE and CdE. Only CdE remained to be found. In 1945, Murray *et al.* found an individual possessing the CDE complex, and the reactions with the four antisera were as predicted. Mourant in 1945 found the predicted antibody η (anti-e) in a person suffering from hemolytic anemia. The reactions were as predicted. The CdE complex was convincingly demonstrated in the mother of a child with erythroblastosis fetalis by van den Bosch in 1948. Fisher carried the genetic argument for the scheme a step further. Noting that the complexes CDe, cDE and cde were relatively common in Britain, that cDe, cdE, Cde and CDE were quite rare, and that CdE was extremely rare, Fisher said that the less common types were maintained by occasional crossing over in the more usual heterozygotes. A crossover event between the C/c and D/d loci in cDE/CDe would produce cDe and CDE combinations. All the so-called second order combinations (low frequency) could originate in this way, but the rare complex CdE would have to be produced by a crossover event involving a second order combination, which is itself a product of a crossover event, if the theory is correct. Carrying the argument still another step, Fisher used the frequencies of the combinations, which, according to this thinking, should be related to the crossover frequencies between the loci, to postulate that the locus order on the chromosome was D/d, C/c, E/e. These arguments were developed by Fisher and Race (1946) and by Fisher (1947). The antiserum notations, anti-D, anti-C, etc., were put forth by Cappell in 1944, and by Fisher and Race in 1946.

Wiener's conception of the system was different from the outset. He looked upon the locus responsible for the Rh factors as a single, immutable locus which could be occupied by a number of different alleles. Wiener and Landsteiner (1943) identified four allelic genes using two antisera, anti-Rh (presently anti-Rh_o), and anti-Rh_1 (presently anti-rh'), and a serum which contained both of these. A new antiserum was mentioned, containing an antibody, then called anti-Rh_2 (presently anti-rh''), and a full paper appeared describing its characteristics (Wiener, 1943a). Wiener (1943b) gave a full description of the eight Rh phenotypes (only seven of which had been found) based on six genes, which could give rise to 21 different genotypes. Family studies were carried out by Wiener *et al.* in 1944 to confirm the six allelic gene hypothesis. Levine (1943) mentioned that he and Javert had encountered an antiserum reacting with some Rh negative bloods, very similar to Race and Taylor's (1943) "St" serum. He called it an anti-Hr serum, to indicate that it seemed to be the reverse of the different "anti-Rh" sera. Levine (1945) noted that the "Hr" designation was a good one for the gene producing the antigen that reacted with the "anti-Hr" sera, and he said that he had first proposed this designation in 1941. Wiener adopted the "Hr" symbolism and incorporated it into his system of Rh nomenclature (Wiener, 1945; Wiener *et al.*, 1945). In 1948, Wiener and Hyman described families in which the rare genes R^z (CDE in Fisher-Race terms) and r^y (CdE) were segregating. Around 1945, Wiener began taking to task both the Fisher theory of inheritance, and the Fisher-Race nomenclature scheme, and he continued to do so for the next 30 years.

22.3 Rh Nomenclature

The basic structure of the Rh system was worked out in the 1940's (see, for example, Race, 1948). Numerous additional complexities within the system have come along since that time, and some of these will be discussed below. The subject of nomenclature is considered here because it will be needed for the remainder of the discussion, and because it has always been something of an issue in discussions of Rh. The nomenclature has changed a number of times, and it would be more confusing than informative to review its history in detail. Some of the older designations are mentioned in the text. Much has been written about Rh nomenclature, because the two schemes reflected real differences in the conception of the system at the genetic level. Wiener has argued strenuously and long for the universal adaptation of his system of nomenclature. Some of his papers on the subject are collected in his several books (Wiener, 1954, 1961, 1965a and 1970). The Fisher-Race nomenclature is clearly presented and used in Race and Sanger (1975), and in all previous editions, and it is used by most English workers. In Table 22.1 are shown the genes, gene products and antigens, or blood factors, along with the reactions of these products with the five common antisera. Fisher-Race usage is indicated in square brackets. The more common phenotypes of the Rh system detectable with the five common antisera, and the genotypes with which they are associated, are indicated in Table 22.2 The "usual" or shorthand notation for the phenotypes is given in the Table as well. The nomenclature has become somewhat more complicated as unusual Rh factors and phenotypes have been found. These will be mentioned briefly below.

Efforts to arrive at a universally acceptable symbolism for the Rh factors and their antisera have been made, but without success. Some of the difficulty derives from the fact that neither system has been able to digest very easily the multitude of complexities that have come along since the eight "basic" Rh factors were discovered in the 1940's. Nomenclature discussions are always inextricably tied directly, or by implication, to the genetics of the system. The subject will be returned to again in a subsequent section.

Table 22.1 Genes, Gene Products and Reactions in the Rh System With the Five Common Antisera

Gene	[Gene Complex]	Agglutinogen	Blood Factors	[Antigens]	Anti-Rho [Anti-D]	Anti-rh' [Anti-C]	Anti-rh'' [Anti-E]	Anti-hr' [Anti-c]	Anti-hr'' [Anti-e]
r	cde	rh	hr', hr''	c,e	−	−	−	+	+
r'	Cde	rh'	rh', hr''	C,e	−	+	−	−	+
r''	cdE	rh''	hr', rh''	c,E	−	−	+	+	−
R^0	cDe	Rh_0	Rh_0, hr', hr''	c,D,e	+	−	−	+	+
R^1	CDe	Rh_1	Rh_0, rh', hr''	C,D,e	+	+	−	−	+
R^2	cDE	Rh_2	Rh_0, hr', rh''	c,D,E	+	−	+	+	−
R^z	CDE	Rh_z	Rh_0, rh', rh''	C,D,E	+	+	+	−	−
r^y	CdE	rh_y	rh', rh''	C,E	−	+	+	−	−

Usage according to Fisher-Race indicated by square brackets.

Table 22.2 Rh Phenotypes and Genotypes ★

Name ☆	Usual Designation ⊙	Reaction with Anti- Rh_o [D]	rh' [C]	rh'' [E]	hr' [c]	hr'' [e]	Genotypes ☆	[Genotypes]
Rh_o	$R_or.R_oR_o$	+	−	−	+	+	R^or,R^oR^o	cDe/cde, cDe/cDe
Rh_1rh	$R_1r.R_1R_o.R_or'$	+	+	−	+	+	R^1r,R^1R^o,R^or'	CDe/cde, CDe/cDe, cDe/Cde
Rh_1Rh_1	$R_1R_1.R_1r'$	+	+	−	−	+	R^1R^1,R^1r'	CDe/CDe, CDe/Cde
Rh_2rh	$R_2r.R_2R_o.R_or''$	+	−	+	+	+	R^2r,R^2R^o,R^or''	cDE/cde, cDE/cDe, cDe/cdE
Rh_2Rh_2	$R_2R_2.R_2r''$	+	−	+	+	−	R^2R^2,R^2r''	cDE/cDE, cDE/cdE
Rh_1Rh_2	$R_1R_2.R_1r'',R_2r'$	+	+	+	+	+	R^1R^2,R^1r'',R^2r'	CDe/cDE, CDe/cdE, cDE/Cde
Rh_2rh	$R_2r.R_oR_2.R_or^Y$						R^2r,R^2R^o,R^or^Y	CDE/cde, cDe/CDE,cDe/CdE
Rh_2Rh_1	$R_2R_1.R_2r',R_1r^Y$	+	+	+	−	+	R^2R^1,R^2r',R^1r^Y	CDE/CDe,CDE/Cde,CDe/CdE
Rh_2Rh_2	$R_2R_2.R_2r'',R_2r^Y$	+	+	+	+	−	R^2R^2,R^2r'',R^2r^Y	CDE/cDE, CDE/cdE, cDE/CdE
Rh_2Rh_2	$R_2R_2.R_2r^Y$	+	+	+	−	−	R^2R^2,R^2r^Y	CDE/CDE, CDE/CdE
rh	rr	−	−	−	+	+	rr	cde/cde
rh'rh	r'r	−	+	−	+	+	$r'r$	Cde/cde
rh'rh'	r'r'	−	+	−	−	+	$r'r'$	Cde/Cde
rh''rh	r''r	−	−	+	+	+	$r''r$	cdE/cde
rh''rh''	r''r''	−	−	+	+	−	$r''r''$	cdE/cdE
rh'rh''	r'r''	−	+	+	+	+	$r'r''$	cdE/Cde
rh_yrh	rY_r						r^Yr	CdE/cde
rh_yrh''	rY_r''	−	+	+	+	−	r^Yr''	CdE/cdE
rh_yrh'	rY_r'	−	+	+	−	+	r^Yr'	CdE/Cde
rh_yrh_y	rY_rY	−	+	+	−	−	r^Yr^Y	CdE/CdE

★ The number of genotypes and phenotypes can be expanded if anti-rh^W [anti-C^W] is included — see sections 22.5.2 and 22.6.1

☆ According to Wiener

⊙ Designations not restricted to either system. Until recently, Race and Sanger used R' for r' and R'' for r'', but have now gone to the lower case r for those phenotypes which do not react with anti-D (anti-Rh_o), a practice long favored by Wiener.

[ ] Square brackets denote Fisher-Race symbols and designations

22.4 The Incomplete Rh Antibody

In the early years of Rh serology, investigators were puzzled by the observation that mothers of children with erythroblastosis fetalis were mainly Rh negative, and that in many cases no anti-Rh could be found in their sera. In 1944, Race and Wiener simultaneously elucidated the nature of this phenomenon. Race (1944) showed that some sera contained what he called "incomplete" antibody. The presence of incomplete anti-D could be demonstrated by sensitizing D+ cells with this antibody, washing and then showing that the cells were no longer agglutinable by an anti-D agglutinin. The "incomplete" antibody could, thus, bind the red cell receptor, but did not bring about agglutination in saline. Wiener (1944b) obtained identical results in his experiments, and referred to the nonagglutinating antibody as a "blocking" antibody. The inhibition of agglutination by sensitization of cells with the nonagglutinating anti-Rh_o was referred to as the "blocking test".

Somewhat better approaches to the detection of incomplete antibodies were soon found. Diamond and Abelson (1945) devised a rapid slide test for the detecton of anti-Rh in serum. During these studies, they noticed that multiple saline washing of Rh+ cells sometimes inhibited agglutination, i.e., the reaction seemed to be better in the presence of some serum or plasma. It was soon shown that serum albumin medium, as well as serum or plasma, allowed the agglutination of the cells by some "incomplete" antibodies (Diamond and Denton, 1945; Cameron and Diamond, 1945). The use of colloidal media for the detection of incomplete antibodies has since been widely confirmed. Wiener thought for a time that an additional component of plasma, which he called "conglutinin" was required for agglutination by the blocking antibody in colloidal media, and that this was

the basis for the observations (Wiener and Hurst, 1947; Wiener *et al.*, 1947).

Detection of "incomplete" Rh antibodies by means of anti-human globulin serum was introduced by Coombs *et al.* (1945a and 1945b). Cells sensitized with the incomplete antibody and washed were agglutinated by the addition of the serum of a rabbit immunized with human serum or human serum globulin. The test is often called the "anti-human globulin test" (AHG test) or the "Coombs test", and the AHG serum is sometimes called "Coombs serum".

In 1946, Pickles discovered that the treatment of Rh+ cells with the filtrate of a culture of *Vibrio cholera* made them agglutinable by incomplete anti-Rh. Pickles believed that an enzyme in the filtrate was responsible for this effect, and in 1947, Morton and Pickles showed that trypsin treatment had a similar effect. In 1950, Kuhns and Bailey found that papain treatment was effective in rendering Rh+ cells agglutinable by saline-incomplete antibodies. Stratton (1953) confirmed the papain findings, and devised a slide method with papainized cells, primarily for screening Rh negative mothers for Rh antibodies. In 1955, Löw proposed a routine papain cell test which is still sometimes quoted. A number of workers prefer papain to other proteolytic enzymes (e.g. Boorman *et al.*, 1977). Detailed instructions for the preparation of papain solution, papain treatment of cells, and trypsin, bromelin and ficin procedures may be found in Boorman *et al.* (1977) as well.

Many antibodies have since been found that are "incomplete" in the original (Race, 1944) sense of the term. There is further discussion of this subject in section 1.3.4.

22.5 Complexities of the Rh System—Further Rh Factors

Some of the complexities of the Rh system will be described. Many of the unusual Rh factors or conditions are very rare, but instructive in yielding information about the nature of the system. Not enough detailed information about the biochemical and immunological nature of the system is yet available to reconcile the complicated array of facts that have accumulated.

22.5.1 Subdivisions of D or Rh_0

In 1946, Stratton described a blood whose cells were agglutinated by anti-c, anti-E and anti-e, but not by anti-C. The results with a number of anti-D reagents were variable, 12 of 32 sera giving agglutination. Some of the anti-D reagents which did not cause agglutination contained incomplete anti-D. Stratton suggested that this weak sort of D was caused by a new allele which was called D^u, and the reactions in this case indicated that these cells were cD^uE/cde. This characteristic was studied in detail by Stratton and Renton (1948) and independently by Race *et al.* (1948a and 1948b). There was variability in different examples of D^u blood, some being agglutinable by some anti-D reagents, and others being detectable only by an AHG test with incomplete anti-D. It is possible to miss D^u in typing, so that a blood grouped as cde/cde (rr), for example, might in reality be cD^ue/cde. Wiener denotes the D^u condition in his nomenclature by a Germanic script upper case R (ℜ). At the other extreme, there are cells with abnormally strong D antigen. A case was reported by Renton and Hancock (1955) in which the cells were agglutinated by some incomplete (against ordinary cells) anti-D. There was something peculiar about the C in the cells as well.

In 1951, Shapiro reported an anti-D in the serum of the blood of a South African Bantu mother, whose cells grouped as D^u. Argall *et al.* (1953) reported another case of anti-D in the serum of a patient whose cells were D^u. Other examples of this behavior were soon found, and since the people did not have hemolytic anemia, the concept of complexity of the D ($\mathbf{Rh_0}$) antigen had to be invoked. Allowing the antigen to be complex, that is, to possess more than a single potential kind of antigenic specificity then allowed a person to have the "parts" of the antigen to which the serum did not contain antibodies, and to lack those "parts" to which it did. A number of examples of this sort of blood have been described, and have been studied by Dr. P. Tippett in London, and by Wiener and his collaborators in New York (Tippett and Sanger, 1962; Wiener *et al.*, 1957b; Unger and Wiener, 1959; Wiener and Unger, 1959; Unger *et al.*, 1959; and see Wiener and Gordon, 1967). The reactions of people with $\mathbf{Rh_0}$ (D)-like receptors on their cells, and an anti-$\mathbf{Rh_0}$ (D)-like antibody in serum, turn out to be different in different cases. Tippett and Sanger (1962) could classify the different forms of D antigen they studied into six categories. Detailed descriptions may be found in Race and Sanger (1975). Wiener enlarged his nomenclature to accommodate the several forms, which were regarded as further factors belonging to the Rh_0 agglutinogen. These were designated $\mathbf{Rh^A}$, $\mathbf{Rh^B}$, $\mathbf{Rh^C}$, etc. A person of type Rh_1rh who could be shown to lack $\mathbf{Rh^A}$ was designated Rh_1^arh. If the $\mathbf{Rh_0}$ factor is weakly reacting (D^u) as well, the Germanic R indicates this in the symbol, e.g. a woman in the studies of Unger and Wiener (1959) was classified $ℜh_2^crh$, indicating that her cells had a weak $\mathbf{Rh_0}$ and lacked the $\mathbf{Rh^C}$ factor associated with Rh_0.

Alter *et al.* (1962 and 1967) reported an antibody in the blood of a mother detecting an antigen which was called Go^a (Gonzales). Further studies by a number of workers indicated that Go^a is associated with Rh. Anti-Go^a apparently reacts with a part of D, the part that is missing in Tippett and Sanger's Category IV. The antigen has also been called D^{cor}, and is somewhat more common in Blacks than in Western European Caucasians.

22.5.2 Variations in C or rh′

In 1946, Callender and Race found a number of antibodies in the serum of a multiply transfused woman, one of which was an Rh agglutinin. The antibody was first called "anti-Willis", and later, anti-C^w. It was detecting an antigen C^w, which was present in the cells of one of the donors to the patient. The antigen was regarded as being due to an allele C^w at the C/c locus. Wiener calls the antibody anti-$\mathbf{rh^w}$ (or anti-$\mathbf{rh^{w1}}$). The common alignment C^wDe is

designated R^{1w} by Wiener, and the less common alignments (C^wde, C^wDE and C^wdE are designated r'^w, R^{2w} and r^{yw}, respectively. Anti-C^w is more common than it once was, and some laboratories use it in their routine testing procedures. Many anti-C contain at least some anti-C^w and are designated anti-CC^w by Race and Sanger (1975). Frequencies of C^w in different populations vary from less than 1% to as high as 9%. C^w (r'^w) is inherited. Wiener *et al.* (1957a) studied a family in which it was segregating.

In 1954, Stratton and Renton, investigating a case of hemolytic disease of the newborn, found an antibody in the mother which reacted with the cells of her husband and baby. Tested with a selection of anti-C reagents, the cells gave variable results. The antigen was named C^x and the antibody anti-C^x. Prokop and Uhlenbruck (1969) said that a number of anti-C contain anti-C^x. C^x is quite rare in Caucasians of Western European origin.

Race *et al.* (1948c and 1948d) reported what appeared to be two new antigens related to C/c and attributed at the time to alleles of C/c which were called C^u and c^v. C^u was a variably reacting kind of C, and more was said about it by Race and Sanger (1951). It turned out later that c^v reactions represented a kind of "position effect", that is, the anti-c reactions which characterized c^v were, in fact, characteristic of CDE/cde cells, and Race *et al.* (1960) said that the symbol c^v was not needed.

Huestis *et al.* (1964) described a serum from an R_1R_1 donor which had an anti-**hr**′ (c) reacting with most cells containing **hr**′. Occasionally, though, cells could be found which reacted with all anti-**hr**′ except this one. This antiserum was assigned the number 26 in the numerical nomenclature system (see in Section 22.6.2), and the phenotype it identified would be called Rh: w4, w6, -26, meaning that the cells react weakly with anti-c and anti-f (see below), but not with anti-Rh 26.

22.5.3 Variations in E or rh′′ and e or hr′′

Greenwalt and Sanger (1955) found an antibody detecting an antigen which was called E^w. The anti-E^w had caused hemolytic disease in one family. E^w cells react with anti-E^w and with selected examples of anti-E. The antigen is apparently quite rare. In Wiener nomenclature, it is designated rh^{w2}. A family in which E^w was segregating was reported by Henke and Kasulke (1976).

Another form of E, called E^u, which reacted weakly and variably with strong anti-E reagents, was described by Ceppellini *et al.* in 1950.

In 1955, DeNatale *et al.* described an Rh antigen which was relatively common in Blacks and relatively rare in Whites, and called it V. A further antibody, anti-VS (from a Mrs. VS) was described by Sanger *et al.* (1960). It reacted with cells having V, and the characteristic r'^s, both of which are more common in Black than in White people. The r'^s gives rise to weak or negative reactions with anti-C reagents whose main component is anti-Ce (see further below). The anti-VS appears to detect the product of an allele of e called e^s. The anti-V detects e^s as well as a "complex" antigen ce^s in the r'^s type (see below). Shapiro (1964) studied three examples of anti-VS, and one of them did not react with bloods that were V+. Such cells are said to have $\mathbf{hr}^v$ in Wiener nomenclature, which Shapiro uses. These results indicated that "anti-VS" might sometimes be anti-$\mathbf{hr}^v$ and anti-$\mathbf{hr}^H$. In the Bantu of South Africa, Shapiro found that $\mathbf{hr}^v$ and $\mathbf{hr}^H$ occurred with different frequencies.

In 1960, Shapiro found in the serum of a Mrs. Shabalala two antibodies, one of which was called anti-$\mathbf{hr}^s$. The anti-$\mathbf{hr}^s$ reacted with all bloods from Caucasians tested, and all but a few Bantu bloods. The antibody reacts generally like anti-**hr**′′, except in the unusual cases where it gives negative reactions with cells that are otherwise **hr**′′ positive. The exceptional bloods were denoted $\hat{R}h_o$ phenotypically, the characteristic being attributed to an allele denoted $\hat{R}^o$. Shapiro said that most anti-**hr**′′ contain anti-$\mathbf{hr}^s$. It is unfortunate and accidental that $\mathbf{hr}^s$ and e^s designations appeared, and they should not be confused for they are not the same thing.

In 1972, Shapiro *et al.* reported another antibody from the mother of a child with hemolytic disease, detecting a different hr′′-associated factor called $\mathbf{hr}^B$. The antiserum detects what would be regarded by the British workers as an e variant, more common in Black than in White people. Shapiro denotes the phenotypes of cells that fail to react with anti-$\mathbf{hr}^B$ by a dot above the R in Rh, e.g., $\dot{R}h^N$, $\dot{R}h_o$, $\dot{R}h_2rh$, etc. Rosenfield *et al.* (1973) assigned the number Rh 31 to $\mathbf{hr}^B$. The original serum of Mrs. Bastiaan contained an additional antibody which could be absorbed out with R_2R_2 cells. The "total immune response" of Mrs. Bastiaan (the antiserum before absorption) is assigned the number Rh 34 in the numerical designations (see below).

22.5.4 Compound or complex antigens and/or antisera

The terms "compound" and "complex" are used in describing these antigens, because they appear to result from some sort of combined interaction of more than one of the "basic" antigens in the present conception of the Rh system.

In 1953, an antibody was found in the serum of a much transfused hemophiliac man which reacted with bloods having cde, cDe and cD^ue composition (Rosenfield *et al.*, 1953b). The original thinking seemed to be that the antiserum might be detecting an antigen due to an additional closely linked locus of C/c, D/d and E/e, and it was called "anti-f". The possibility of "f" being some product of c and e when they are "cis", i.e., located on the same chromosome, was considered. Jones *et al.* (1954) describing a further example of anti-f, tended to favor the "cis-ce" explanation, but had nevertheless found a few cells that were R_1r (CDe/cde) which were not agglutinated by anti-f. Rosenfield and Haber (1958) favored the suggestion that f was ce (**hr** in the Wiener nomenclature). Although anti-f (anti-**hr**) behaves in most cases as an "anti-ce", there is undoubtedly more to it, because it reacts with cells in rare cases that it would not be expected to react with if it were simply "anti-ce".

In 1954, Race *et al.* described what they called "position effects" with regard to C and E in reactions with various anti-C and anti-E. The terms "cis" and "trans" refer to the genes being located on the same, or on opposite members of the homologous pair of chromosomes, respectively. The terms have meaning only in the Fisher closely linked allele concept of Rh. It was observed that, with selected antisera, C appeared to be inhibited when C and E were cis (as in CdE/cde or CDE/cDe), while E was less strong when they were trans (as in Cde/cdE or CDe/cDE). In 1958, Rosenfield and Haber found a serum "Ba." which detected an antigen called **rh$_i$**, the product of R^1 and r'. The **rh$_i$** would be equivalent to Ce. Some anti-**rh'** (C) sera contained anti-**rh$_i$**. Race and Sanger (1975) said that they now favor the idea that cis C and e give rise to the **rh$_i$** (Ce) antigen, while trans C and e do not, essentially as suggested by Rosenfield and Haber. The c reactions in the original "position effect" studies could be understood if some anti-E contained anti-CE, as has indeed proved to be the case. Examples of anti-cE have been reported as well.

Sturgeon (1960) studied the relationship between what was called anti-**rh'^{N}** and anti-**rh$_i$**. The **rh'^{N}** was designated rh'^{s} (or r'^{s} for the gene) by Race and Sanger (described above in section 22.5.3) to avoid the racial label to which objections had been raised, and because the type is not limited to Blacks. Sturgeon found that so-called anti-**rh'^{N}** and anti-**rh$_i$** were identical. Anti-**rh$_i$** reacts generally with **rh'** when it is the product of R^1 and r', but not when it is the product of R^2 (or r^y). It fails to react with **rh'** in many Black and a few White people, which are then called **rh'^{N}**. Rosenfield *et al.* (1960) reported an **rh'^{N}** blood in a family which had other Rh peculiarities.

In 1958, Allen and Tippett described the reactions of the cells of a Mrs. Crosby in Boston. They reacted as Rh negative with anti-D, anti-C and anti-E, but reacted with a number of "anti-CD" sera. The explanation was given that the cells possessed an antigen "G", and that "anti-CD" reagents had "anti-G" in them. Her genotype was written r^Gr. The "anti-G" could be isolated by absorption and elution with these cells, and was used to show that all the common genes except r and r'' gave rise to G. So-called anti-CD reagents were assumed to have a G component, and could be anti-C + G, anti-D + G or anti-C+D+G. This finding could explain a number of previously puzzling cases of the immunization of mothers by their fetuses, e.g., rr mothers making apparent anti-CD in response to fetuses whose cells had D but not C (the antibody was anti-D+G), and could also explain some previously puzzling absorption results with anti-CD. Samples having D or C without G have been reported, as have samples with G which lack C and D. Levine *et al.* (1961c) reported a case of an r^Gr^G homozygote. The r^G appeared to condition the antigens G, e, **Hr$_o$**, **Hr** and C^G, but not D, C, c, E, C^w, C^x, f, Ce, V or **hr^s**. C^G is like C but not identical to it, and **Hr$_o$** and **Hr** are very high frequency antigens.

In 1953, Davidsohn *et al.* found an antibody in a Mrs. Berrens, who had given birth to a baby suffering from hemolytic disease. The antibody reacted with the cells of her husband and baby, and detected a rare antigen which was called Be^a. The factor was inherited and was segregating in Mr. Berrens' family. The factor is highly immunogenic, and Be(a+) cells caused production of anti-Be^a when injected into volunteers (Stern *et al.*, 1958). A family called Koz., studied by Ducos *et al.* (1974) demonstrated that Be^a belongs to the Rh system. Be(a+) people have an unusual cde complex, which produces weak e, c and ce (f).

In 1971, Giles *et al.* reported a new gene complex of Rh, giving rise to a hitherto undescribed antigen, and for which a specific antibody was found. The complex is of the R^o type, with a weak D and a weak E, a kind of c(D)(E), where the parentheses indicate a weak or depressed antigen (see further below). The antibody, contained in the serum "Hawd.", was designated anti-Rh 33 in the numerical system, and the antigen may also be designated as R^{oHar} after a British blood donor in whose cells it was found.

In 1960, Rosenfield *et al.* discovered two new alleles in a Black family. One of them, r'^{N} (or r'^{s}) was prevously discussed. The other, called $\overline{\overline{R}}^N$, had ordinary D, but much depressed C and e. It was, thus, a kind of (C)D(e), where the parentheses denote depressed antigen. The symbol was chosen because $\overline{\overline{R}}^o$ denotes a complex having only **Rh$_o$**, with no **rh'**-**hr'** or **rh''**-**hr''** factors (–D–). This last will be discussed below. Antisera have been found which, after appropriately absorbing out other antibodies, have anti-$\overline{\overline{R}}^N$ activity. There are only a few examples of these sera. In 1971, Chown *et al.* reported a young Mennonite man who was $\overline{\overline{R}}^N R_2$. His parents were R_1R_2 and R_1R_1 and six sibs were of the parental types. Legitimacy was all but certain, and Chown *et al.* preferred mutation, probably at some control gene locus, but affecting structural gene products, as the explanation. Race and Sanger (1975) thought that not enough families with $\overline{\overline{R}}^N$ have been studied as yet to justify mutation as an explanation. The $\overline{\overline{R}}^N$ was assigned the numerical designation Rh 32 by Allen and Rosenfield in 1972. $\overline{\overline{R}}^N$ is more common in Black than in White people. In 1963, Broman *et al.* described a number of (C)D(e) Swedish people, and the characteristic was shown to be inherited. The (C)D(e) cells are not serologically identical to $\overline{\overline{R}}^N$ cells from the Black families, as indicated by testing with many anti-C and anti-E (P. Tippett, see in Race and Sanger, 1975).

Heiken (1967) has reported several people having an Rh complex with ordinary D, but with suppressed c and E, i.e., (c)D(E).

22.5.5 The LW antigen

The antibody which is now called anti-**Rh$_o$** (anti-D) was first described by Levine and Stetson (1939). In 1940, Landsteiner and Wiener published the studies on the rabbit immune anti-Rhesus monkey red cell serum. The rabbit immune serum appeared to have identical properties to the antibody observed by Levine and Stetson, and the human example acquired the name anti-Rh (later, anti-**Rh$_o$**). It is

now known that the antigen in human cells detected by antisera raised against Rhesus monkey cells in rabbits or guinea pigs is not identical to the clinically significant antigen **Rh_0** or D, detected by anti-**Rh_0** of human origin. By the time this fact was recognized, however, it was too late to change the name.

The first indication that anti-Rhesus monkey red cell serum and human anti-**Rh_0** were not the same came in 1942, but was not understood at the time. Fisk and Foord (1942) observed that the cells of newborns, whether Rh+ or Rh−, as indicated by antiserum of human origin, all reacted with guinea pig immune serum against Rhesus monkey red cells. The antibody raised in guinea pigs is now known to be anti-LW. In 1952, Murray and Clark looked into the possibility of preparing anti-Rh sera in animals using heat extracts of Rh+ red cells. It had been shown that heating saline suspensions of Rh+ red cells to 50° made them "Rh−", and yielded up "Rh substance" to the medium. They were successful in preparing anti-D in guinea pigs using the heat extracts, but observed to their surprise that about half the heat extracts of Rh negative cells caused production of anti-D in the guinea pigs as well. Levine *et al.* (1961a and 1961b) were able to show that human red cells, whether Rh+ or Rh−, as well as Rhesus monkey red cells, possessed what they called a "D-like" antigen. Antibodies prepared in guinea pigs with these cells, or heat extracts of them, reacted as "anti-D-like". Levine *et al.* (1963) suggested that the "D-like" antigen be called "LW" in honor of Landsteiner and Wiener, and that the antibodies detecting it be called "anti-LW", or "anti-Rh(LW)". It turns out that the gene conditioning LW is not part of the genes conditioning the Rh complex. This matter is discussed in the next section.

22.5.6 Suppressions, deletions and modifiers

In 1950, Race *et al.* found a blood with no detectable C, c, E or e on the red cells. The person was homozygous for the deficiency, and the mother was heterozygous for it. The condition was designated -D-/-D-. A number of further examples of the phenotype have since been found. Wiener denotes such cells, missing the **rh′-hr′** and **rh″-hr″** factors, but having **Rh_0**, as $\overline{\overline{Rh}}_0$. People with the -D- or $\overline{\overline{Rh}}_0$ characteristic have more D (**Rh_0**) than people of ordinary D-containing phenotypes. There is also a rather high rate of consanguinity among the parents of these propositi. Cells have been described, too, which have C or c, and D, but which lack E/e, e.g. cD-/cD-. Such a blood is designated $\overline{Rh}_0$ by Wiener (see Sachs *et al.*, 1960).

Among the rarest but most interesting findings in Rh serology has been the description of red cells which lack the Rh antigens altogether. There appears to be more than one kind of basis for the lack of Rh antigens. In 1961, Vos *et al.* found an Australian Aboriginal woman, 37 years old, with no living parents or children, who lacked Rh antigens of any kind regardless of antisera or technique used. The condition was first designated "—/—", but was later called Rh_{null}, as suggested by Ceppellini (Levine *et al.*, 1964). One explanation for the condition is that the person lacked a very common gene which codes for the precursor to all Rh substances on the cell membrane. It was, of course, of great interest to find out whether this person had the LW antigen (section 22.5.5). Levine *et al.* (1962) showed that she did not. Boettcher and Watts (1978) carried out serological studies on the Rh types of a relative of the original Rh_{null} proposita and his immediate family. He and one of his daughters are believed to be heterozygotes for Rh_{null}, and they exhibit weak expression of Rh antigens. The data were consistent with the idea that Rh_{null} represents homozygosity at a genetic locus controlling the synthesis of precursor for LW and Rh antigens. In 1964, a second example of Rh_{null} blood was found in a White American woman (Mrs. LM). Her husband was rr, and her daughter R_1r, and she therefore had at least one, and probably two normal Rh gene complexes which were unexpressed. She was LW− as well. The finding that both known Rh_{null} persons were LW− tempted the suggestion that LW antigen was some sort of basic precursor substance for the Rh antigens. In 1955, however, Cahan and Wallace (see in Race and Sanger, 1975) had found two people, both R_1r, who had a weak, but identifiable, kind of "anti-D", which turned out to be anti-LW. Both families of these LW− people have since been studied, and the results reported in Race and Sanger (1975). Although LW− people are extremely rare, they do have normal Rh antigens, and LW cannot, therefore, be a direct precursor for the Rh substances, nor can the gene responsible for LW be part of the Rh gene complex (Tippett, 1963). The third example of Rh_{null} was found in a Japanese boy (Ishimori and Hasekura, 1967), and was called —/—, or $\overline{\overline{rh}}$. A number of family members were heterozygous for the condition, and the pedigree indicated that this case was different from the other two, most readily explained by the presence of a silent allele for Rh ($\overline{\overline{r}}$). Race and Sanger (1975) refer to the first kind of Rh_{null} as the "regulator" type, caused by a regulator gene, while the kind that appeared in the Japanese family is called the "amorph" type. These findings make more clear Henningsen's (1958) case, in which a ccDEE mother had a CCDee child. This was not a baby mix-up, and the grandmother of the child was apparently rr. The best explanation was that the grandmother was cde/—, the mother cDE/—, and the child CDe/—.

There can be a number of serological abnormalities associated with Rh_{null}, apart from the absence of Rh antigens. Expression of the antigens of the MNSsU system may be affected (Schmidt and Vos, 1967). There is evidence that the condition is associated with membrane abnormalities, as noted by Smith and Sinclair (1977), who used the most unfortunate designation "Rh_0" to refer to Rh_{null}, as though the nomenclature situation in this system were not already complicated enough. A number of cases of Rh_{null} are reviewed by Race and Sanger (1975), and there are not always abnormalities in the MNSsU expression. Likewise, some subjects are anemic while others are healthy. Rh_{null} is reviewed by Seidl (1967).

22.6 Inheritance of the Rh Factors and Further Nomenclature Considerations

22.6.1 Rh Inheritance

It is important to realize that the differences of opinion about the mechanism of Rh inheritance do not affect the routine use of the system at a practical level. The arguments have been over the relationship between "gene" and "antigen". At the level of testing for most routine purposes, the results are translatable from one system to the other, and most importantly, the results are the same. Translation is provided in Tables 22.1 and 22.2. The number of genotypes and phenotypes in Table 22.2 can be enlarged if tests are carried out with anti-C^w (anti-**rh**^{w}). In any one of the genotypes having the "C" of Fisher-Race, C^w can occur instead. Two examples will suffice: Suppose in line 2 of Table 22.2, the cells reacted with anti-C^w (anti-**rh**^{w}) and not with anti-C (anti-**rh**′). The name of the phenotype would then be Rh^wrh, with usual designations R^wr, R^wR_o and r'^wR_o, corresponding to genotypes $R^{1w}r$, $R^{1w}R^o$ and $R^or'^w$ or C^wDe/cde, C^wDe/cDe, and cDe/C^wde, respectively. As a second example, suppose cells reacted only with anti-**rh**^{w} (C^w), anti-**hr**′ (c) and anti-**hr**′′ (e). It would have the name rh'^wrh, the usual designation r'^wr, and the genotype r'^wr or C^wde/cde.

Fisher originally conceived of Rh as being controlled by a series of three closely linked loci, at which codominant alleles C and c, D and d, and E and e operated. Each allele was imagined to code for an antigen on the red cell. Two predictions are made by this hypothesis that have not been supported experimentally. One is that there should have been found anti-d. It appears now that anti-d does not exist, the few reports of it in the literature having been discredited. The other is that crossing over, although expected to be very rare event, should be observed on occasion. If the hypothesis is correct, d must be regarded as a silent allele. There is only one report in the literature of a possible crossover (Steinberg, 1965). The observation was made in a family in a small, culturally and genetically isolated population called the Hutterites. An R_1r father and an rr mother had eight children, of whom four were rr and three were R_1r. The 6th child born, however, was r′r (Cde/cde). The sociological circumstances of these people and the other blood groups essentially excluded illegitimacy as an explanation. Crossing over would provide an explanation for this child, but mutation cannot be excluded either. Steinberg considered a "D suppressor" as a possibility, but thought that it would have been seen more frequently in grouping thousands of these people if it were around. In view of what is now known of suppressors in the Rh system, however, Race and Sanger (1975) were inclined to think it the most likely explanation.

Wiener's conception of the system is that the Rh locus is not complex, that is, that the locus is occupied by only one "gene" at a time. The system, however, is coded for by a series of multiple alleles. The allele codes for what is termed an "agglutinogen", and this occupies the red cell surface. Agglutinogens can be thought of as complex antigens in a way, i.e., they have more than one blood group determinant specificity. The structures in which the blood group specificity resides, and to which antibodies are directed, are called blood factors. The blood factors are denoted by bold face type.

For Fisher, there was a one-to-one correspondence between "gene" and "antigen", and the genetic locus was complex. For Wiener, the Rh locus was not complex, but had multiple alleles, and the agglutinogens for which they coded were complex antigens. Neither of the systems has been able to assimilate easily the growing list of complicated new phenomena, and in some of the arguments over nomenclature, it is not clear whether the issue is notation or genetics.

It seems quite clear that an understanding of the biochemical genetics of Rh will ultimately settle the issues involved. The membrane structures responsible for Rh activity have been more resistant to study than have their counterparts in the ABO, Lewis and MNSsU systems, in part because they are apparently lipoproteins, and in part because soluble forms of them do not exist.

22.6.2 The numerical system of nomenclature

The notion of a numerical system of notation which describes the serological reactions observed with the Rh antisera used is not a new one. Murray (1944a and 1944b) proposed it as a simple means of recording reaction results. The system was simple, reflecting the state of knowledge at the time. Antisera would be assigned standard numbers, and one would then indicate by subscripts of the symbol "Rh" which ones had agglutinated the cells, e.g. Rh_4 would mean that the cells had reacted only with serum number 4. Murray's suggestions were apparently not very widely adopted.

In 1962, Rosenfield *et al.* resurrected the idea of numerical designation, taking into account a number of complexities of Rh which were by then apparent. Their major point was that a notation should give the reader the essential information about the reactions observed with the antisera without carrying implications about fundamental assumptions about the genetics of the system. The paper should be read, for it points up clearly the deficiencies of both Fisher-Race and Wiener nomenclature, and the underlying assumptions which gave rise to them. The assumptions about the relationships between mutational sites, antigenic structural groupings and specificities in either view could not be supported, it was argued, by the available immunochemical data. The term "agglutinogen" to denote a total gene product, and "blood factor" to denote a serological specificity were regarded as undesirable, because this terminology is not used anywhere else in general immunology or immunochemistry, and because they represent assumptions about the immunochemical nature of the Rh system which have yet to be proven. Further, since the pathway between the genetic locus and the antigen may prove to be complex, a one-to-one correspondence between "gene" and "antigen", as postulated by Fisher, is not yet warranted. Twenty-one

antisera defining Rh specificities were given in the 1962 paper. By 1972, Allen and Rosenfield could list 33 Rh antisera, to which has been added the serum of Mrs. Bastiaan before absorption (see in Section 22.5.3), Rh 34 = Bas (Rosenfield *et al.*, 1973). Race and Sanger (1975) said they thought that the designation Rh 35 had been given to the product of the (C)D(e) complex detected by the FBC serum (serum 1114) of Giles and Skov (1971).

The numerical designations are given in Table 22.3, along with Wiener and Fisher-Race equivalents. There are a few simple rules for the use of the numerical nomenclature system. Antisera are simply called anti-Rh 1, anti-Rh 2, etc. A phenotype is denoted by the symbol "Rh", followed by a colon, and a list of the factors tested for, separated by commas, and preceded by a minus sign if the results were negative. Thus, the types rh (cde/cde) and R_1R_2 (CDe/cDE), if tested with the five common antisera, would be designated Rh:-1,-2,-3,4,5 and Rh:1,2,3,4,5, respectively. If an unexplained weak reaction is encountered, a lower case "w" may precede the number. Thus, (C)D(e)/cDE may be designated Rh:1,w2,3,4,w5,-6,-7,-8,-9,-10,12. Alleles are indicated by an italic capital R, with the factors determined (and not determined) by the allele shown as superscripts. Thus the allele R^{1w} (or C^wDe) would be designated $R^{1,2,-3,-4,5,8}$, and, if necessary, could be designated $R^{1,2,-3,-4,5,-6,7,8,-9,-10,12,(19),(21)}$. Factors given in parentheses mean that only a single example of the specificity has been described so far. Examination of Table 22.3 illustrates the present complexity of the Rh system, and the problems of equivalency between Fisher-Race and Wiener usage.

In 1979, Rosenfield *et al.* (1979a) transferred the task of keeping track of new numerical assignments in the Rh system to Mr. P.D. Issitt then in Cincinnati, now in Miami. Observers of "new" Rh specificities were urged to consult Mr. Issitt concerning the assignment of numbers. Some recently assigned numbers, and the antigens they represent: Rh 36 is Be^a, an antigen defined by Mrs. Berrens serum, and discussed in section 22.5.4. The informative Koz family (Ducos *et al.*, 1974) showed that Be^a belonged to Rh. Rh 37 is the antigen 'Evans', studied by Contreras *et al.* (1978). Rh 38 is 'Duclos', described by Habibi *et al.* (1978). Rh 39 has no other name, and was briefly described by Issitt *et al.* (1978) and more fully by the same authors in 1979. Rh 40 is 'Targett', first described in 1975. The family Nie allowed the assignment of 'Targett' to the Rh system (Lewis *et al.*, 1979).

The nomenclature issue, which was brought up frequently by Wiener, has been the subject of many papers. Wiener felt that his nomenclature should be adopted internationally, as had been done for the ABO system nomenclature years earlier. But efforts to bring this about always failed, and at times led to strong reactions in print. In 1957, the Committee on Medicolegal Problems of the A.M.A. reported out a set of recommendations, which included exclusive use of the Rh-Hr nomenclature. The subcommittee responsible for the recommendations consisted of Wiener and three adherents of his nomenclature. A great number of other workers objected to this report (see Allen *et al.*, 1958c). Wiener (1965b) objected to the numerical designation system as well, and Rosenfield and Kochwa (1965) answered the objections.

22.7 Biochemical Studies on the Receptors—Biochemical Genetics

22.7.1 The number of Rh antigenic sites on red cells

It has been known for quite some time, and has been demonstrated quantitatively, that the amount of Rh antibody with a particular specificity that can be bound to the cells is a function of the Rh genotype of the cell. Although there are a number of different quantitative methods for assessing the amount of antibody bound, the results that have been obtained with radioactive iodine-labelled Rh antibodies will be mentioned here.

Masouredis (1960) showed that more ^{131}I-anti-D was bound to DD than to Dd cells (about 1.6 times more) when both types of cells were also cc. Cells having a CD makeup took up less anti-D than those with a cD one as well. The data indicated that there was heterogeneity in the D antigen, and Masouredis said that the data could be regarded as accurate only for the particular antibody actually used in the experiments, since different antibody might give very different results. Kochwa and Rosenfield (1964) and Rosenfield and Kochwa (1964) carried out similar kinds of studies, and found that there were large differences in the uptake of ^{131}I-anti-D by cells of various D-containing phenotypes. The magnitude of the differences was seen to increase as the amount of available antibody increased. In 1965, Rochna and Hughes-Jones found that there were no differences in the equilibrium constant for the binding of ^{125}I-anti-D to cells of various phenotypes, indicating that there was no qualitative difference in the D receptor. The number of D sites per cell was found to be 9,900–14,600 for CcDee, 12,000–20,000 for ccDee, 14,000–16,600 for ccDEe, 14,500–19,300 for CCDee, 23,000–31,000 for CcDEe and 15,800–33,300 for ccDEE. Masouredis and Sturgeon (1965) found that the uptake of ^{131}I-anti-D by D^u cells was less than 10% of that in control cells, but that papain treatment of the D^u cells resulted in a 3-fold increase in anti-D uptake. Several techniques were used to measure anti-D uptake and the results were in accord, but the values varied according to the technique used. Hughes-Jones *et al.* (1971) measured the number of c, D and E sites with ^{125}I-labelled antibodies and found that the homozygotes always gave higher values than the heterozygotes, except that with anti-E, the results were somewhat variable. The number of D sites on -D- cells was measured, and found to be very much higher than on ordinary D cells (110,000–202,000 sites/cell). Skov and Hughes-Jones (1977) measured available C sites with radioactive anti-C, and found 45,700–56,400 for CDe/CDe, 42,200 for Cde/Cde, 25,500–39,700 for CDe/cDE, 31,100 for Cde/cde, 8,500–9,800 for CDE/cDE, 21,500–40,000 for C^wDe/cde and 7,200 for Cde^s/cde.

Table 22.3 Numerical Designations of Rh Factors and Their Equivalents *

Antigen	Wiener Equivalent	Fisher-Race Equivalent	Antigen	Wiener Equivalent		Fisher-Race Equivalent
Rh1	Rh_o	D	Rh19	hr^s		
Rh2	rh'	C	Rh20	—		VS; e^s
Rh3	rh''	E	Rh21	—		c^G
Rh4	hr'	c	Rh22	—		cis-CE
Rh5	hr''	e	Rh23	—		D^W (Wiel)
Rh6	hr	f; cis-ce	Rh24	—		E^T
Rh7	hr_i	cis-Ce	Rh25	—	LW	—
Rh8	rh^{W1}	C^W	Rh26	—	Deal	"c-like"
Rh9	rh^X	C^X	Rh27	—		cis-cE
Rh10	hr^V	V; ce^s	Rh28	hr^H		—
Rh11	rh^{W2}	E^W	Rh29	—	"total Rh"	—
Rh12	rh^G	G	Rh30	Rh_o^{Cor}	Go^a	D^{Cor}
Rh13	Rh^A	—	Rh31	hr^B		
Rh14	Rh^B	—	Rh32	Product of $\frac{=}{R}N$		
Rh15	Rh^C	—	Rh33		R^oHar	
Rh16	Rh^D	—	Rh34		Bas.	
Rh17	Hr_o	—	Rh35		FBC	(C)D(e)
Rh18	Hr	—				

* Designations written in between the "equivalent" columns do not belong to one or the other of the two older systems

22.7.2 Relationship of the Rh antigens to the erythrocyte membrane

Nicolson *et al.* (1971) used a technique for isolating RBC membrane surfaces from D+ cells to which had been bound anti-D and ferritin-labelled AHG. The preparations were studied in the electron microscope, and the number of sites on the membrane, as judged from the electron micrographs, correlated well with the estimates based on ^{125}I-labelled anti-D (which was used throughout the experiments). The electron micrographs also showed that the receptor sites were dispersed randomly in a two dimensional array. The evidence was further consistent with a generalized model of biological membrane structure called the fluid mosaic model (Singer and Nicolson, 1972). This model supposes that the membrane consists of various globular proteins embedded in a matrix of phospholipid. The phospholipid material is arranged as bilayer, and the globular proteins are arranged so that their polar groups are directed outward on either surface toward the aqueous exterior of the membrane, with apolar, hydrophobic groups buried in the interior of the membrane. The entire structure is fluid, analogous to a two-dimensionally oriented solution of integral proteins in a viscous phospholipid bilayer solvent. Singer and Nicolson (1972) discussed the evidence favoring their model. It may be necessary to think about the mechanism of proteolytic enzyme modification of cells, and the agglutination of protease-modified cells by incomplete antibodies, in terms of this membrane model. Knox (1977) carried out a re-analysis of the data of Nicolson *et al.* (1971) and said that it showed that the arrangement of antigen sites was orderly, rather than random.

Masouredis *et al.* (1977) carried out ^{125}I-labelled anti-D binding studies and electron microscope studies on ferritin-labelled anti-D sites with normal and protease modified D+ cells. Papain and neuraminidase treatment of cells brought about significant increases in the quantity of ferritin-anti-IgG bound to anti-D, although only papain induces agglutination of the cells by the IgG anti-D. The findings were interpreted to mean that the role of the proteolytic enzyme treatment in the enhancement of agglutination might have to do with alterations that are brought about in the biophysical structure of the membrane. The detailed studies of Margni *et al.* (1977) on the mechanism of agglutination of trypsin treated Rh+ cells by incomplete antibodies (and immunochemical fragments derived therefrom) do not appear to be inconsistent with the thinking of Singer and Nicolson, although they were not discussed in these terms. Morley (1978) conducted a study of the binding characteristics of two different anti-D antibodies to a series of cells of different phenotype. A positive correlation between site density and association constant of the antibody was demonstrated. It was suggested that there might be two "types" of antigenic sites on the cell-surface, recognizable only by affinity differences in the antibody. A higher affinity site would be capable of binding both arms of an IgG, whereas a low affinity site would bind only one arm.

22.7.3 Isolation and purification of the Rh antigens

Efforts to obtain "pure" preparations of Rh antigens, or substances, have not been nearly as successful as those directed toward ABH, Lewis or MNSs substances. The ABH and Lewis work was made easier by the existence of the secreted, soluble glycoproteins, and glycoproteins have generally been easier subjects for detailed biochemical study than have lipoproteins. Membrane-resident proteins are notoriously intractable in terms of their isolation in "pure" form with retention of the biological activity.

The earlier efforts reflect attempts to reason by analogy from what was being learned about ABH, Lewis and MNSs substances. There are, in effect, two approaches to the study of the nature of the antigenic determinant. In the direct approach, the biologically active molecule or substance is isolated and characterized. An indirect approach, which paid handsome dividends in the studies on ABH and Lewis substances (Section 19.9.2), was the use of inhibitor substances thought to be structurally related to the antigen. A small molecule which inhibits the binding of antibody to antigen may be doing so, the reasoning goes, because of its structural similarity to the immunodeterminant which thereby allows competition for the antibody binding site.

A number of studies were done using the inhibition approach, some of them with sugars or sialic acid because of the roles these appeared to have in the immunodominant end group structures of ABH, Lewis and MN substances. In 1958, Hackel *et al.* looked at the inhibition of agglutination by anti-Rh and anti-Lu sera by a large number of compounds (60 in all) representing various classes of biologically active molecules. Specific inhibition of the anti-C, anti-D, anti-E, anti-Lu^a and anti-Lu^b reactions was obtained with four compounds derived from RNA: cytidine sulfate, and adenylic, cytidylic and uridylic acids. These findings were interpreted to mean that the antigenic structures of the Rh and Lu determinants bore at least some resemblance to these nucleotides, a conviction that was further strengthened by the finding that the anti-c and anti-e reactions were also inhibited (Hackel and Spolyar, 1960) and that ribonuclease treated red cells showed significant decreases in titration scores with anti-Rh and anti-Lu antisera (Hackel and Smolker, 1960). Hackel extended his studies in 1964. Boyd *et al.* (1959) could not at first confirm Hackel's findings, but said later in a personal communication to Hackel that they had observed the effect. Boyd *et al.* (1959) got weak inhibition of Rh reactions with L-glucose, L-mannose and D-glucose, and speculated that L-sugars or less common D-sugars might be involved in the immunodeterminant structure. Chattoraj and Boyd (1965) found some inhibition with amino acids. In 1960, Dodd *et al.* reported inhibition of the anti-D reaction by crude and pure preparations of N-Ac-neuraminic acid. A sialic acid-containing brain ganglioside and a polysaccharide from a *Pseudomonas* showed inhibition as well. These studies were extended using other sialic acid compounds and a purified ganglioside (Dodd *et al.*, 1964). Johnson and McCluer (1961) were unable to confirm the NANA inhibition, using pure or crude preparations, and said further that neuraminidase treatment of D cells made them more, not less, agglutinable by anti-D. Prager and Lowry (1966) showed that enzymatic desialidation of D red cell membranes did not significantly decrease the activity with anti-D. Rule and Boyd (1964) saw some NANA inhibition of the anti-D reaction. Prager *et al.* (1963) observed weak inhibition of the anti-D reaction with cytidine sulfate and cytidylic acid, but other organic phosphates were found to be more potent inhibitors, α-tocopherol-phosphate being the strongest. These effects were considered to be nonspecific since the compounds did not affect the Coombs test, and acid and alkaline phosphatase, RNase and phosphodiesterase treatment of cells had no effect on Rh agglutination.

In 1950, Moskowitz *et al.* reported the preparation of a complicated fraction from red cell membranes which contained the ABH and Rh activity of the cells from which it was derived. The fraction was called "elinin". Several other fractions derived from membrane stroma contained no blood group substance activity (Moskowitz and Calvin, 1952). Wolf and Springer (1965) did not like the name "elinin", and called the fraction with blood group activity "AF" (active fraction). It was heat labile, sensitive to ethanol, inhibited by pH very far from 8, and inactivated by periodate and iodoacetamide, implicating carbohydrate and sulfhydryl groups as part of the immuno-active material. Proteases did not abolish the activity, and the fraction reacted with *Vicia graminea* lectin.

Various procedures involving butanol extraction of red cell stroma did not yield fractions possessing Rh activity (Rega *et al.*, 1967; Poulik and Lauf, 1965 and 1969). Green (1965) found that lyophilized red cell stroma derived from C or D containing cells had antigenic activity (would take up anti-C or anti-D). Reactivity was temperature and pH dependent, inhibited by high concentrations of sulfhydryl reagents, and abolished by $-SH$ binding reagents. High concentrations of sulfhydryl reagents afforded some protection against the abolition of activity by $-SH$ reagents. In 1967, Green showed that lyophilized membranes that had been solubilized and disaggregated by anionic detergents and SDS had lost detectable Rh activity, even following the removal of the detergent on an ion exchange resin. In 1968, Green showed that a butanol extractable component was required for Rh activity of membrane preparations, and that Rh antigenic activity could be reconstituted in butanol extracted membranes by adding back the contents of the butanol extract. Chloroform-methanol extracts would also restore activity to butanol extracted membranes. The extractable component, required for C or D activity was found to be a lecithin (phosphatidyl choline), and it was thought to be associated with a sulfhydryl protein in the membrane (Green, 1968a and 1968b). These findings helped to explain the earlier studies in which activity had not been found in solvent extracts of membranes. The evidence also suggested that the lecithin had to contain at least one unsaturated fatty acid residue. In 1972, Green showed that reconstitution of Rh antigenic activity to butanol extracted membrane fractions was correlated with the binding of lecithin to the membrane material. The evidence suggested that the phospholipid was associated with the membrane protein by hydrophobic forces.

In 1968, Weicker reported that he had obtained a small peptide, with a MW between 6000 and 12,000, by an exhaustive dialysis procedure, followed by gel filtration chromatography, which had D-activity as assessed by hemagglutination inhibition. The peptide contained 12 different amino acids, including Cys. With a refined purification pro-

cedure, Weicker and Metz (1971) isolated a peptide with MW about 10,000, with a small amount of bound phospholipid, and containing 14 amino acids. The antigenic activity was assessed by an anaphylactic shock procedure using isolated muscle, and was said to be established specifically as D-activity (Weicker and Roelke, 1971). Hemagglutination inhibition and gel precipitation were not thought to be satisfactory methods for detecting antigenic activity because the antigen was small and the freeze drying procedure affected reactivity. Jackson *et al.* (1972) could not confirm Weicker's findings using hemagglutination inhibition of the anti-D reaction with bromelin treated D cells as an assay procedure.

Loruso and Green (1975) reported obtaining D-active membrane fractions by solubilization with deoxycholate, followed by removal of the detergent and slow addition of Mg^{++} by dialysis. The "reaggregated vesicles" had D activity. In 1977, Loruso *et al.* extended these studies, using sodium dodecylsulfate in place of deoxycholate, and showing that Ca^{++} as well as Mg^{++} worked in the reconstitution stage. D activity was separable from A activity and did not reside on glycoprotein 1.

Beginning in 1974, Abraham and Bakerman have reported the solubilization and partial purification of Rh antigens D, C, E, c and e. The procedure was similar in each case. Hemoglobin free membranes (Limber *et al.*, 1970) were solubilized using EDTA and then NaCl solutions. The material was fractionated on ultrafilter membranes and further purified by isoelectric focusing in pH gradients. Immunological activity was tested by hemagglutination inhibition and by formation of specific antibodies after immunization of animals with the preparations. All the purified antigens migrated as single bands on disc electrophoresis. Antigen c had a MW in the 20,000–30,000 range (Abraham and Bakerman, 1974), antigen C in the 50,000–100,000 range (Abraham and Bakerman, 1975a), antigen D in the 10,000–20,000 range (Abraham and Bakerman, 1975b), antigen E in the 50,000–100,000 range (Abraham and Bakerman, 1976) and antigen e in the 20,000–30,000 range (Abraham and Bakerman, 1977), as judged by the exclusion limits of the ultrafilters. More recent studies on the isolated D antigen have been carried out to test the effect of various enzymes on the preparation (Litten *et al.*, 1978). A modification of the isolation procedure was introduced, and activity was measured by the ability of the preparation to inhibit hemagglutination. Because of the findings implicating phospholipid in the antigenic structure, the preparation was treated with phospholipases A and C, alkaline phosphatase, neuraminidase, leucine aminopeptidase and carboxypeptidases A and B. None of these affected hemagglutination inhibition, however. Activity was abolished by pronase, trypsin, chymotrypsin and papain, suggesting that protein was most important in the antigenic determinant structure in the solubilized preparation. Lipid molecules may play a role in maintaining the receptor in its structural conformation in the membrane, but is apparently not required for activity in the isolated preparation.

Plapp *et al.* (1979) partially purified D antigen by affinity chromatography of sodium deoxycholate-solubilized membrane material. Similar quantities of active material were obtained from Rh positive and Rh negative cells. This intriguing result suggests that Rh negative people make D antigen, but that it is somehow not exposed to the external cell surface. The authors said that their results would explain why neither d nor anti-d has ever been found. Interestingly too, the D antigen fraction contained the LW antigen. Toma *et al.* (1979) obtained what appear to be related results, using wholly different techniques. They used ^{99}Tc-pyrophosphate-labelled anti-D to label D^+, D^u and D^- cells, and observed binding in all cases. They said that d was not an allele of D, but rather the weakest in the antigen series $D > D^u > d$. They also employed uranyl-labelled anti-D for immunoelectron microscopy, and said that anti-D was bound to Rh negative red cell membranes. It seems reasonable to suppose that the results of Plapp *et al.* (1979) could provide a basis for the observations made by Toma *et al.* (1979).

Folkerd *et al.* (1977) estimated the *in situ* MW of D antigen as $174{,}000 \pm 10{,}000$ using an indirect radiation bombardment procedure.

22.7.4 Biochemical genetics of Rh

Before a detailed understanding of the biochemical genetics of Rh can be attained, more will have to be known about the exact nature of the antigens, and its relationship to red cell membrane structure. Knowledge of the antigen structure will enable a fuller investigation of the nature of the Rh gene products. Since there is not very much detailed information about the immunochemical structures involved, biochemical genetic schemes proposed for Rh are speculative. A number of models have been put forth, having to do with the order of activity of the genes in an Rh antigen synthetic pathway, with gene structure itself, and with structural complexity for both antigens and antibodies. Some references are Nijenhuis (1961), Lauer (1964), Boettcher (1964), Knox (1966), Edwards (1968, Hirschfeld (1973) and Rosenfield *et al.* (1973). The last mentioned is an effort to explain all the available serological and genetic data with a kind of Jacob-Monod operon model, which in certain up to date ways, retains some of Fisher's original notions. Some of the current thinking about Rh has recently been reviewed by Stroup (1977).

22.8 Medicolegal Applications of Rh

The Rh system has been in use for a long time in cases of disputed parentage. Wiener (1944a) mentioned that he was using Rh typing in some of his cases. The chances of excluding a true nonfather using Rh are better than for most other systems taken individually. Wiener (1950) reported on a large series of cases from New York, and Silveira (1967) reported on a case in which an exclusion was obtained in the Rh system, genotypes having been deduced from family studies.

The chances of excluding a falsely accused father with the Rh system, using the five common antisera, are about 18% for Blacks and about 27% for Whites (Chakraborty *et al.*, 1974; AABB, 1978). Ordinarily, Rh genotype cannot be determined from the phenotypes which represent multiple genotypes without family studies. Sometimes, special antisera (e.g. anti-ce, anti-Ce) can help to distinguish different genotypes within phenotypes. For example, anti-ce (anti-f) could distinguish between CDE/cde and CDe/cDE. The investigator must be aware of the complexities of the Rh system (section 22.5) in interpreting results in paternity cases, even though many of them are very rare. Tippett, in AABB (1978), has discussed the pitfalls in the use of the Rh system in parentage cases.

Rh typing in bloodstains did not come up in the literature until the late 1940's, and determination of Rh antigens in bloodstains on anything like a routine basis is still largely restricted to a few specialized laboratories.

In 1949, Closon carried out experiments on the detection of the "Rh antigen" (presumably $\mathbf{Rh_0}$ or D) in dried blood using an inhibition technique. The amounts of dried blood that were needed were large by present day standards, 100 mg being used with 0.6 mℓ antisera for the observation of unequivocal inhibition by Rh^+ dried blood. 100 mg dried blood corresponded to the residue from about 0.4 mℓ whole blood. Good results were obtained in dried blood up to 15 days old, but in samples older than this, activity was greatly diminished. Ruffié and Ducos refined the technique for the determination of Rh antigens in dried blood to a high degree. An inhibition method, modified from that of Holzer, was employed. Detection of C, D and E in bloodstains was reported in 1952, these being reliably detectable in dried blood up to 4 days old. In 1953 and 1954, c and C^w antigens were added to the list (Ruffié and Ducos, 1953; Ducos and Ruffié, 1954). Fairly large amounts of dried blood were needed for the tests, and Rh antigens were undectable in stains that were very old. Detailed methods were given, and the importance of selecting antisera of known specificity was stressed. Apparently, it was difficult to get anti-C which had no anti-D in it.

Ducos (1958) reviewed the status of antigen grouping in dried blood, and included the results of a survey of 27 laboratories in 18 countries, which had asked which antigens were grouped routinely. Two did D routinely, and four others on occasion, two did C and E routinely and one on occasion, and only one lab did C^w and c routinely.

The next step was taken when the elution method of Kind was introduced in 1960. The elution method is probably employed almost universally for Rh at present. In 1962, Nickolls and Pereira reported that the D antigen could be reliably determined in bloodstains by elution. In 1967, Bargagna and Pereira reported in detail on the determination of D, C, E, c, e and C^w in dried bloodstains by the elution method. Absorption was carried out at 37°, as was the washing step. Studies were done with saline agglutinating antisera as well as with incomplete antisera, using enzyme or AHG techniques. Both complete and incomplete antisera had certain advantages. Fixation of the stained material was not only unnecessary but deleterious to some of the antigens. There were some problems with anti-c, but properly diluted antisera always gave specific reactions by the AHG test. Some incomplete anti-e sera failed to react with e stains. The elution technique was believed to be very suitable for the Rh antigens in dried bloodstains, though it was said that some slight refinements were still needed. In 1968, Lincoln and Dodd put forth an elution procedure suitable for C, C^w, D, c, E and e (as well as S) (Lincoln and Dodd, 1968b). Certain antigens were detectable longer than others in experimental bloodstains, D, C, C^w and c being stable for 6 months, while E and e were stable for 4–6 months. Successful typing of still older stains has subsequently been reported (e.g. McDowall *et al.*, 1978b). Papain treated cells were used for the detection of all the antigens. It could be shown that the spurious anti-c reactions seen by Bargagna and Pereira did sometimes occur with bromelin-treated cells, but they were not observed if enzyme technique was carried out with papain. The most serious problem was the availability of suitable antisera. Reagents specific and suitable for cell grouping are not necessarily specific or suitable for stain typing by absorption-elution. Suitable anti-C were difficult to obtain. These sera often contain anti-D and anti-G. Depending upon the characteristics of the separate antibodies, the anti-D problem may be gotten around, but the anti-G problem is much more difficult. Anti-C reagents must be carefully tested with R_2R_2 or R_2r cells to insure specificity. Only a few suitable anti-E sera could be found. The careful selection and evaluation of specific and potent antisera was stressed. The matter was discussed in section 19.10.3.4. Goryanina (1973) discussed the selection of antisera for $\mathbf{Rh_0}$ determination in stains by absorption-elution. Denault *et al.* (1978) tested the survival of the Rh antigens in bloodstains on a number of substrata, using the elution procedure. D was detectable up to 26 weeks, but e up to only about two weeks, regardless of substratum or humidity. The antigens C, c and E were adversely affected by high humidity. Indications were that C was somewhat more stable at 20% relative humidity than were c or E. Antigens E and e could be missed in stains, and false positive C, c and E reactions were occasionally observed.

Martin (1977) presented a technique for determination of the Rh antigens C, c, D, E, e and C^w which required only 1 cm long stained threads. The technique consisted of affixing threads to a polycarbonate sheet for the absorption and washing stages, and then transferring them to tubes for elution and detection of the eluted antibodies. This represented an improvement over the older techniques which generally required larger amounts of material. Incomplete antisera were used, and papain-treated red cells were employed for detection of eluted antibody. Elution of Rh antibodies cannot be carried out directly into test cells, because the higher temperatures have a deleterious effect on the cellular antigens.

Autoanalyzer techniques have been applied to bloodstain grouping with successful results. The autoanalyzer tech-

niques were introduced in the 1960's, and are very helpful in large scale typing and screening of cells and of sera. There are many technical problems associated with blood grouping by autoanalyzer. Details will not be discussed here. A few references to autoanalyzer procedures are Rosenfield and Haber (1965), Sturgeon *et al.* (1965), Morton and Pickles (1965) and Kliman and Smith (1966). At the simplest level, the autoanalyzer serves as a device for mixing red cells with antisera and other necessary reagents, such as colloids, enzyme solutions, etc., and has a detection device for telling whether agglutination has occurred. Autoanalyzers are used for bloodstain analysis because they can detect very small amounts of antibody.

Douglas and Stavely (1969) reported successful results in grouping artificial bloodstains for D and c by autoanalyzer. In bloodstain grouping, absorption and elution are generally carried out manually, but the eluate is processed in the autoanalyzer. In 1970, Pereira (see in Culliford, 1971) described in some detail the autoanalyzer technique being used at the time for the Rh antigens. An advantage to the autoanalyzer, apart from the time saved and the objectivity introduced in the evaluation of agglutination results, was that D^u would ordinarily be missed if only a saline agglutinating anti-D were used in the test, and it is usually necessary to do an AHG test to check for the presence of D^u. The high sensitivity of the autoanalyzer in detecting antibodies is achieved by introducing colloidal media into the system which brings about rouleaux formation and allows for more efficient agglutination. The rouleaux are then dispersed by addition of saline, and the agglutinates remain. Lincoln (1973) carried out some collaborative experiments with Pereira, comparing the strength of the antigen-antibody reaction as measured by manual titration of the eluates with the peak area seen in the autoanalyzer for the same samples. The correlation between the results of the two methods was very close indeed. Refinements in autoanalyzer techniques for determination of the Rh antigens in stains, based on the use of low ionic strength media (see below), and so forth, have been discussed by Martin *et al.* (1975) and by Brewer *et al.* (1976). McDowall *et al.* (1978b) recently compared manual and autoanalyzer techniques for detecting antibodies eluted from bloodstains. Anti-D, anti-C, anti-E and anti-c were used in the experiments. The autoanalyzer system was a low ionic strength polybrene (LISP) system, described in Boorman *et al.* (1979). The autoanalyzer did not give significantly more sensitive detection than the manual technique. Furthermore, with stains older than about 4 weeks, the manual detection procedure actually gave more conclusive results. It is to be noted, however, that the manual procedure uses papain treated test cells whereas this particular autoanalyzer system does not.

Akaishi (1965) reported that $\mathbf{Rh_0}$ (D) could be grouped in stains by mixed agglutination if bromelin treated test cells were employed. Hasebe (1962a) said that D could be detected in red cell smears by the fluorescent antibody technique.

The Rh antigens do not survive aging in dried bloodstains as well as the ABH antigens generally speaking, but can often be determined in stains that are a number of months old depending upon the antisera, the technique, and the condition of the stain. The rate of disappearance of the different Rh antigens in dried blood is not necessarily the same, probably due in part to the different densities of sites on the red cells to begin with (Section 22.7.1). The interpretation of negative results is, therefore, something of a problem.

McDowall *et al.* (1978a) have recently shown that the enhancement of red cell antigen-antibody reactions by low ionic strength media can be productively exploited in bloodstain grouping. In 1964, Hughes-Jones *et al.* looked at the effect of several parameters, known to influence the equilibrium constant for antigen-antibody reactions, on the reaction between anti-D and D cells (Hughes-Jones *et al.*, 1964a). Using ^{131}I-labelled anti-D, it could be shown that the association constant was increased 1000-fold by reducing the ionic strength from 0.17 to 0.03. Atchley *et al.* (1964) obtained similar results using low ionic strength media made up with glycine or a number of nonelectrolytes such as sucrose. This effect could be shown to apply to a variety of antibodies, and is clearly a way of increasing the sensitivity of methods of detection of blood group antibodies. Low ionic strength solutions were useful in enhancing the titer of a number of antibodies if used in the first stage of an AHG test (Hughes-Jones *et al.*, 1964b; Elliot *et al.*, 1964). The effect was more pronounced with antibodies for factors other than ABO or Lewis, although there were exceptions. Low ionic strength media also leads, in some cases, to the nonspecific uptake of antibody. In 1974, Löw and Messeter indicated that a low ionic strength solution (LISS) consisting of 0.03M NaCl containing 0.24M sodium glycinate and 5 mM phosphate buffer, pH 6.7, gave the enhancement effect while virtually eliminating the nonspecific uptake of antibody. One of the main applications of LISS was to the first stage of the AHG test. By increasing both the rate and the amount of antibody uptake, the incubation times were shortened and antibodies present in small amounts, or those of low avidity, were more easily and quickly detected. The results were confirmed by Moore and Mollison (1976). The value of low ionic strength media in detecting antibodies, and the many variations in procedure, have now been studied extensively (Rosenfield *et al.*, 1979b; Jorgensen *et al.*, 1979a and 1979b; Fitzsimmons and Morel, 1979). Lincoln and Dodd (1978) demonstrated the applicability of LISS technique, in combination with the use of enzyme treated cells, to the detection of small amounts of blood group antibodies, and showed the technique to be especially useful for eluted antibodies. McDowell *et al.* (1978a) applied the technique to bloodstain grouping. The sensitivity of the elution technique of Lincoln and Dodd (1973) was increased markedly by the LISS-enzyme treated cell method of detecting Rh antigens and S. As with other serological procedures, the selection of appropriate antisera is an essential ingredient in obtaining good results.

The issue of interpretation of the results of Rh grouping

in bloodstains was raised recently by Martin (1977), although it is neither a new issue nor necessarily one which is restricted to the Rhesus system. The question which arises is two-fold: First, should negative results with a bloodstain be interpreted to mean that the antigen is in fact absent from the stain being examined, and second, should the results be reported in a way that states or implies a genotype or most probable genotype. The two are related, of course, and if one does not interpret negative findings to mean that the antigen is absent, the second part of the question never comes up. There are differences of opinion about the interpretation of negative results. As to the way results are reported, however, there is some agreement that only the results of the reactions with the antisera used in the tests should be reported. Thus in cases where a phenotype can be the result of a number of genotypes, no genotypical implications would be given in the report, according to this way of thinking. One can never give more than a *probable* genotype, if the phenotype contains more than one genotype without family studies in any case, even if fresh cells have been grouped. In cases where a phenotype is the result of a unique genotype, e.g. C^w-, C−,D−,E−,c+,e+, it would be up to the individual to decide whether this bloodstain was in fact rr or not. The possibility that a weak antigen was present (e.g. D^u) in the blood, or that one of the antigens originally present in the stain was not detected because of the age or condition of the stain, should not be overlooked. There is considerable value in reporting the positive and negative reactions because it is possible, then, to select informative results, and leave aside those results which would be meaningless or confusing, or which are unsatisfactory. An example would be the reporting of a D+ in a stain, but a D− result in the red cells of a suspect. This result is exclusionary, regardless of the results obtained with other antigens.

22.9 Frequency of Rh Phenotypes in U.S. Populations

Frequencies for Rh phenotypes in some U.S. populations are shown in Table 22.4. Phenotypes have been designated in Wiener nomenclature, as in column 1 of Table 22.2, and studies where only one or a few antigens were studied (e.g. D+ and D−) have been omitted.

Table 22.4 Rh Phenotypes in U.S. Populations

Population	Total	Frequency — Number (Percent) Rh_o	Rh_1rh	Rh_1Rh_1	Rh_2rh	Rh_2Rh_2	Rh_1Rh_2*	rh	Rare Phenotypes See Note:	Reference
CAUCASIAN										
New York, NY	2,390	69 (2.89)	798 (33.39)	489 (20.46)		349 (14.6)☆	330 (13.8)	321 (13.43)	1	Wiener and Gordon, 1951
University of Iowa	2,181	91 (4.17)	723 (33.15)	382 (17.51)	319 (14.63)		303 (13.89)	338 (15.5)	2	Buckwalter et al., 1962
Southeastern GA	331	9 (2.7)	112 (33.8)	65 (19.6)	31 (9.4)	13 (3.9)	44 (13.3)	46 (13.9)	3	Cooper et al., 1963
San Francisco, CA Bay Area Mothers	4,926	(2.66)	(34.66)	(19.66)	(11.75)	–	(13.52)	(13.82)		Reed, 1967
Children		(2.29)	(34.60)	(20.41)	(11.85)	–	(12.56)	(13.80)		
San Francisco, CA Bay Area All Caucasians	8,962	206 (2.3)	3,086 (34.4)	1,728 (19.3)	1,033 (11.5)	212 (2.4)	1,189 (13.3)	1,365 (15.2)		Reed, 1968
"Western European" Caucasians	5,056	99 (2.0)	1,748 (34.5)	912 (18.0)	594 (11.7)	109 (2.2)	661 (13.1)	853 (16.8)		
New York, NY	500	11 (2.2)	162 (32.4)	111 (22.2)	47 (9.4)	9 (1.8)	80 (16.0)	60 (12.0)	4	Wiener, 1969
South Central WV	1,412	(2.1)	(33.4)	(17.4)	(12.8)	(3.9)	(14.4)	(14.3)	5	Juberg, 1970
Tecumseh, MI	6,963	188 (2.1)	3,051 (34.04)	1,452 (16.19)	1,124 (12.54)	208 (2.32)	1,124 (12.54)	1,412 (15.75)	6	Schreffler et al., 1971
Detroit, MI	505	(2.6)	(32.3)	(13.5)	(9.3)	(2.4)	(13.1)	(23.2)	20	Shaler, 1978 ◇
Miami/Dade Co., FL	370	10 (2.7)	138 (37.3)	64 (17.3)	41 (11.1)	12 (3.2)	43 (11.6)	51 (13.8)	15	Stuver, 1979 and see Shaler, 1978
Los Angeles, CA Case material	250	11 (4.4)	80 (32.0)	56 (22.4)	24 (9.6)	14 (5.6)	34 (13.6)	31 (12.4)	18	Sigler, 1979 and see Shaler, 1978
NEGRO										
New York, NY	200	(46.5)	(24.5)	(2.0)		(16)☆	(5)	(5.5)	7	Miller et al., 1951
Washington, D.C. Howard University Students	937	(44.5)	(24.6)			(17.3)	(5.1)	(7.3)	22	Moore, 1955
Baltimore, MD	580	(43.8)	(25.8)			(15.4)	(6.3)	(7.2)	8	Glass and Li, 1953
Southeastern GA	304	161 (53.5)	58 (19.3)	4 (1.3)	49 (16.3)	4 (1.3)	9 (3.0)	16 (5.3)	9	Cooper et al., 1963
San Francisco, CA Bay Area Mothers	1,453	(47.83)	(22.85)	(2.68)	(14.11)	–	(3.72)	(6.13)		Reed, 1967
Children		(48.04)	(23.81)	(2.13)	(14.38)	–	(3.1)	(5.71)		
Birmingham, AL	613	320 (52.2)	132 (21.5)	2 (0.3)	77 (12.6)	13 (2.1)	18 (2.9)	40 (6.5)	10	Casey et al., 1968
San Francisco, CA	3,146	1,514 (48.1)	713 (2.7)	69 (2.2)	458 (14.6)	33 (1.0)	108 (3.4)	201 (6.4)		Reed, 1968
New York, NY	500	229 (45.8)	104 (20.8)	10 (2.0)	93 (18.6)	1 (0.2)	20 (4.0)	34 (6.8)	11	Wiener, 1969
South Central WV	133	(47)	(28)	(2)	(13)	(2)	(3)	(7)	12	Juberg, 1970
Detroit, MI	505	(50.3)	(20.8)	(1.6)	(13.5)	(1.6)	(3.6)	(7.3)	21	Shaler, 1978 ◇
Miami/Dade Co., FL	350	179 (51.1)	73 (20.9)	7 (2.0)	48 (13.7)	5 (1.4)	14 (4.0)	22 (6.3)	16	Stuver, 1979 and see Shaler, 1978
Los Angeles, CA Case material	125	63 (50.4)	26 (20.8)	5 (4.0)	12 (9.6)	3 (2.4)	10 (8.0)	6 (4.8)		Sigler, 1979 and see Shaler, 1978

Table 22.4 Cont'd.

Population	Total	Frequency — Number (Percent) Rh_o	Rh_1rh	Rh_1Rh_1	Rh_2rh	Rh_2Rh_2	Rh_1Rh_2 ★	rh	Rare Phenotypes See Note:	Reference
CHINESE										
New York, NY	103	(1)	(11.7)	(53.4)	(8.7) ☆		(24.2)	(1)		Miller et al., 1951
New York, NY	400	1 (0.3)	30 (7.5)	213 (53.3)	6 (1.5)	19 (4.8)	126 (31.5)		13	Wiener, 1969
New York, NY	946 ⊙	3 (0.32)	70 (7.4)	506 (53.49)	21 (2.22)	42 (4.44)	284 (30.02)	2 (0.21)	14	Wiener, 1974
HISPANIC										
San Francisco, CA "Mexican"	335	8 (2.4)	103 (30.7)	81 (24.2)	33 (9.9)	13 (3.9)	59 (17.6)	20 (6.0)		Reed, 1968
Miami/Dade Co., FL	364	25 (6.9)	124 (34.1)	56 (15.4)	43 (11.8)	9 (2.5)	49 (13.5)	52 (14.3)	17	Stuver, 1979 and see Shaler, 1978
Los Angeles, CA Case material	111 □	1 (0.9)	33 (29.7)	26 (23.4)	12 (10.8)	7 (6.3)	29 (26.1)	3 (2.7)	19	Sigler, 1979 and see Shaler, 1978

★ Includes R_1R_2, R_1r'', R_2r', R_2r'', R_2R_o and R_or_y

☆ Anti-hr" (e) was not used — Rh_2rh and Rh_2Rh_2 indistinguishable

⊙ Includes the 400 persons in the Wiener (1969) study

□ Primarily Mexican

◇ Data of Stolorow and collaborators

1. 22(0.92) were rh'rh, 11(0.46) were rh"rh", and 1 was Rh_zRh_1
2. 25(1.15) were rh'rh" (or rh_yrh)
3. 7 were rh"rh, 2 were rh'rh, 1 was Rh_zRh_2; 3 of the 9 Rh_o had D^u, 2 of the 112 Rh_1rh had C^u and 1 of the 65 Rh_1 was C^w (i.e. Rh_1^w)
4. 4 were rh'rh, 4 rh"rh, 1 rh_yrh, 5 $Rh_1^wRh_1$, 5 Rh_1^wrh and 1 Rh_zRh_1
5. 0.3% were rh'rh, 0.6% were rh"rh, 0.3 were $CcD^{u}ee$, 0.1 were $ccD^{u}Ee$, 0.2 were Rh_zRh_1 and 0.3 were Rh_zRh_2
6. 10(0.11) were Rh_zRh_2, 1 was $ccD^{u}EE$, 5 were Rh_zRh_1, 37(0.41) were $Rh_1^wRh_2$, 15(0.17) were $ccD^{u}Ee$, 79(0.88) were $Rh_1^wRh_1$, 4 were $CCD^{u}ee$, 109(1.22) were Rh_1^wrh, 33(0.37) were $CcD^{u}ee$, 16(0.18) were $ccD^{u}ee$, there was one each of rh"rh", rh'rh' and rh'rh" (or rh_yrh), 54(0.6) were rh"rh and 38(0.42) were rh'rh.
7. 0.5% were rh'rh'
8. 1.4% were rh'rh'
9. 3 were rh'rh
10. 1 was Rh_zRh_2, 9 were rh'rh and 1 was $ccD^{u}ee$
11. 5 were rh'rh, 3 were Rh_o and 1 was Rh_1^wrh
12. 1% were rh'rh
13. 4 were Rh_zRh_1 and 1 was Rh_zRh_2
14. 15(1.59) were Rh_zRh_1, 2(0.21) were Rh_zRh_2 and 1(0.1) was rh'rh
15. 2 were Rh_zRh_1, 1 was Rh_zRh_2, 3 were rh'rh, 3 were rh"rh, 1 was rh'rh' and 1 was rh_yrh".
16. 2 were rh'rh
17. 2 were Rh_zRh_1, 1 was Rh_zRh_2 and 3 were rh'rh.
18. 2 were Rh_zRh_2, 1 was Rh_zRh_2, 1 was rh"rh and 2 were rh'rh.
19. 1 was Rh_zRh_2, 2 were Rh_zRh_2, 1 was rh"rh, and 2 were rh'rh.
20. 0.2% were Rh_zRh_2, 0.4% were Rh_zRh_2, 0.2% were Rh_zRh_1, 0.6% were rh'rh', 1.2% were rh'rh, 1.0% were rh"rh and 0.2% were $CcD^{u}ee$
21. 1.2% were rh'rh
22. 1.2% were rh'rh' or rh'rh

SECTION 23. THE KELL, DUFFY AND KIDD BLOOD GROUP SYSTEMS

23.1 Kell System

23.1.1 The K and k antigens

In 1946, Coombs *et al.* reported their results on investigations of 15 cases of children with hemolytic disease of the newborn, most of which had been caused by Rh incompatible pregnancies. In one case, the baby's cells gave a positive direct AHG test, and the mother's serum was capable of sensitizing the father's cells, but not those of 13 randomly selected persons representing all known Rh types. The antibody did not appear to belong to any known system. Race (1946) called the antigen "Kell", after the woman's surname, and said that anti-Kell serum reacted with about 7% of random blood samples. In 1947, Wiener and Sonn Gordon reported a hemolytic transfusion reaction in a woman that could not be accounted for on the basis of known antibodies. Her name was Singer, and they called the antibody "anti-Si". "Si" appeared to be inherited as a simple Mendelian dominant, and could be found in the cells of 19 out of 148 bloods from Caucasians. Some anti-Si was sent off to Dr. Mourant in England, and he showed that "anti-Si" and "anti-Kell" were identical. The name "Si" was abandoned, and the antigen was to be called "Kell", shortened to "K" (Wiener *et al.*, 1953a).

In 1949, Levine *et al.* found an antibody in a mother who had had a baby suffering from hemolytic disease. It reacted in saline at 37° and detected a very high incidence antigen (99.8% of 2,500 random blood samples reacted). The antibody was called "anti-Cellano" after the patient's name. Calculations on the expected percentage of occurrence of the allele of the Cellano gene suggested a possible association with Kell or Lutheran, and family studies using anti-Cellano and anti-K soon established that Kell and Cellano were determined by alleles. Cellano was thus called "k", and the Kell system consisted of two alleles, *K* and *k*, giving rise to the genotypes *KK*, *Kk* and *kk*. This simple situation persisted until 1956. The k antigen is found in fetuses aged 9 weeks or older in the same strength as in adults, and with the same frequency, while K may be found in 14–16 week old fetuses (Toivanen and Hirvonen, 1969b).

23.1.2 Complexities of the Kell system

In 1956, Allen found an antibody in the serum of a Mrs. Penney that agglutinated about 2% of randomly selected cells. Certain informative families revealed that 'Penney' was related to the Kell system, and the antigen was named "Kp^a". In another serum from a person called 'Rautenberg' was found an antibody which appeared to be detecting the antigen governed by the allele of Kp^a. Further studies were reported by Allen and Lewis in 1957. In 1958, Allen *et al.* showed that the 'Rautenberg' serum was indeed detecting Kp^b (Allen *et al.*, 1958a). Kp(a+) is quite rare (2% in 5,500 people). Allen *et al.* (1958a) said that, in theory, there could be four kinds of gene complexes: KKp^a, kKp^a, KKp^b and kKp^b, and these were denoted K^a, k^a, K^b and k^b, respectively, in shorthand. KKp^a had not been found. In 1960, Lewis *et al.* screened 14,611 bloods for K, and then examined the 1,221 K+ samples with anti-Kp^a in a search for KKp^a. 22 K+Kp(a+) samples were found, but family studies revealed that *K* and Kp^a were on different chromosomes in every case. In 1969, Dichupa *et al.* conducted another search for KKp^a, this time by screening for Kp(a+) first. 274 Kp(a+) bloods were found in 11,239 samples, of which 11 were also K+. 9 families were studied, but without finding KKp^a (K^a), which in this latter paper was denoted K^p.

In 1958, Giblett found an antibody in a White male cancer patient who had received transfusions of blood from Black donors. The antibody reacted with the cells of 33 out of 172 Black people, but not with any samples from 240 White people. Further investigation showed that the antibody did not appear to be part of any known system, and the antigen being detected was provisionally called Js. In 1959, Giblett and Chase studied the characteristic further, and called the antigen Js^a. It appeared to be part of a new blood group system, which was to be called 'Sutter', and it was supposed that an antibody detecting Js^b would be found. Js^a was inherited as a simple Mendelian dominant. A second example of anti-Js^a was found in a multiply transfused patient in Detroit by Jarkowski *et al.* (1962), who found that 34 of 244 examples of red cells from Black people reacted with it, but that none of the cells of 103 White people did so. In 1962, Greenwalt *et al.* found the expected anti-Js^b in the serum of a Black woman in Memphis, who was being treated for complications associated with her 11th pregnancy. Further studies were done by Walker *et al.* (1963). Js^b is of relatively high frequency, while Js^a is relatively low. In 1965, Stroup *et al.*, studying the Js^b characteristic, found that the cells of two people who lacked all Kell antigens, i.e., were K–k–Kp(a–b–) (see below), were also Js(a–b–). Studies on informative families established that the *Js* locus was closely, if not absolutely, linked to the Kell locus.

In 1957, Chown *et al.* found two sisters in a family of Polish extraction, the results of a marriage between second cousins, who lacked K, k, Kp^a and Kp^b. Their phenotype was written K–k–Kp(a–b–). Allen *et al.* (1958a) designated this condition as K^o. The serum of the proposita (Peltz) contained an antibody which reacted with cells of all Kell

phenotypes except K^o, and came to be known as anti-Ku. Using this serum, Kaita *et al.* (1959) found another K^o person (Kan.), also the result of a consanguineous marriage. In 1966, Nunn *et al.* found another K^o person with a second example of anti-Ku in her serum. Studies on this, and some other families indicated that K^o must be controlled by an allele at the Kell locus, rather than by an independent suppressor. If the Kell locus is thought of in terms of an operon model, the K^o gene could be regarded as an operator mutation. K^o is extremely rare, the population studies thus far indicating its occurrence on the order of 5 per 100,000. Its theoretical significance, however, far exceeds its frequency in the population. More will be said about K^o below.

In 1961, Allen *et al.* described a new Kell phenotype in a healthy man called McLeod. His red cells reacted very weakly with anti-k and anti-Kp^b, and very weakly with anti-Ku. They did not react with anti-K or anti-Kp^a. After Stroup *et al.* (1965) had shown that Sutter belonged to the Kell system, McLeod cells were found to be very weakly reactive with anti-Js^b. Thus, the McLeod phenotype, as it is usually called, is K-kwKp(a–bw)Js(a–bw), where "w" indicated "weak". McLeod's family was not informative as to the genetic background of this condition. In 1968, another case of depressed Kell antigens was found in a Dutch boy who was five years old in 1965 when the studies were done (van der Hart *et al.*, 1968). He had the McLeod phenotype, and his serum contained an antibody which reacted with every kind of cell tested. It did not react with his cells, though, and it did not react with McLeod's cells. This serum, "Claas", is now said to contain anti-KL. Anti-KL reacts with K^o cells, and may be a mixture of antibodies. By selective absorption and elution, the activity reacting with K^o cells can be separated from that which reacts with cells of the common Kell type. Marsh *et al.* (1975a) have shown the identical thing, and the antibody activity which is directed at the antigen present in large amounts on K^o cells was called anti-Kx. Marsh believes that the substance being detected by anti-Kx may be the precursor substance for all the Kell antigens. This point is returned to in section 23.1.4.

23.1.3 Numerical notation and nomenclature for the Kell system

In 1961, Allen and Rosenfield proposed a numerical notation for Kell antigens and antibodies. They thought that such a system should be put into practice at the time, since Kell was starting to reveal some of its complexities, before an unsystematic nomenclature, or several systems, became entrenched and it became difficult to make changes with general agreement, as had happened with Rhesus. At the time, there were five Kell antigens; there are now about 18. The numerical designations, along with their common name equivalents, are indicated in Table 23.1. Most of the antigens have been discussed in section 23.1. Those which have not: Ul^a was defined by an antibody found in a transfused Helsinki man in 1967. It is inherited as a dominant character, and occurs in about 2.6% of Helsinki donors, but is apparently quite rare in other populations. The exact relationship of Ul^a to the other genes in the complex is not completely clear. Wk^a is an antigen of low frequency controlled by a gene which has been shown to be allelic to a gene controlling the high frequency antigen Côté. Another high frequency antigen of the Kell system, K18, has been described by Barrasso *et al.* in 1975, this work being cited in Marsh *et al.* (1975a). In 1979, Sabo *et al.* reported on an antiserum to another Kell specificity, anti-K19. The antibody defined a high frequency antigen. Yamaguchi *et al.* (1979) found four Japanese sisters who were Kp(a–b–) but who had normal Kell antigens otherwise. The most likely explanation was a third allele, Kp^c. In 1946, Callender and Race had found three previously unknown antibodies in the serum of a multiply transfused woman. One was anti-C^w (section 22.5.2), a second was anti-Lu^a (section 24.2.1), and the third was called anti-Levay. The Levay antigen was named after the donor whose cells had elicited the antibody. Gavin *et al.* (1979) have now demonstrated that Levay belongs to Kell and is identical to the Kp^c seen in Japan by Yamaguchi *et al.* (1979).

23.1.4 Genetics of the Kell system

At present, there are four allelic loci belonging to Kell: Kk, $Kp^aKp^bKp^c$, Js^aJs^b, and K11 K17 (Wk^a Côté). Some antigens are on record whose association with the Kell system is not in doubt, but whose relationship to the other allelic loci is not clear. Race and Sanger (1975) refer to these latter as "para-Kell" antigens. Any theory of the biochemical genetics of the system must accommodate all the observations, and a fully acceptable understanding of Kell has not been attained. Interestingly, some of the clues about the biochemical genetics of the system have been gathered from studies of the very rare phenotypes.

In 1971, Giblett *et al.* noted that several boys from different places suffering from a rare, inherited disease called chronic granulomatous disease (CGD) all showed Kell system irregularities, having either K^o or the McLeod phenotypes. The occurrence of two very rare conditions in a number of patients is most improbable if the conditions are independent and simultaneous occurrence of the conditions is due to chance alone. There were also 13 cases of CGD people with no abnormality in Kell. CGD is an inherited defect of neutrophil metabolism, in which phagocytic activity is normal, but in which post-phagocytic bacterial destruction is impaired (see McKusick, 1975; catalogue number 30640). There is a body of evidence that CGD is an X-linked characteristic, but apparently the issue is not completely settled in every mind, as can be seen in reading the letters to the Editor of *The Lancet* (Windhorst, 1969). Marsh and his collaborators have taken up studies on the relationship between CGD and Kell antigens. As noted above, van der Hart and her colleagues had indicated that anti-KL serum appeared to have two different specificities which could be separated by selective absorption and elution procedures. Marsh *et al.* (1975a) confirmed these findings. Absorption of anti-KL (anti-K9) with cells of the common Kell phenotype, K−k+Kp(a−b+)Js(a−b+), removes the

Table 23.1 Kell System Numerical Nomenclature

Number	Common Name Equivalent	Number	Common Name Equivalent
K1	K	K10	Ul^a
K2	k	K11	Côté
K3	Kp^a	K12	Bøc
K4	Kp^b	K13	Sgro
K5	Ku	K14	San
K6	Js^a	K15	Kx
K7	Js^b	K16	—
K8	K^w	K17	Wk^a
K9	KL	K18	—

antibody directed at these, but leaves behind an anti-K° activity. Overabsorption with common phenotype cells removes all activity. Conversely, absorption of anti-KL with K° cells removes "anti-K°", and leaves behind the antibody directed against the common type. The absorbed "anti-K°" can be eluted, and this activity, directed against the antigen present in large amounts on K° cells, is called anti-Kx (anti-K15). Marsh (1975a) and Marsh *et al.* (1975a) have shown that leucocytes, neutrophils in particular, have the Kell antigens, and that normal neutrophils absorb anti-Kx. In studying a number of CGD patients with Kell defects (K° or McLeod phenotypes), they found that the neutrophils failed to absorb anti-Kx. It is postulated, therefore, that Kx is required for the expression of the Kell antigens on white cells or red cells. Marsh *et al.* (1975b) have tested the leucocytes of mothers of CGD patients and found that the Kx activity is intermediate between normal and absent. Further, these maternal leucocytes show mosaicism, i.e., there is a population of Kx deficient cells and a population of normal cells in equal mixture, just as would be predicted by the Lyon Hypothesis (section 1.2.4.4) if the gene governing the synthesis of Kx were X-linked. Marsh *et al.* believe, therefore, that the synthesis of Kx is under the control of an X-linked gene called X^1k. A genetic model for the Kell system is proposed in which the X^1k gene acts first, giving Kx in red cells and white cells. Kx is then acted upon by the products of the Kell locus genes (*K, k, Kp,* Ul^a, K^{17}, K^o, etc.) to give the Kell antigens, according to the individual genotype (Marsh *et al.*, 1976). In this view, the McLeod phenotype is due to a variant gene at the X^1k locus. Several variant alleles are postulated at this locus to explain the variations seen in Kell irregularities in red cells and/or white cells in people with and without CGD, and these are denoted X^2k, X^3k, etc. While the argument is put forth on the basis of the X^1k gene being X-linked because of the present evidence, the model does not stand or fall on the location of X^1k. The essence of the model is that Kx is the product of an allele not associated with the Kell locus, and that it serves as precursor substance for Kell locus gene products. If the X^1k locus were autosomal, and its product modified in some way by an X-linked gene product, or sex-modified in some other way, the data would still fit.

23.1.5 Kell antibodies

Anti-K is relatively common, while anti-k is much less so, because the KK genotype is relatively rare, and it is these people who can make anti-k. Anti-Kp^a may be relatively common, but Kp(a+) cells must be used to find it. Anti-Kp^b is comparatively rare because Kp^a homozygotes are comparatively rare. The rest of the Kell antibodies are fairly rare.

23.1.6 Medicolegal applications of Kell

The inheritance of the established allelic pairs of genes in the Kell system is well established, and the system can be used in disputed affiliation cases. Because of the distribution of phenotypes, however, few exclusions of nonfathers are to be expected on the basis of Kell alone. The probability

of excluding a true nonfather with anti-K and anti-k is about 3% for Whites and about 0.5% for Blacks (Chakraborty *et al.*, 1974; AABB, 1978). The probability for Whites, using anti-Kp^a and anti-Kp^b, is about 1%, while that for Blacks, using anti-Js^a and anti-Js^b, is about 6%. In the case of Kell, as with the other blood group systems, the rare peculiarities must be kept in mind in using the system for paternity testing.

There is little in the literature on Kell grouping in bloodstains. There is no doubt that K can be determined in bloodstains by inhibition or by elution techniques. Perhaps because anti-k is relatively difficult to obtain, and in part because the determination of k is not especially informative in most cases, no reports on the determination of k in bloodstains were found. If one were to do such determinations at all, they would probably be done to show that k is active when K is negative, or occasionally on K+ stains to determine zygosity.

At around the same time, and independently, Jones and Diamond (1955) in this country and Ducos in France (Planques and Ducos, 1957; and see Ducos, 1958) reported determination of K in bloodstains by inhibition techniques. Jones and Diamond (1955) carried out the determination on exhibits in a homicide case, and were able to distinguish the victim from the assailant. The amounts of material required for the inhibition tests were relatively large by today's standards. In 1969, Douglas and Stavely showed that K could be determined in bloodstains by an absorption-elution technique, the eluates being tested in an autoanalyzer. Amounts of material as small as 0.5 cm long threads from 27–34 day old bloodstains could be grouped correctly. In 1975, Lincoln and Dodd described a microelution procedure which needed a 2 mm square fragment of bloodstained material for each antigen to be tested, and was applicable to K. Incomplete anti-K was used, with incubation at 37°, washing at 4°, elution for 10 min at 60°, and detection by the AHG method. Stains up to 10 months old could be correctly grouped for K. Selection of appropriate, specific, high titer anti-K was important. Detection of K was successful in bloodstained fragments that gave negative results with the inhibition technique (Lincoln and Dodd, 1975a and 1975b). McDowall *et al.* (1978b) recently compared manual and autoanalyzer techniques for the detection of anti-K eluted from bloodstains. The manual technique gave more reliable results and was preferred.

Denault *et al.* (1978) could detect K in bloodstains by elution-AHG procedure only up to about 2 weeks of aging. And K could be missed in a one week old bloodstain on cotton. Burke and Tumosa (1978) could detect K in a 4 year old bloodstain using the microelution procedure of Lincoln and Dodd (1975b).

23.2 Duffy System

23.2.1 Discovery and development

In 1950, Cutbush *et al.* found an antibody in the serum of a 43 year old hemophiliac, who had received a number of transfusions, which reacted with about 2/3 of randomly selected red cells from English donors. By its reactions, the antibody could be shown to be defining a new antigen. With the patient's permission, the system was named Duffy, and the antigen being detected by his serum, Fy^a. Further studies (Cutbush and Mollison, 1950) showed that 64.9% of 205 English blood samples reacted with anti-Fy^a. Such cells were designated Fy(a+) by analogy to the nomenclature for Lutheran and Lewis (see in section 24.2.1). The antigen was inherited in a straightforward manner, was fully developed in embryos, and no Fy^a could be found in saliva. The predicted hypothetical allele was called Fy^b. Race *et al.* (1951a) and Race and Sanger (1952) carried out family and population investigations on Duffy, using anti-Fy^a, in a number of unselected families, and the data were in agreement with the postulation that Fy(a+) people could be Fy^aFy^a or Fy^aFy^b the Fy^b being hypothetical at the time. Ikin *et al.* (1951) and Blumenthal and Pettenkofer (1952) found in the serum of a Mrs. Hahn in Berlin the anticipated anti-Fy^b, and it gave the reactions to be expected if it were detecting the product of the Fy^b allele. Tests with anti-Fy^a and anti-Fy^b revealed that English blood donors were distributed approximately as 20% Fy(a+b−), 48% Fy (a+b+) and 32% Fy(a−b+).

In 1955, Levine *et al.* found a second example of anti-Fy^b in a Mrs. MF (Levine *et al.*, 1955c). Some of the serum was provided to Sanger *et al.* (1955) who used it, along with anti-Fy^a, to test a series of 125 bloods from Black donors from New York City. To everyone's astonishment, 68% of the cells did not react with *either* serum, i.e., they were Fy(a−b−). The remainder of the sample was 8.8% Fy(a+b−), 1.6% Fy(a+b+) and 27% Fy(a−b+). The Fy(a−b−) reactions could not be understood on the basis of the two alleles Fy^a and Fy^b. A third allelomorphic gene was postulated, and called *Fy*. This allowed Fy(a−b+) to be either Fy^bFy^b or Fy^bFy and Fy(a+b−) to be either Fy^aFy^a or Fy^aFy, genotypically, while Fy(a−b−) represented the *FyFy* genotype. Some support for this view was generated using a particular anti-Fy^a serum which showed a dosage effect, and whose reactions indicated that most of the Fy(a+) cells from the Black donors had a single dose of Fy^a. If *Fy* turned out to be coding for another antigen—which would only be known if and when an anti-Fy was found—it would then be promoted to Fy^c. The anti-Fy would be expected to be found in a Caucasian who had been transfused with blood from Black donors, but would have to be looked for using test cells from Black donors who had the *Fy* gene.

In 1965, Chown *et al.* found that the explanation for the rare Fy(a−b−) phenotype in Caucasians was not the same as it is in Black people (Chown *et al.*, 1965a). A new Duffy locus allele, Fy^x, was found to be the usual reason for the condition in Whites. Fy^x is not qualitatively different from Fy^b, but it is different quantitatively. With carefully selected anti-Fy^b and AHG sera, a small amount of Fy^b can be found in Fy^x people. The gene is quite rare, and unless special testing is done with selected anti-Fy^b, Fy^aFy^x people will routinely be classified as Fy(a+b−), when in fact they are Fy(a+bw). Lewis *et al.* (1972) found a person who was

probably Fy^xFy^x. The gene *Fy* does occur in Caucasians but is extremely rare. Libich *et al.* (1978) described a family of gypsy origin in Czechoslovakia which had 5 Fy(a−b−) members due to homozygosity for the *Fy* allele. The red cells of these people were Fy(−3) (see in section 23.2.2) as well. The *Fy* gene may reach appreciable frequencies in the gypsies, the authors said.

23.2.2 Extension of the Duffy system

In 1971, Albrey *et al.* found an antibody in an Fy(a−b−) Australian White woman which agglutinated the cells of every kind of blood except Fy(a−b−). Her cells did not give the Fy^x reactions. The age of numerical notation having arrived (see sections 22.6.2 and 23.1.3), the antibody was called anti-Fy3. It reacted with Fy(a+b−), Fy(a−b+) and Fy(a+b+) cells from White or Black donors. But for its behavior with papain treated cells, anti-Fy3 could have been regarded as anti-Fy^aFy^b. Treatment of Fy(a+) or Fy(b+) cells with papain abolishes their reactivity with anti-Fy^a and anti-Fy^b, whereas it enhanced their reactivity with anti-Fy3. The antibody may be detecting some precursor substance for Fy^a and Fy^b. Anti-Fy4 was found in a 12 year old Black sickle cell anemia patient who was Fy(a+b+) by Behzad *et al.* (1973). The antibody was weak, but reacted with all Fy(a−b−) cells, and with most Fy(a+b−) and Fy(a−b+) cells from Black donors. It appeared in general to be the long sought for anti-Fy^c of Sanger *et al.* (1955). Its reactions, however, were enhanced by papain treatment of cells, as are those of anti-Fy3. Anti-Fy5 was found in a Black Fy(a−b−) donor by Colledge *et al.* (1973). The antibody reacted very much like anti-Fy3, except that it reacted with the cells of the person in whom anti-Fy3 had first been found. It did not react with Fy(a−b−) cells from Black donors, and curiously, it did not react with Rh_{null} phenotype cells which had ordinary Duffy antigens. The last observation led to speculation that anti-Fy5 might be an antibody to some combination of Rh and Duffy gene products.

23.2.3 Other aspects of the Duffy system

The great majority of anti-Fy^a and anti-Fy^b are incomplete antibodies, best detected by the AHG technique and having a 37° temperature optimum. Both anti-Fy^a and anti-Fy^b are inactive with enzyme-treated cells. Ficin, papain and bromelin markedly reduce or abolish the reactivity of the Duffy receptors. This characteristic can be exploited in identifying Duffy and non-Duffy antibodies in a human serum which contains a mixture of antibodies of differing blood group specificity. Anti-Fy^a is quite a bit more common than anti-Fy^b. Fy^a is, by one measure, about 40 times less immunogenic than the Kell antigen, and Fy^b is probably still less so, as only about 1 example of anti-Fy^b is found for every 20 examples of anti-Fy^a (Marsh, 1975b). Duffy antigens are well developed at birth, and can be found in 6–7 week old fetuses (Toivanen and Hirvonen, 1969b and 1973).

There have recently been suggestions that Fy^a or Fy^b receptors on red cells may also be receptors for one type of organism that causes malaria. *FyFy* erythrocytes could be shown to be resistant to infection by one species of *Plasmodium*, and it is possible that malaria infection may be an important selective force in the maintenance of the *Fy* gene in parts of West Africa (Miller *et al.*, 1975; Gelpi and King, 1976).

The Duffy system has recently been extensively and well reviewed by Marsh (1975b).

23.2.4 Medicolegal applications

The Duffy system is well established as a genetic marker in parentage investigations. Using anti-Fy^a and anti-Fy^b, the probability of excluding a falsely accused father is about 18% for Whites and about 4% in Blacks. Great caution must be used in the interpretation of results involving Black people because of the very high frequency of the silent allele *Fy*. Ordinarily, Fy^aFy and Fy^aFy^a people will be indistinguishable, as will Fy^bFy and Fy^bFy^b people. Morel, in AABB (1978) has noted that exclusions of the second order have no meaning when Black people are involved in the case. Even in non-Black populations, *Fy* can occur rarely, suggesting that caution should be exercised in general with second order exclusions in the Duffy system (Libich *et al.*, 1978).

In 1957, Ruffié and Ducos reported successful determination of Fy^a in dessicated blood by an inhibition technique. The anti-Fy^a they had available was incomplete, and its reactions best detected by AHG technique. Following absorption of 0.3 mℓ by 40 mg dried blood specimen for 36 hrs at 37°, the absorbed serum was titrated with Fy(a+) cells using AHG to bring about agglutination. Anti-Fy^a was used at a titer of about 1:8 and Fy^a-containing dried blood up to six days old gave an inhibition of about three dilutions. Planques and Ducos (1957) mentioned Fy^a in the list of antigens then being determined in dried blood. Ducos (1958) reported that the results of his survey of a number of laboratories (see in Section 22.8) indicated that only one laboratory was carrying out determination of Fy^a in dried blood.

In 1975, Lincoln and Dodd (1975a and 1975b) reported successful determination of Fy^a and Fy^b in small amounts of bloodstained material using an absorption-elution technique. The antisera were incomplete, and the reactions were detected using AHG. No difficulty was experienced in the determination of both antigens in relatively fresh bloodstains. The technique was very similar to that employed by these investigators for s (Section 21.9.5) and for Kell (Section 23.1.6). Denault *et al.* (1978) could detect Fy^a in 1–2 week old bloodstains by elution-AHG technique, whether the stains were on cotton or on wool. Burke and Tumosa (1978) detected Fy^a in a 4 year old bloodstain, although they said that the eluate gave a weak reaction. They used the microelution procedure of Lincoln and Dodd (1975b).

23.3 Kidd System

23.3.1 Discovery and development

In 1951, Allen *et al.* found an antibody in the serum of a recently delivered mother, Mrs. Kidd, whose baby was suffering from hemolytic disease of the newborn. She had had

five previous unremarkable pregnancies, and no known transfusions. Her serum contained anti-K in addition to the new antibody. The new antibody was active in saline at 37°, but gave a higher titer by AHG technique. The saline reacting antibodies disappeared upon storage of the antiserum. The antigen being detected by this antibody was called Jk^a after Mrs. Kidd's son. People having the antigen were denoted Jk(a+), those lacking it, Jk(a−). Race *et al.* (1951b) found that 76–77% of people in Boston and London were Jk(a+) and studies on 51 families indicated that the antigen was governed by a Mendelian dominant gene which could be present in the homozygous or heterozygous condition. In the same year, another example of anti-Jk^a was found in the serum of a Mrs. McGimpsey. She had been recently delivered of her first child, who was healthy, and she had no history of transfusions (Hunter *et al.*, 1951). The third example was found in a Scottish mother, whose serum also contained anti-E and a cold reacting anti-P (Milne *et al.*, 1953). Rosenfield *et al.* (1953a) reported three additional examples of anti-Jk^a, and van der Hart and van Loghem (1953) found another one. Many examples have since been found.

In 1953, Plaut *et al.* found the expected anti-Jk^b in the serum of a mother of two children, who had had two miscarriages, and previous transfusions. None of her children had been affected by hemolytic disease. Her serum contained an anti-Fy^a in addition to the anti-Jk^b. In 1951, Unger had found that the sensitivity of the AHG test could be substantially improved by carrying out the test with trypsin-treated red cells. This study was carried out primarily on different examples of incomplete anti-Rh_0, and the technique was recommended for the detection of antibodies present in very low titer in serum. Plaut *et al.* (1953) found that the technique was very helpful in the detection of the new anti-Jk^b. A second example of anti-Jk^b was identified by Sanger *et al.* (1953) in the serum of a volunteer who had been immunized to produce anti-C, anti-D and anti-E. In addition, he produced anti-s and anti-Jk^b. Van Loghem *et al.* (1953) reported a third example of anti-Jk^b in a serum which also contained anti-C and anti-M. The titer was improved by carrying out the AHG test with trypsin or papain treated cells.

A large number of family studies have established beyond much doubt that the inheritance of Kidd can be explained most easily on the basis of two alleles Jk^a and Jk^b. Race and Sanger (1968), in the 5th edition of *Blood Groups in Man*, analyzed 1197 matings with 3116 children, and the data fit very well. They noted that their data show an excess of Jk^a Jk^b heterozygotes, and they said that there may be something more to the anti-Jk^b reagent than is presently obvious. Chown *et al.* (1965b) did not observe any excess of heterozygotes in their data. Kidd antigens are well developed at birth. Jk^a has been reported in fetuses 10–11 weeks old and Jk^b in 6–7 week old ones (Toivanen and Hirvonen, 1973).

23.3.2 The Jk(a−b−) phenotype

In 1959, a Phillipine woman of some Spanish and Chinese ancestry was found to have an antibody in her serum that reacted with all cells tested except her own. She had given birth to two children, neither of whom showed any sign of hemolytic disease. Investigation showed that her cells were Jk(a−b−), and that her serum contained an antibody which was a kind of anti-Jk^aJk^b, with some separable anti-Jk^b (Pinkerton *et al.*, 1959). The condition could be accounted for on the basis of a third allele, *Jk*, or of a modifying gene. In 1965, Day *et al.* reported the Jk(a−b−) phenotype in a Chinese mother of six, and her serum contained the cross-reacting anti-Jk^aJk^b with a separable anti-Jk^a. Day *et al.* mentioned that seven examples of Jk(a−b−) had been described in addition to the original case and the one being reported in their paper. Some further examples of the phenotype were found in a family in Hawaii by Yokoyama *et al.* (1967), and another family with three Jk(a−b−) members was studied by Arcara *et al.* (1969). Most Jk(a−b−) people have anti-Jk^aJk^b in their sera, sometimes with separable anti-Jk^a or anti-Jk^b, but not always. The Jk(a−b−) sister of the propositus in the family studied by Arcara *et al.* (1969) had no Kidd antibodies, although she had been pregnant seven times. All the Jk(a−b−) people described thus far have been of Asian ancestry. The phenotype can occur in Europeans, however. Crawford *et al.* (1961) described a family in which a Jk(a−b+) father and Jk(a+b−) mother had three Jk(a+b−) children. This could be explained most easily by the presence of a segregating *Jk* allele. The case was complicated by the fact that the rare Lutheran phenotype Lu(a−b−) was segregating in this family as well (section 24.2.2). Race and Sanger (1975) mentioned that a few other European families had been tested in which *Jk* appeared to be present. It is not clear whether the genetic background of the European and Asian kind of *Jk* are the same or not.

23.3.3 Kidd antibodies

There is a tendency for Kidd antibodies to become inactive both *in vivo* and *in vitro*. Sometimes, the antisera work both in saline and by AHG technique, and only the saline reactivity decreases. It is helpful in many cases to carry out the AHG test with trypsin treated cells (Unger, 1951) or papain treated red cells. Complement can be involved in the anti-Jk^a reaction. Stratton (1956) found that refrigerated, and even frozen, anti-Jk^a becomes less active with time, but that there is some restoration of activity if fresh serum is added. Restoration of activity by addition of fresh serum is indeed the result of adding complement. Freeze dried sera appeared to be stable. Polley and Mollison (1961) examined the role of complement in the reactions of Kidd and several other antisera. Anti-Jk^a can be the cause of hemolytic disease of the newborn on rare occasions (see, for example, Greenwalt *et al.*, 1956), and so can anti-Jk^b (Kornstad and Halvorsen, 1958).

The anti-Jk^aJk^b antibodies made by Jk(a−b−) people appears to be more than a mixture of anti-Jk^a and anti-Jk^b, or an antibody cross-reacting with both antigens. Marsh *et al.* (1974) have found that neutrophils will absorb anti-Jk^aJk^b (anti-Jk3) made by Jk(a−b−) people, but that they do not absorb either anti-Jk^a or anti-Jk^b. These data suggest

that "Jk^aJk^b" is a distinct and separate antigen, which should perhaps be called Jk3, and Marsh *et al.* (1974) did not find it on lymphocytes nor on platelets.

23.3.4 Medicolegal applications

Anti-Jk^a and anti-Jk^b can be used in disputed affiliation cases, although the antisera are relatively scarce, and the matings which can show exclusions are relatively infrequent. The probabilities of exclusion for a true nonfather are about 18% for Whites, and about 15% for Blacks (Chakraborty *et al.*, 1974; AABB, 1978). Anti-Jk^b is very scarce, however, and may not be available. The probability of exclusion is obviously less if only anti-Jk^a is used. In Whites, for example, the value is about 3%. The possible occurrence of the rare, silent allele, *Jk*, should not be forgotten in interpreting exclusions with Kidd, even when both antisera can be used (Morel, in AABB, 1978).

There does not appear to have been any effort to determine Kidd antigens in bloodstains until the work of Lincoln and Dodd (1975a and 1975b). They showed that Jk^a could be determined in experimental bloodstains a few days old using an AHG reacting anti-Jk^a in a micro-elution procedure. There does not appear to be any reason why Jk^b could not be determined as well, except that, as these authors noted, suitable examples of anti-Jk^b are quite rare and difficult to obtain. Denault *et al.* (1978) detected Jk^a in week-old bloodstains on cotton or on denim by an elution-AHG procedure.

23.4 Distribution of Kell, Duffy and Kidd Phenotypes in U.S. Populations

The distribution of Kell phenotypes in some U.S. populations is given in Table 23.2 and that for Duffy and Kidd phenotypes in Table 23.3.

Table 23.2 Kell Phenotypes in U.S. Populations

Population	Total	Frequency — Number (Percent) KK	Kk	kk	Kp(a+b−)	Kp(a−b+)	Kp(a+b+)	Js(a+)	Js(b+)	Reference
CAUCASIAN										
Boston, MA	210 ★	21 (10.0)		189 (90.0)						Allen et al., 1951
Minnesota	300 ★	(11)		(89)						Matson et al., 1954
Boston, MA	1,925	(0.3)	(9.5)	(90.2)	(2.0)					Allen and Lewis, 1957
Seattle, WA	240 ○								240	Giblett, 1958
Boston, MA	2,709 ✪									Allen et al., 1958a
Seattle, WA	500 ○								500	Giblett and Chase, 1959
Boston, MA	6,830					6,830 (100)				Walker et al., 1961
Southeastern GA	333 ★	28 (8.4)		305 (91.6)						Cooper et al., 1963
Eastern San Francisco Bay Area, CA										
Mothers	4,928 ★	(8.26)		(91.74)						Reed, 1967
Babies		(8.56)		(91.44)						
NC and VA										
Mongoloids	585 ★	44 (7.5)		541 (92.5)						Goodman and Thomas, 1968
Controls	585 ★	36 (6.2)		549 (93.8)						
Donors	253 ★	19 (7.51)								
San Francisco Bay Area, CA										
All Caucasians	8,962 ★	768 (8.6)		8,194 (91.4)						Reed, 1968
Caucasians of "Western European" origin	5,056	452 (8.9)		4,604 (91.1)						
New York, NY	500 ★	42 (8.4)		458 (91.6)						Wiener, 1969
South Central WV	1,412	(0.4)	(8.1)	(91.4)						Juberg, 1970
Tecumseh, MI	8,443 ☆	6 (.07)	594 (7.04)	7,842 (92.88)		8,312 (98.45)	130 (1.54)			Schreffler et al., 1971

Table 23.2 Cont'd

Population	Total	Frequency — Number (Percent) KK	Kk	kk	Kp(a+b−)	Kp(a−b+)	Kp(a+b+)	Js(a+)	Js(b+)	Reference
NEGRO										Miller et al., 1951
New York, NY	200 ★		(3.5)	(96.5)						Race and Sanger, 1955 cited by Mourant et al., 1976
New York, NY	126 ★		2 (1.6)	124 (98.4)						
Seattle, WA	172 ○							33 (19.2)	139 (80.8)	Giblett, 1958
Seattle, WA	440 ○							86 (19.5)	354 (80.5)	Giblett and Chase, 1959
Southeastern GA	303 ★		3 (1.0)	300 (99.0)						Cooper et al., 1963
Philadelphia, PA								18 (0.9)	2,048 (99.1)	Lovett and Crawford, 1967
Eastern San Francisco Bay Area, CA										
Mothers	1,453 ★		(1.58)	(98.42)						Reed, 1967
Babies			(2.13)	(97.87)						
San Francisco Bay Area, CA	3,146 ★		52 (1.7)	3,094 (98.3)						Reed, 1968
New York, NY	500 ★		5 (1.0)	495 (99.0)						Wiener, 1969
South Central WV	133	(0)	(1.0)	(99.0)						Juberg, 1970
CHINESE										
New York, NY	103 ★		(0)	(100)						Miller et al., 1951
New York, NY	160 ★		0	160						Sussman, 1956
New York, NY	946 ★		2 (0.11)	944 (99.89)						Wiener, 1974
HISPANIC										
San Francisco, CA										Reed, 1968
"Mexican"	335 ★		14 (4.2)	321 (95.8)						

★ Tests done with anti-K

☆ Of 8,312 Kp(a−b+), 7,717 were kk, 589 Kk and 6 KK; Of 130 Kp(a+b+), 125 were kk and 5 were Kk; 1 person was kk Kp(a+b+)

✪ Approximate frequencies were: kKp^a 0.011, KKp^b 0.048, kKp^b 0.936

○ Tests done with anti-Js^a

Table 23.3 Duffy and Kidd Phenotypes in U.S. Populations

Population	Total	Fy(a+b−)	Fy(a+b+)	Fy(a−b+)	Fy(a−b−)	Jk(a+b−)	Jk(a+b+)	Jk(a−b+)	Reference
CAUCASIAN									
Boston, MA	189 ★☆					146 (77.2)		43 (22.8)	Allen et al., 1951
New York, NY	726 ☆					557 (76.72)		169 (23.28)	Rosenfield et al., 1953a
Minnesota	100 ✪		68 (68)		32 (32)				Matson et al., 1954
Southeastern GA	333 ✪		221 (66.4)		112 (33.6)	92 (27.6)	173 (52.0)	68 (20.4)	Cooper et al., 1963
Eastern San Francisco Bay Area, CA									
Mothers	4,928 ✪		(66.52)		(33.48)				Reed, 1967
Babies			(65.87)		(34.13)				
San Francisco Bay Area, CA									
All Caucasians	8,962 ✪		6,007 (67.0)		2,955 (33.0)				Reed, 1968
"Western European" Caucasians	5,056 ✪		3,405 (67.4)		1,651 (32.6)				
South Central WV	1,016 ☆	(15.4)	(46.2)	(38.3)			(77.4)		Juberg, 1970
Tecumseh, MI	8,946 ✪		6,009 (67.17)		2,937 (32.83)				Schreffler et al., 1971
	6,546 for Jk^a and 1,892 for Jk^b								
NEGRO									
New York, NY	305 ☆					283 (92.79)		22 (7.21)	Rosenfield et al., 1953a
New York, NY	125	11 (8.8)	2 (1.6)	27 (21.6)	85 (68)				Sanger et al., 1955
New York, NY	179	19 (10.6)	9 (5.0)	43 (24)	108 (60.4)				Race and Sanger, 1968
New York, NY	67					38 (57)	23 (34)	6 (9)	Race and Sanger, 1968
Southeastern GA	304 ✪		27 (8.9)		277 (91.1)	166 (54.8)	118 (38.9)	19 (6.3)	Cooper et al., 1963

Table 23.3 Cont'd.

Population	Total	Fy(a+b−)	Fy(a+b+)	Fy(a−b+)	Fy(a−b−)	Jk(a+b−)	Jk(a+b+)	Jk(a−b+)	Reference
San Francisco Bay Area,CA									
Mothers	1,453		(17.41)		(82.59)				Reed, 1967
Babies			(17.14)		(82.86)				
San Francisco Bay Area, CA	3,146 ✪		564 (17.9)		2,582 (82.1)				Reed, 1968
Detroit, MI	404 ✪		85 (21.04)		319 (78.96)				Gershowitz, 1968, cited by Reed, 1968
South Central WV	103	(17)	(5)	(78)			(86)	(14)	Juberg, 1970
CHINESE									
New York, NY	103 ☆					54 (52.43)		49 (47.57)	Rosenfield et al., 1953a
HISPANIC									
San Francisco, CA									
"Mexicans"	335 ✪		268 (80)		67 (20)				Reed, 1968

★ All subjects were K−

☆ Only anti-Jk^a used in tests

✪ Only anti-Fy^a used in tests

● 4,974 (75.99) were Jk(a+) and 1,572 (24.01) were Jk(a−); 1,398 were Jk(b+) and 494 (26.11) were Jk(b−)

SECTION 24. THE P AND LUTHERAN SYSTEMS

Although the P and Lutheran systems have become somewhat more complicated than they first appeared, they will be described fairly briefly here. They have not yet been applied to any appreciable extent to the analysis of antigens in dried blood.

24.1 P System

24.1.1 Discovery and development

Landsteiner and Levine (1927b) first described the antigen which they called P, based on reactions of an immune rabbit serum they had prepared against human red cells. These were the same experiments that resulted in the discovery of the M and N antigens, as noted in Section 21.1. Anti-P divided bloods into two categories, P+ and P−. The characteristic appeared to be inherited as a Mendelian dominant, but because some bloods reacted weakly with the original serum, it was difficult to establish the frequency of P with certainty. It was clear that the frequencies were not the same in Black and White people (Landsteiner and Levine, 1929). An anti-P was soon found in a human serum (Landsteiner and Levine, 1930), and normal, non-immune sera from some horses, cattle, pigs and rabbits contained it as well. Landsteiner and Levine (1931) suggested the symbols P_i, P_n and P_a to denote the P being detected on human cells by the rabbit immune serum, the naturally occurring human agglutinin, and the naturally occurring animal serum agglutinins, respectively. These distinctions have not persisted, because the conception of P is somewhat different today than it was at the time. The antigen that Landsteiner and Levine first described is now called P_1, and its inheritance as a Mendelian dominant characteristic has been widely confirmed.

In the first communication, Landsteiner and Levine (1927b) mentioned that there appeared to be two kinds of reactions of cells with anti-P, strong and weak. In 1929, Landsteiner and Levine said that the reactions of different examples of red cells with anti-P could be divided into four categories, based upon the strength of reaction. They regarded the division of cells into categories on this basis, however, as arbitrary. The question of the strength of P_1 reactions has been investigated by a number of workers. Henningsen (1949a), who used a titration technique to study P_1 in a large number of people, did not think that the antigen could be divided up into distinct categories, based upon the strength of its reaction. The results showed a continuous normal distribution. Grosjean (1952), however, interpreted the results of his tests on 1,000 people to mean that the differences were discontinuous, and controlled by several different alleles. Cazal and Mathieu (1950) carried out quantitative studies as well, but the results were somewhat complicated by the problem of the Q blood factor (see below). Henningsen (1949b) carried out extensive studies on the inheritance of P_1 and on the strength of the receptor in the cells of a number of people. The data indicated to him that antigen strength might well be inherited. Fisher (1953) did a statistical genetic analysis of Henningsen's (1949b) data, and concluded that homozygosity could provide a solid basis for the variation in reaction strength, and that it would be premature to invoke other genetic origins as a foundation for the differences. The inheritance of P was studied in a considerable body of family material from Germany by Dahr (1940 and 1942a) and from Scandinavia by Henningsen (1949a and 1949b).

24.1.2 Extension of the P system

In 1951, Levine *et al.* found an antibody in the serum of a woman, whose blood was being cross matched in preparation for some surgery, which detected a very high incidence antigen (Levine *et al.*, 1951a). Her name was Mrs. Jay, and the Jay antigen being detected by her serum was denoted Tj^a. The gene responsible for it was called Tj^a, and the antibody, anti-Tj^a. Mrs. Jay's sister was also Tj(a−), and it was supposed that Tj^a had an allele, Tj^b, for which these sibs were homozygous. In 1952, Zontendyk and Levine found a second example of anti-Tj^a in a South African woman 37 years old. She had been pregnant four times, but never transfused. The South African antibody was an agglutinin when found, while the original Jay antibody was hemolytic when fresh, but behaved as an agglutinin when inactivated. In 1952, Hirszfeld and Grabowska found anti-Tj^a in a person called Fran Z., and they called it anti-Z^a at first. In 1954, Levine *et al.* found the antibody in 2 of 3 sibs in another family (Levine *et al.*, 1954a). In the same year, anti-Tj^a was identified in the serum of a 22 year old Australian woman with no history of pregnancy or transfusion. Her 19 year old sister had the antibody as well (Walsh and Kooptzoff, 1954). The rare (Tj(a−) people were assumed to be Tj^bTj^b, and their parents Tj^aTj^b. By 1955, 14 Tj(a−) people had been identified, and Sanger (1955) noticed that three unrelated examples of Tj(a−) cells were P−, an improbable result, since about 1 in 5 people in Europe is P−. A look at the literature revealed that three other cases of Tj(a−) cells had been found to be P−, and Sanger said that the probability of six unrelated Tj(a−) people being P− by chance alone was too great to be considered reasonable. It was then found that absorption of anti-Tj^a with P− cells left an anti-P antibody in serum. Absorption of anti-Tj^a with P+ cells removed all the antibody. These observations were readily understandable, she said, if Jay were part of P, and if the P reactions were thought of by analogy to the A_1A_2O reactions. If P_1 designated "old" P+ and P_2 designated "old" P−, then anti-Tj^a could be regarded as an anti-PP_1, like

anti-AA_1. Just as absorption of anti-AA_1 by A_2 cells leaves anti-A_1 behind, absorption of anti-PP_1 with P_2 cells left anti-P_1 behind, while absorption of anti-PP_1 with P_1 cells removed all activity, just as absorption of anti-AA_1 with A_1 cells does. The designation "p" was applied to the rare cells which did not react with anti-PP_1, i.e., to Tj(a−) cells. At the least, the analogy is useful for remembering the relationships within P. Salmon *et al.* (1979) have reported on the segregation of the *pp* genotype in two generations of a highly inbred Tunisian family.

In 1959, an antibody was found in Mrs. Mys. in Minnesota by Matson *et al.* which was first thought to be anti-PP_1, because it did not agglutinate p cells. She would have thus been *pp*. But her cells were agglutinated by anti-PP_1 and by anti-P_1, so the simple explanation was not workable. It had to be supposed that the cells of this proposita (and her sister) possessed a new antigen, which was called P^k, and that antisera previously regarded as being anti-PP_1 and anti-P_1 were also anti-P^k. A second family having P^k was described by Kortekangas *et al.* (1959). P^k was envisaged as being controlled by an allele P^k, but this allele was not straightforwardly inherited. It is expressed only when homozygous, or in combination with *p*, but not in the presence of P_1 or P_2. P^k people have the P^k antigen, but lack P. Most have P_1 (phenotype $P_1{}^k$) but some do not (phenotype $P_2{}^k$). Anti-P regularly occurs in the serum of P^k subjects. Extensive studies on P^k were carried out by Kortekangas *et al.* (1965). The relationships of P_1, P_2, P^k and p are given in Table 24.1, as adapted from Race and Sanger (1975). Anti-P^k can be left behind in the sera of some *pp* people after absorption with P_1 cells. Kato *et al.* (1978) have recently studied the anti-P and anti-P^k antibodies in p sera, and the anti-P in P^k sera, by complement fixation reactions using the purified antigen-active glycosphingolipids (see in section 21.1.5). Naiki and Kato (1979) have shown that P^k can be detected on P_2 cells if a p serum absorbed with P glycolipid to remove anti-P_1 is used. This fact had not been previously appreciated because P_1 cells (which contain trihexosyl ceramide, now known to be P^k) had always been used to absorb the P sera. Anti-P^k with different affinities for trihexosyl ceramide and its derivatives could also be prepared by partial absorption of p sera with P_1 cells, and elution.

Wiener (1968) did not agree with the explanation of P^k on the basis of a separate allele P^k. He said that the failure of anti-PP_1P^k serum, after absorption with P_1 cells, to agglutinate P_1 cells, and retention of its ability to agglutinate P^k cells, could be explained in other ways. His explanation was based on a different conception of the P system, which was proposed in the paper. In his view, four allelic genes and two antisera were involved. The genes *p*, *p'*, *P* and P_1 give rise to the corresponding agglutinogens p, p', P and P_1, and these have associated with them the blood factors **p'** and **P** as follows: p' has **p**; P has **P**; P_1 has **p'** and **P**; and p has none. There are two antisera, anti-**p'** and anti-**P**. The relationships are summarized in Table 24.2. In this scheme, P^k corresponds to the phenotype p', and anti-PP_1P^k to anti-

Table 24.1 P System Relationships

Phenotype	Anti-P_1	Anti-PP_1P^k	Anti-P	Anti-P^k
P_1	+	+	+	−
P_2	−	+	+	−
p	−	−	−★	−
P_1^k	+	+	−	+
P_2^k	−	+	−	+

Anti-P_1 is found in P_2 persons and some animal sera. Anti-PP_1P^k (anti-Tj^a) is found in pp persons. Anti-P is found in P^k people. Anti-P^k is prepared by absorbing certain anti-PP_1P^k with P_1 cells.

★ Some examples of anti-P react weakly.

Table 24.2 P System Relationships According to Wiener (1968)

Phenotype	Reaction With Anti-P	Reaction With Anti-p′	Genotypes	Isoantibodies that may be present in serum
p	–	–	*pp*	Anti-P, Anti-p′
p′	–	+	*p′p′, p′p*	Anti-P
P	+	–	*pp, Pp*	Anti-p′
P_1	+	+	*P¹P¹,P¹P,P¹p,P¹p′,Pp′*	None

p′ + anti-P. Absorption of this serum with P_1 cells could leave behind anti-p′ which would be reactive. Wiener admitted, however, that the data of Kortekangas *et al.* (1965) did support the notion of an additional specificity, except that he wanted to regard it as being associated with his p′ phenotype, and to call it p^k.

24.1.3 Blood factor Q

In 1935, Imamura and Furuhata found an antibody in the serum of pigs which agglutinated the red cells of some people, but not of others. The factor being detected was called "Q", and was shown to be inherited. The papers are written in Japanese, and references may be found in Prokop and Uhlenbruck (1969) and in Race and Sanger (1975). There was a suspicion that Q might be P, and the Japanese investigators apparently obtained some anti-P from Dr. Landsteiner, but did not find the anti-P and anti-Q reactions to be identical. Cazal and Mathieu (1950) arrived at the same conclusions. Furuhata and Hasebe (1955) reported on population and family studies on the Q factor. In 1955, Henningsen and Jacobsen compared an anti-Q from Japan with a series of anti-P reagents, and said that P and Q were identical. Race and Sanger (1975) and Wiener (1943c) regarded this problem as solved, but Prokop and Uhlenbruck (1969), while admitting that P may very well be Q, were less certain.

21.1.4 Additional notes about the P system

Another complication of the P system came to light in 1965, when Tippett *et al.* found a very peculiar agglutinin in the serum of Mr. Luke P. in Oklahoma City. He had Hodgkins disease, and had died in 1960. The agglutinin gave the following reactions with red cells that were tested: most samples were Luke(+) or Luke (w); a few rare ones were Luke(–), including p and P^k people's cells. Of the remaining P(–) people, the type was commoner in P_2 than in P_1 people, and commoner in A_1 and A_1B than in other ABO types. Other examples of the antibody have been found.

It is known that P_1 is not necessarily developed in fetuses or in children, and this circumstance can, of course, lead to serious errors if children are being tested. Ikin *et al.* (1961) found the antigen to be present in a higher proportion of younger than of older fetuses, and the reactions were stronger, but the numbers were still much lower than those for adults. Heiken (1966) said that the antigen could be undeveloped in children several years old, and he did not think that P_1 grouping in children less than one year old should be considered reliable.

There is a rare inhibitor gene called *In(Lu)* which gives rise to the dominant kind of Lu(a–b–) phenotype of the Lutheran system (Section 24.2.2), but which also inhibits the antigens Au^a (Section 25.5) and P_1 (Crawford *et al.*, 1974). Because of the P_1 inhibition, apparent contradictions to P inheritance can occur in Lu(a–b–) families, $P_2 \times P_2$ matings giving rise to P_1 offspring (Contreras and Tippett, 1974).

24.1.5 Biochemical studies on the P system

The P substances, whose occurrence is not restricted to the red cell membrane, have turned out to have carbohydrates as their immunochemical determinant groups. Considerable progress has been made in understanding the structure of the P substances, although the biochemical

genetic pathways do not as yet appear to have been completely worked out.

In 1957, Cameron and Staveley noticed that two patients being treated for hydatid cyst disease had developed potent anti-P_1 in their sera. The finding led them to search for P_1 or P_1-like substances in the cyst fluid. Hydatid cysts are fluid filled cysts which form in the livers of human beings and of ruminants due to the presence of the larvae of *Echinococcus* tapeworms. It was found that cyst fluid from sheep liver cysts contained an anti-P_1 inhibiting substance, which varied in inhibitive titer, and which was present only in those cysts which contained scolices. The substance was stable to boiling for 10 min. Staveley and Cameron (1958) went on to show that the fluid substance partially inhibited anti-P + P_1 sera. The presence of P_1 or P_1-like substance in these cyst fluids enabled Watkins and Morgan to initiate structural and immunochemical studies on the material (Watkins and Morgan, 1962; Morgan and Watkins, 1962). Partial purification of the hydatid cyst fluid substance gave three glycoprotein fractions. These materials could be precipitated by a number of examples of anti-P_1 reagent, a mixture of glycosidases from *T. fetus* destroyed the activity, and the reaction between P substance and anti-P_1 was inhibited by compounds having terminal α-D-galactosyl residues. If the substances were combined with a conjugated protein from *Shigella shigae*, and injected into rabbits, powerful anti-P_1 precipitin and agglutinin sera could be obtained.

Prokop and Schlesinger (1965a and 1965b) have shown that saline extracts of the bodies of certain worms possess P_1 substance (as well as ABH substances in some cases). P_1 substance or P_1-like substance could be isolated from *Lumbricus terrestris* and from *Ascaris suum*, and the authors speculated that immunization of people who have anti-P_1 in their serum might have resulted from infection by these or similar parasitic worms.

In 1974, Cory *et al.* purified a P_1-active glycoprotein material from sheep hydatid cyst fluid, and found that the terminal carbohydrate structure was D-Gal-α-(1→4)D-Gal-β-(1→4)NAc-D-GlcNH_2. The next steps have been taken primarily by Marcus and his collaborators. Their studies concentrated on purification and structure determination of P-active glycosphingolipids from the red cell membrane. A host of glycosphingolipids can be isolated from the erythrocyte membrane by various solvent extraction procedures, and purification of the extracts on silicic acid columns and thin-layer chromatographic plates commonly follows (see Vance and Sweeley, 1967). Marcus first isolated a P_1-active fraction from lyophilized red cell stroma in 1971, and it contained two glycosphingolipids. In the discussion which follows, it will be helpful to refer to Table 24.3, which gives the specialized terminology for the glycosphingolipids used by the workers in this field, taken from Schwarting *et al.* (1977).

Ando and Yamakawa (1973) and Ando *et al.* (1973) isolated and structurally characterized three glycosphingolipids from erythrocyte stroma, which they called A-30-III, A-60-III and G_{M3}. These had the structures of the paragloboside, sialosylparagloboside, and hematoside shown in Table 24.3, respectively. Siddiqui and Hakomori (1973) likewise isolated and characterized the first two of these compounds. In 1974, Naiki and Marcus identified the P antigen as globoside and the P^k antigen as trihexosyl ceramide. It was suggested that P^k is a precursor of P_1, and that P^k people lack the enzyme required to add the needed NAc-galactosyl residue. P_1-active glycosphingolipid was isolated and characterized by Naiki *et al.* (1975) and found to have the structure shown in Table 24.3. Treatment of the material with α-galactosidase produced galactose and paragloboside. Neither P nor P^k were therefore thought to be precursors of P_1. It will be noted that the terminal trisaccharide structure of P_1 glycosphingolipid is identical to that of the P_1 glycoprotein isolated by Morgan and Watkins and their collaborators (Cory *et al.*, 1974) above. This work was extended and further discussed by Marcus *et al.* (1976). A biosynthetic scheme (Fig. 24.1) could be proposed based upon this work in which lactosyl ceramide is converted to P^k by a transferase controlled by the P^k gene, and P^k in turn converted to P by a transferase under the control of the P_2 gene. P_1 is made on a different pathway from paragloboside by the intervention of an α-galactosyl transferase. If the α-galactosyl transferases converting lactosyl ceramide to P^k on the one hand, and paragloboside to P_1 on the other, are identical, and the product of the P^k gene, then it had to be supposed that P_1 gene makes a product which somehow modifies this enzyme's action in P_1 cells. Otherwise, the fact that P_2 people make P^k but not P_1 could not be understood. In this view, the primary biochemical feature of p cells is the absence of the α-galactosyl transferase which converts lactosyl ceramide to P^k and paragloboside to P_1.

Fellous *et al.* (1974) studied the P, P_1 and P^k antigens in cultured somatic cells, and found that P^k, which is so rare on red cells, was almost universally present in fibroblasts. Its absence from red cells was suggested to be the result of the control of P^k expression in red cells by an independent genetic locus, called F/f. P^k would be expressed in red cells only in people of *ff* genotype. The data also indicated that the *P* locus genes (P_1 and P_2) act before the P^k gene, and a scheme was proposed incorporating these ideas. If the structures of the P substances are correct, however, the scheme shown in Fig. 24.1, or one similar to it, would make better sense biochemically.

There have been two reports of an anti-p. The first, by Engelfriet *et al.* (1971) described a biphasic hemolysin in the serum of a 72 year old woman who was P_1. The serum also contained an agglutinin which acted optimally at lower temperatures and on *pp* cells. The characteristics of the antibody changed over the course of time, and activity eventually disappeared. Recently, Metaxas, Metaxas-Bühler and Tippett (cited by Schwarting *et al.*, 1977) found a serum called "Föl." which reacted strongly with p cells, and to a lesser extent with P_2, P_1 and P_1^k cells. Schwarting *et al.* (1977) studied the specificity of this serum and found it to be specifically inhibited by sialosylparagloboside. This observation supports the suggestion above that p cells lack the

Table 24.3 Names and Structures of Glycosphingolipids of Erythrocytes, after Schwarting et al., 1977

Lactosylceramide	Gal- β (1—4)-Glc-Cer
Trihexosyl ceramide	Gal- α -(1—4)Gal- β (1—4)-Glc-Cer
Globoside	GalNAc- β -(1—3)-Gal- α -(1—4)Gal- β (1—4)-Glc-Cer
Sialosylparagloboside	NANA- α -(2—3)-Gal- β -(1—4)-GlcNAc- β -(1—3)-Gal- β -(1—4)-Glc-Cer
Paragloboside	Gal- β -(1—4)-GlcNAc- β -(1—3)-Gal- β -(1—4)-Glc-Cer
P_1	Gal- α -(1—4)-Gal- β -(1—4)-GlcNAc- β -(1—3)-Gal- β -(1—4)-Glc-Cer
Hematoside	NANA- α -(2—3)-Gal- β -(1—4)-Glc-Cer
G_{DIa}	NANA- α -(2—3)-Gal- β -(1—3)-GalNAc- β -(1—4)-Gal- β -(1—4)-Glc-Cer ↑ α -(2—3) NANA

Abbreviations: Gal — D-galactose; Glc — D-glucose; GalNAc — N-acetyl-D-galatosamine; GlcNAc — N-acetyl-D-glucosamine; NANA — N-acetylneuraminic acid; Cer — ceramide (N-acylsphingosine)

α-galatosyltransferase(s), and accounts for the accumulation of paragloboside and its sialosyl derivative in these cells. While the reactivity of *pp* cells with Föl. serum is thus explained, the fact that this serum reacts more strongly with P_2 than with P_1 cells is not. The relationships as outlined by Naiki and Marcus (1975) and by Schwarting *et al.* (1977) are shown in Fig. 24.1. Kundu *et al.* (1978) described a normal Chinese man, whose cells were weakly reactive with anti-P_1PP^k serum. His red cell glycosphingolipids were analyzed, and his P antigen and P^k antigen content were found to be way less than normal. His ganglioside and sialosylparagloboside content were many fold above normal. The combination of features suggested that he represented a new phenotype, probably due to homozygosity for an allele of P^k that produces defective, or abnormally small amounts of α-galactosyl transferase.

Some further discussion of the genetic model, based on measurements of the glycosphingolipid content of red cell membranes, is given by Fletcher *et al.* (1979).

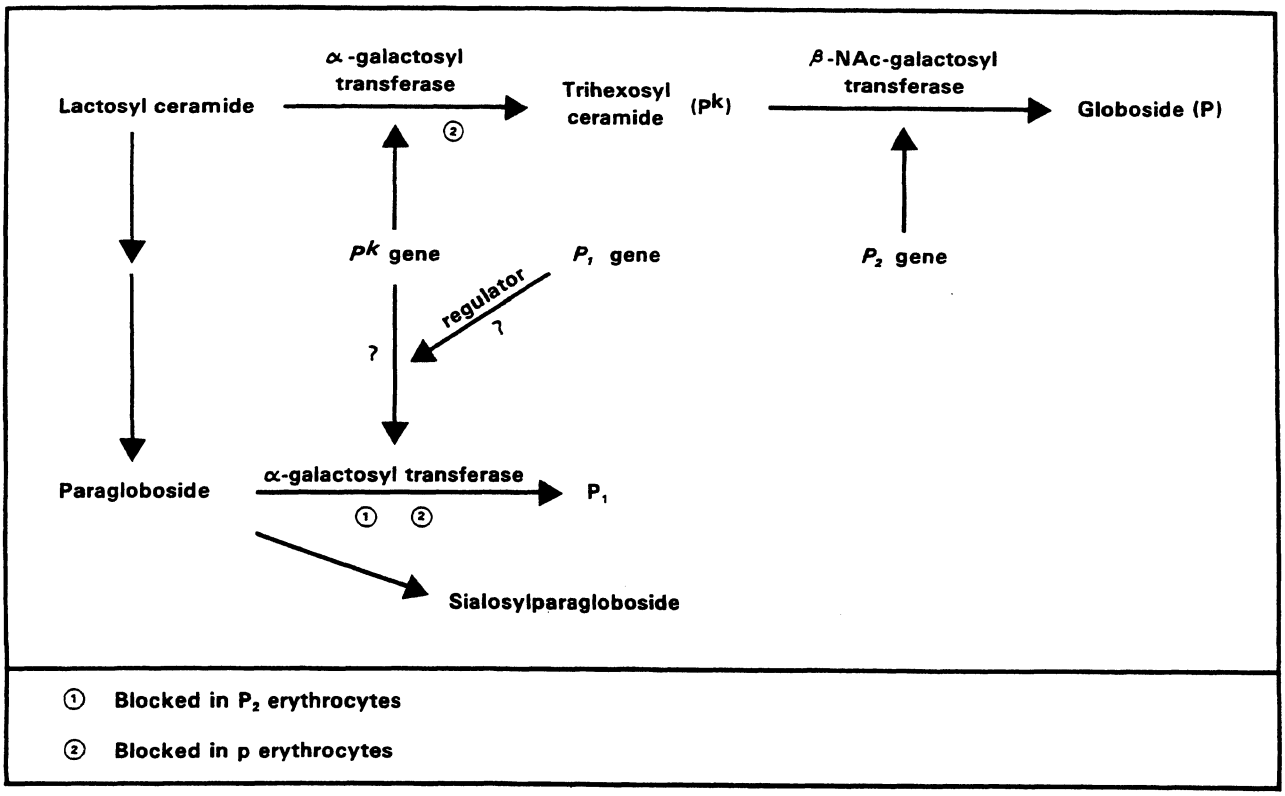
Watkins (1978) reviewed the biochemistry of the P system.

24.1.6 Medicolegal applications

The P system has not been applied to medicolegal problems to the same extent as have a number of the other systems. It can be a problem in disputed parentage cases because of the slow development of P_1 in children. The P system is useful in paternity cases only when a P_1+ child is seen as the result of an alleged $P_1- \times P_1-$ mating, and the situation does not arise very often. The chances of excluding a falsely accused father are about 2% in Whites, and less than 1% in Blacks. Difficulty can also be experienced because of the variability of anti-P_1 sera (Chakraborty *et al.*, 1974; Morel, in AABB, 1978).

The first efforts to detect P_1 in dried blood were apparently those of Roesgen in his Inaugural Dissertation of 1942 (cited by Schnug, 1952). The antigen could be detected in 2 day old bloodstains on linen, but not on wool or silk, by an inhibition technique. Schnug (1952) used a pig serum anti-P for determination of P in dried blood by inhibition. From 40 to 60 mg dried blood was incubated with 0.15 mℓ antiserum for 24 hrs at room temperature. Strong P_1 test cells were used, and the antigen could be detected in some samples of several month old bloodstains on a number of different substrata. Certain substrata interfered with the determination. Weaker P antigen was less reliably detectable and decayed faster over time. The test was said to have been used in casework. Mishakova (1961) reported that P could be determined in dried blood. Pettenkofer and Bickerich (1955) tested the P content of various organs and did not find it in muscle or kidney tissue, except that heart muscle from human or guinea pig sources gave some inhibition of anti-P.

Figure 24.1 Biosynthetic Scheme for the P System Antigens



24.2 Lutheran System

24.2.1 Discovery and development

In 1945, Callender *et al.* reported that a young woman, diagnosed as having lupus erythematosus diffusus, had made a remarkable series of antibodies in response to a series of transfusions. Among them were antibodies defining three antigens not previously encountered, and these antigens were named after the blood donors whose cells had apparently elicited the response. The antigen called Willis later became C^w (section 22.5.2), and a rare antigen called Levay, now known to be Kp^c (section 23.1.3) was detected. The third new antigen was Lutheran. A full report by Callender and Race followed in 1946. The symbols *L* and *l* were originally used to denote the genes for Lutheran(+) and Lutheran(−). It will be recalled the symbols *L* and *l* had been used to designate the first Lewis antigen too, however (Section 20.2). The ambiguity was cleared up at a conference of a group of interested people (Andresen *et al.*, 1949). It was decided to assign the symbol *Le* to Lewis, and *Lu* to Lutheran. The Lewis genes would be denoted Le^a and Le^b, the genotypes Le^aLe^a, Le^bLe^b, etc., the phenotypes Le(a+b−), Le(a−b+), etc., and the antisera, anti-Le^a and anti-Le^b. Lutheran would be handled similarly, the genes being Lu^a and Lu^b, genotypes Lu^aLu^a, Lu^aLu^b, etc., and phenotypes, Lu(a+b−), Lu(a+b+), etc. This convenient notation has been applied to most of the systems discovered since the conference. Dr. Ford suggested that the first antigen discovered be given the superscript "a", e.g., Lu^a, but that the product of the hypothetical allele not be called "b" until the antibody defining it was actually found. Thus, the hypothetical allele of Lu^a would be called *Lu* until an anti-Lu^b was found, and so forth for any system.

The antigen "Lutheran", defined by the serum of the patient discussed above, became Lu^a. The gene controlling this antigen was inherited as a simple Mendelian dominant character. This fact was established in the original work, and confirmed by a number of subsequent studies. About 7.7% of British people were Lu(a+) (Mainwaring and Pickles, 1948; Lawler, 1950; Bertinshaw *et al.*, 1950). In 1956, Cutbush and Chanarin found the expected anti-Lu^b in the serum of a Mrs. R. It gave the expected reactions, and a second example was soon reported by Greenwalt and Sasaki (1957). Tests on families and populations showed that the Lu^a and Lu^b antigens were indeed governed by an allelic pair of genes, giving rise to the phenotypes Lu(a+b+), Lu(a+b−) and Lu(a−b+). Lu(b−) people are fairly uncommon. In the United States, a few studies have been done which give an idea of the phenotypic frequencies. Dublin *et al.* (1964) found 82 Lu(a+b+), 1,116 Lu(a−b+) and 3 Lu(a+b−) in 1,201 Boston Caucasians. This population consisted of control and rheumatic people in a study designed to test for correlations between rheumatic heart disease and blood groups. Juberg (1970) found 25

Lu(a+b+), 291 Lu(a−b+) and 2 Lu(a+b−) in 318 Caucasians in South Central West Virginia. Molthan and Crawford (1966) found 27 Lu(a+b+) and 529 Lu(a−b+) in 556 Black donors in Philadelphia. Juberg (1970) reported 6 Lu(a+b+) and 29 Lu(a−b+) in 35 Black people in the West Virginia study.

24.2.2 The Lu(a−b−) phenotype

In 1961, Crawford *et al.* reported finding six people in three generations of a Caucasian family who were Lu(a−b−), the proposita in this case being Mary Crawford herself. This minus-minus phenotype, unlike those of any other known system, behaved as a dominant characteristic. Lu^b was obviously inhibited in this phenotype, and it was reasonable to suppose that Lu^a would be as well. Additional examples of Lu(a−b−) cells were found (e.g. Stanbury and Francis, 1967). It seemed likely that this phenotype was controlled by a gene coding for the absence of some important precursor substance. It became clear from the studies of Tippett (1971) and Taliano *et al.* (1973) that Lu^a was inhibited in this phenotype as well as was Lu^b, and that the inhibitor locus was different from the Lutheran locus. Taliano *et al.* (1973) suggested the symbol *In(Lu)* for the dominant inhibitor gene at the inhibitor locus, and *in(Lu)* for the normal, recessive allele. The symbol may be something of a misnomer, as it turns out, since it is now clear that *In(Lu)* affects more than the Lutheran alleles. There are disturbances in the expression of P_1, of i in the Ii system (Section 25.2) and of Au^a (Section 25.5) as well (Tippett, 1963; Contreras and Tippett, 1974; Crawford *et al.*, 1974).

In 1963, Darnborough *et al.* found a patient with Lu(a−b−) red cells who had come to their attention because of an anti-Lu^aLu^b in the serum. This antibody was the first example of its kind, and the anti-Lu^a and anti-Lu^b activities were inseparable, all activity being removed by absorption with either Lu(a−b+) or Lu(a+b−) cells. This serum was used to screen the cells of 18,000 additional people, and one more Lu(a−b−) blood was found. There was a suspicion that this Lu(a−b−) was not the same as the dominant kind described above, and this proved to be the case when informative families were found (Brown *et al.*, 1974). This recessively inherited kind of Lu(a−b−) appears to be best explained by the presence of an allele *Lu* at the Lutheran locus which is either silent, or coding for an as yet undiscovered antigen.

24.2.3 Extension of the Lutheran system

In recent years, a number of antibodies have been found which detect antigens related to Lutheran. Most of them are of high frequency, but a few are not. Numerical designations have been used in naming these new antigens. The designation anti-Lu3 has been applied to the anti-Lu^aLu^b first found in the Lu(a−b−) person by Darnborough *et al.* in 1963 (Bove *et al.*, 1971). Bove *et al.* (1971) described anti-Lu4. Anti-Lu5, anti-Lu6 and anti-Lu7 were reported by Marsh (1972). All define high incidence antigens present on almost all Lu(a+b+), Lu(a−b+) and Lu(a+b−) cells, but absent in both the dominant and recessive kinds of Lu(a−b−) cells. Lu4 is inherited, but not controlled by an allele of Lu^a and Lu^b (Bove *et al.*, 1971). It is also clear that Lu5 (Bowen *et al.*, 1972) as well as Lu6 and Lu7 (Marsh, 1972) are inherited. Lu8 was described by MacIlroy *et al.* (1972) and it, too, is inherited. Lu9 is a low incidence antigen, and has been shown to be controlled by a gene allelic to that controlling Lu6 (Wrobel *et al.*, 1972; Molthan *et al.*, 1973a). Dybkjaer *et al.* (1974) have, however, described a family in which the proposita is Lu:–6, and, therefore, presumably Lu^9Lu^9, but her cells reacted only weakly with anti-Lu9. The sister of this proposita was also Lu:–6 and reacted as expected (strongly) with anti-Lu9. Anti-Lu10 is, according to Race and Sanger (1975), mentioned occasionally, but no full description of it has been published. There is apparently a suspicion that Lu^{10} may be an allele of Lu^5. Lu11 is a high incidence antigen described by Gralnik *et al.* (1974). The designation Lu12 has been applied to the antigen defined by the "Much." serum, described by Sinclair *et al.* (1973). The authors agreed to the suggestion that it be called anti-Lu12, although they do not do so in the paper. The Much. serum reacts negatively with Lu(a−b−) cells of either type, and with some but not all Lu(a−bw) cells. The evidence suggested that this antigen is not controlled by a gene in the Lutheran complex locus. Lu14 was described by Judd *et al.* (1977) and their data indicated that Lu^{14} is allelic to Lu^8. It appears, therefore, that there are three allelic pairs at the Lutheran complex locus, Lu^aLu^b, Lu^6Lu^9,, and Lu^8Lu^{14}. The relationships of the other Lutheran antigens are not as yet clear.

24.2.4 Development of Lutheran antigens

Greenwalt *et al.* (1967) found that Lu^a is weakly expressed in Lu(a+b+) newborns, and increases in strength for many years, to about age 15. Lu(a+) cells can be detected, however, in fetal blood. Lu^a was detected in a 12 week fetus by Race and Sanger (1975), and in a 14 week fetus by Toivanen and Hirvonen (1973). Lu^b is weaker in fetal cells than in adult ones (Greenwalt *et al.*, 1967) and Toivanen and Hirvonen (1969b and 1973) could detect it in one 10 week fetus but not in one 9 week old one.

24.2.5 Medicolegal applications

The Lutheran system can be used in disputed affiliation cases, although its power of discrimination is fairly low. There is about a 3% probability of excluding a falsely accused father, whether he be Black or White, using both antisera. Anti-Lu^a and anti-Lu^b are quite scarce, and the system is not commonly included in paternity tests for this reason (Chakraborty *et al.*, 1974; Morel, in AABB, 1978). If the system is employed, the possible occurrence of the dominant *In(Lu)* gene which produces Lu(a−b−) as well as of the silent *Lu* allele must be kept in mind, even though both are very rare (AABB, 1978).

Perhaps because the antisera are rare, no papers on the determination of Lutheran antigens in bloodstains were found in the literature.

SECTION 25. SOME OTHER BLOOD GROUP SYSTEMS

25.1 Introduction

There are a number of other blood groups besides those discussed thus far in Unit V. There has been little, if any, application of these to the individualization of bloodstains. Some of them will be discussed briefly in this section for the sake of completeness.

25.2 The Ii System

What has come to be known as the Ii system derived from observations on the "cold" type of agglutinins found in the sera of patients suffering from acquired hemolytic anemia. The "system" no longer appears to be simple, and is related in some way to ABH and Lewis.

In 1956, Wiener *et al.* gave the name anti-I to a high titer cold autoagglutinin in the serum of a patient suffering from hemolytic anemia. The antigen I, defined by this serum, was of extremely high incidence, but rare I-negative people (denoted "i") could be found. Only five people of the i phenotype were found in the first 22,000 bloods examined. There was variability in the anti-I reaction with different examples of cells, and it was suggested that I might consist of a number of subgroups, I_1, I_2, etc. Jenkins *et al.* (1960) studied the original serum along with another anti-I serum called "Steg.", and a great range of I strength was noted, which could be fitted to a normal distribution curve. The I antigen was feeble in the cells of newborns and in cord blood cells. Tippett *et al.* (1960) extended these studies. A few more i people were found, and they had anti-I in the serum. Not all the anti-I found were quite the same. In 1960, Marsh and Jenkins found an anti-i in the serum of a patient. It reacted weakly with the patient's and most other red cells, but strongly with i cells and cord cells. Like its partner, anti-i was a cold reacting antibody. Marsh (1961) extended these studies, and suggested that five phenotypic categories could be distinguished, based on the strength of the I antigen and the presence of any anti-I in the serum. Most adults, it was said, develop I from i, since all infants have i, and the transition was suggested to be under genetic control. Infants have a strong i antigen, and very little I, but over the course of about 18 months, the relationship reverses itself. Dzierzkowa-Boradej and Voak (1979) have suggested that four subtypes of i can be distinguished based upon a complete array of evidence. Joshi and Bhatia (1979) found several Asian Indian blood donors who were I-i- in a large survey.

There is evidence of more than one kind of serologically distinguishable I (Marsh *et al.*, 1971; McGinnis *et al.*, 1974). As mentioned, anti-I is often the culprit in cases of acquired hemolytic anemia. Anti-i may sometimes be involved as well (Bell *et al.*, 1967).

It has been clear for some time that I is in some way associated with ABO, and it appears that this relationship as well as the complicated serological characteristics of the system may be clarified by the immunochemical studies on the antigens. Marcus *et al.* (1963) studied the effect of treating red cells with β-galactosidase from *Clostridium tertium* on anti-I reactivity. The data suggested the involvement of β-galactose and NAc-neuraminic acid in the immunodeterminant structure of the I receptor. Gardas and Koscielak (1974) isolated an I-active material from erythrocyte stroma and found it to be immunochemically indistinguishable from ABH-active substance. This finding is in conformity with the immunochemical studies of Feizi *et al.* (1971a and 1971b). They studied a partially purified I-active substance, isolated from milk. It was active only with selected examples of anti-I, and these were used to carry out quantitative precipitation studies. Different examples of anti-I were found to contain different antibody specificities against the milk-derived I substance, Le^a substance, precursors of ABHLe substances, and molecules resulting from various stages of chemical degradation of ABH substances. The most striking finding was the apparent heterogeneity of both different anti-I sera and of the I determinants. Mild degradation of ABH substances appeared to enhance I-activity generally, and suggested that I determinants are concealed in the interior of the ABHLe molecules, and of various biosynthetic stages of them. At the least, these findings suggest an immunochemical basis for the observed serological variability of I reactivity. These studies have been refined and extended more recently (Feizi *et al.*, 1978; Watanabe *et al.*, 1979). It may be mentioned that wheat germ agglutinin reacts with I substance purified from papain treated red cells (Oppenheim *et al.*, 1977). Maniatis *et al.* (1977) said that the red cells of sickle cell trait and sickle cell anemia patients show higher Ii reactivity than cells with normal hemoglobin.

Ducos *et al.* (1969b) said that I and i antigens could be diagnosed in bloodstains by an inhibition procedure with appropriate antisera, and that this fact could be useful in investigating bloodstains thought to be of infant or fetal origin. Tumosa (1977b) demonstrated that cold agglutinins of the anti-I type would readily absorb onto, and elute from bloodstains. There is no doubt that this fact would cause false reactions if there were cold agglutinin contamination of routine grouping reagents used for bloodstains. No such contamination was noted with several commercial ABH grouping antisera.

25.3 Diego

Diego was first mentioned by Levine *et al.* (1954b), who had tested a number of bloods from Caucasians with anti-Diego, and found them to be uniformly negative. The anti-

gen was, therefore, described as a low incidence antigen. The antibody was found in a mother in Caracas, whose baby suffered from hemolytic disease of the newborn, and further investigation showed that the antigen being detected occurred in appreciable frequencies in certain South American Indian populations (Layrisse *et al.*, 1955; Levine *et al.*, 1956). The antigen was denoted Di^a, and the gene responsible for it, Di^a, behaved as a Mendelian dominant characteristic (Layrisse *et al.*, 1955). Layrisse and Arends (1956) indicated that Di^a occurs with reasonable frequency in Chinese and Japanese populations, in addition to the South American Indian populations that have it, but it is extremely rare in Caucasians and in Negroes. An extensive study of Venezuelan Indian populations by Layrisse and Wilbert (1966) included Di^a frequencies in the various populations. Family studies suggest that Diego is probably an independent system. In 1967, Thompson *et al.* reported the first two examples of anti-Di^b in the sera of women of Mexican Indian ancestry. Other examples have since been found, but anti-Di^b is rarer than anti-Di^a. The Diego system appears to consist, therefore, of two alleles, Di^a and Di^b, which can give rise to three phenotypes. As noted above, however, the system is not significantly polymorphic in Caucasian and Black populations.

25.4 Yt

In 1956, Eaton *et al.* reported an antibody in the serum of a young woman who had had several pregnancies and had been transfused. She had died of cancer in 1955. The antigen being detected was called Yt^a, and was of very high incidence in Europeans. The antibody reacted weakly, and was best detected by a Coombs test using trypsinized cells. Papain appeared to destroy the receptor. It appeared that the gene could be present in single or double dose. In 1964, Giles and Metaxas found the expected anti-Yt^b. The system is understandable on the basis of two codominant alleles, Yt^a and Yt^b. Evidence thus far indicates that Yt is independent of other systems. Yt(b+) occurs in about 8% of Europeans. Giles *et al.* (1967) indicated that the phenotypic frequencies in 1030 people in London were: 946 Yt(a+b−), 82 Yt(a+b+) and 2 Yt(a−b+). In addition, they tested 69 U.S. Black people and found only one to be Yt(b+). Wurzel and Haesler (1968) tested 714 Blacks in the Philadelphia area, however, and found 60 to be Yt(b+). The system makes fairly useful distinctions, but antisera are not common, and in some cases the antibodies have been found in sera containing other antibodies, making it more difficult to obtain workable antibody preparations.

25.5 Auberger

The antibody defining Au^a was found in a multiply transfused woman in Paris by Salmon *et al.* (1961). A number of other antibodies were present in her serum as well. Another example of the antibody was found 10 years later. Both sera were AHG-reactive with papain treated cells. Europeans appear to be about 82% Au(a+). Au^a is inherited as a dominant characteristic, and is not thus far associated with any established system. Au^a is affected by the *In(Lu)* gene responsible for the dominant kind of Lu(a−b−) (see in section 24.2.2).

25.6 Dombrock

In 1965, Swanson *et al.* found the antibody defining Do^a in the serum of Mrs. Dombrock. The antibody reacted by the AHG test using papain treated cells, but AHG sera varied in their ability to demonstrate the reaction. The serum reacted with about 64% of cells from Europeans, and family studies indicated that the Do^a gene was dominant, and independent of a number of other systems. Polesky and Swanson (1966) extended the population studies, and found that fewer Blacks than Whites were Do(a+), about 55% of 161 as against 64% of 814, respectively. Tippett (1967) and Tippett *et al.* (1972) carried out family and population studies on Dombrock and confirmed its dominant mode of inheritance as well as establishing its independence from most other blood group systems. In 1973, anti-Do^b was found in the serum of a Mrs. Pam. by Molthan *et al.* (1973b). She was the mother of monozygotic twins and had been transfused a number of times. The antibody reacted best by the AHG test using enzyme treated cells. Dombrock consists, therefore, of the alleles Do^a and Do^b. If antisera become more available, Dombrock can be quite useful since it makes very good distinctions in populations.

25.7 Colton

In 1967, three examples of sera containing an antibody to a high incidence antigen were reported by Heisto *et al.* The antigen being detected was named after the first patient in whom the antibody was found, and was called Co^a. Family studies indicated a dominant pattern of inheritance, and dosage effects with the antiserum indicated that single or double doses of the Co^a gene could be present. Co(a−) people are very rare in Caucasian and Negro populations, something of the order of 1 or 2 per 1,000. Giles *et al.* (1970) found anti-Co^b in a multiply transfused patient, whose serum contained a number of other antibodies. Colton appeared to consist of the two alleles, Co^a and Co^b. Moulds *et al.* (1974) briefly reported a family, however, containing Co(a−) members whose reactions with anti-Co^b were weaker than expected, and the best explanation for which was a silent allele, *Co*, segregating in three generations. In addition, three Co(a−b−) people have been found by Rogers *et al.* (1974). The serum of one of them contained an inseparable anti-Co^aCo^b (anti-Co3, perhaps).

25.8 Sid and Cad

In 1967, Renton *et al.* reported an antibody which agglutinated most of the cells of Europeans but with widely varying strength. The antigen, named after the first strong reactor, was called Sd^a, and the system was called "Sid". About 91% of Europeans were Sd(a+) but the range of reactivity was striking, and the antibody was not easy to work with. Macvie *et al.* (1967) reported on Sd^a simultaneously. The AHG test appeared to be the best way of detecting reactions

with this serum. *Sd*^{a} was inherited as a dominant characteristic, and its allele was, for the time being, called *Sd*. Sd^a is found in the saliva of people whose red cells are Sd(a+), but the amounts present show wide variation. Macvie *et al.* (1967) said that it was not always easy to distinguish Sd^a "secretors" because of this variability. Morton *et al.* (1970) showed that Sd^a was present in most human secretions, urine being the richest source. The saliva of newborns has more Sd^a than that of adults. About half the people whose cells are Sd(a−) secrete some Sd^a in urine. Further, the occurrence of Sd^a is not limited to human beings, the antigen being found in guinea pig kidneys and urine, and in the urine of a number of other animals as well.

In 1968, Cazal *et al.* found an antigen of very low occurrence in a family of Indian extraction from Mauritius Island. The cells of certain members of this family were agglutinated by every example of human serum tested, i.e., were polyagglutinable. The polyagglutinability was not related to T nor to Tn. Extracts of *Dolichos biflorus*, previously thought to have only anti-A_1 specificity, agglutinated these cells, too, even though they were not of group A. The cells were said to have the "Cad" antigen after the name of the family. Cad was inherited, but very rare. This was the first example ever of an apparently inherited kind of polyagglutinability. Cazal *et al.* (1968) said that 250,000 examples of cells from Europeans were found to be Cad(−)! Cazal *et al.* (1971) extended their studies and found that certain other anti-A agglutinins from snail sources agglutinate non-A Cad(+) cells too, e.g. the anti-A from *Helix pomatia* or *Helix aspersa*. Sanger *et al.* (1971) found several *Dolichos* reacting non-A members of a Danish family, and noticed that they reacted very strongly with anti-Sd^a. Other members of the family whose cells did not react with *Dolichos* lectin showed weak or negative reactions with anti-Sd^a. The strength of Sd^a in Cad(+) cells was shown to be the highest ever observed, and the link between Sid and Cad was thus established. The conclusion was that Cad amounted to Sd^a in the extreme. Race and Sanger (1975) used the designation Sd(a++) to denote Cad(+), and Cad may be called "super Sid". Uhlenbruck *et al.* (1971) have shown that several anti-A agglutinins from snails, including *Helix pomatia*, *Helix aspersa* and *Cepea nemoralis*, react with Cad(+) cells whether they are of group A or not, but the *Phaseolus* (lima bean) agglutinin does not. Bird and Wingham (1971) found the very same thing. That *Phaseolus* lectin fails to react is a puzzle, since it is thought that it, like *Dolichos* lectin, is specific for terminal α-NAc-galactosaminyl residues. *Salvia farinacea* and *Salvia horminium* seed extracts contain an anti-Cad activity, separable from their anti-Tn activity (Bird and Wingham, 1974). Myllylä *et al.* (1971) found that the electrophoretic mobility of Cad(+) cells was not much reduced compared to normal ones, in contrast to T and Tn red cells. Race and Sanger (1975) noted that they had suggested previously that the polyagglutinability of Sd(a++) cells is not polyagglutinability in the usual sense, but is probably due to the presence of anti-Sd^a in the great majority of sera. The evidence indicates that the Cad agglutinating power of a serum is in approximately inverse proportion to the strength of the Sd^a antigen of the serum donor's cells. Some studies on the biochemical properties of a partially purified Sd^a substance from urine were reported by Morton and Terry (1970). The material behaved as if its serological specificity depended on a terminal oligosaccharide structure. Prokop *et al.* (1976) have recently reviewed Sid and Cad.

25.9 Some General Considerations on Blood Groups

There are many antigens known besides those which have been discussed in Unit V. Some of them belong to what are already, or are close to being established as independent blood group systems. Examples are Sm and Bu^a which are controlled by allelic genes, and make up the Scianna system, and Wright. Many other antigens exist whose relationship to one another and to established systems is not yet clear. The antigen Xg^a is of interest because it is the only blood group antigen coded for by an X-linked gene. This antigen has its own chapter in Race and Sanger (1975).

There are many antigens which are either of very high incidence or very low incidence. Some workers call these "public" and "private" antigens, respectively. Such antigens do not make useful distinctions in the population very often and are not, therefore, usually included in medicolegal tests. Antisera for these antigens are usually very scarce as well. If one happens to have antisera for rare antigens, tests might be done in an occasional case, since if a rare phenotype is found, it gives a very strong indication of exclusion or of inclusion. Thus, while the rare types are seldom encountered, they can be very valuable when they do occur in a case. It should be kept in mind that an antigen can be fairly well distributed in certain populations, and not in others. The Diego groups (Section 25.3) are a good example of this behavior.

The number of known antigens is now quite large. There are at least nine established systems: ABH, Rh, MNSs, P, Lutheran, Kell, Ii, Duffy and Kidd. Diego, Yt, Dombrock, Colton, Auberger, Scianna, Sid, Wright and Xg may sooner or later be added to the list. Issitt and Issitt (1975) list almost 400 antigens in their book. Some of these may be identical. Establishing the independence of a new antigen is not easy. Antisera to the public antigens are very rare, while cells having the private antigens are equally rare. Only a few laboratories have very extensive collections of these materials, and testing new cells or sera against dozens and dozens of sera or cells is very tedious work indeed.

The list of blood group antigens which have been successfully determined in dried blood continues to grow. The subject was excellently reviewed by Dodd in 1972. Theoretically, any antigen which is known to be inherited, and which makes a useful distinction in the population, could be useful in individualizing a bloodstain, provided that a technique is available for its reliable determination. In many cases, there are practical reasons for the inability to make use of a particular antigen or system in bloodstain analysis on a large

scale. The availability of antisera is sometimes a problem. In addition, the interpretation of negative results is not a trivial problem when grouping bloodstains. At the present time, the ABO, Rh and MNSs antigens along with Kell, Fy^a, Fy^b and Jk^a are well established as bloodstain markers. Leaving aside the problems with MN determinations in bloodstains, it appears that an informative discrimination could be obtained in most cases with anti-A, anti-B, anti-H, anti-D, anti-C, anti-E, anti-c, anti-e, anti-M, anti-N, anti-S, anti-s, anti-K, anti-Fy^a, anti-Fy^b and anti-Jk^a. Using very rough estimates of the phenotypic frequencies in U.S. populations, a White person having all the most common types, e.g. O, R_1r, MNs or MNSs, K-, Fy(a+b+), Jk(a+) would occur once in 85 people, while one having all uncommon types, e.g. AB, R_oR_o, MNS or NSs, K+, Fy(a+b−), Jk(a−), would occur only once in about 5 million people. A Black person with the groups O, R_oR_o, MNs, K-, Fy(a−b−), Jk(a+) would be expected about once in 25 people, while one with the groups AB, R_1R_1, MS or NSs, K+, Fy(a+b+), Jk(a−) would be seen only once in about 200 million people. Clearly, in most cases the discrimination will fall somewhere between the extremes. Good discrimination can also be obtained in many cases if only some of the antigens mentioned are used.

There are many excellent reference works in blood group serology. Race and Sanger (1975), Prokop and Uhlenbruck (1969) and Giblett (1969) make up an encyclopedic reference set, the latter two also containing considerable information on the material of Units VI and VII as well. Issitt and Issitt (1975) is a very good review, in addition to being a detailed reference to whole blood grouping techniques. Boorman, Dodd and Lincoln (1977) provide an excellent review of all the systems, and give a variety of detailed procedures which have been thoroughly checked in their own laboratory. The book has the additional advantage of containing substantial information about the medicolegal applications of blood grouping, based upon the authors' own considerable contributions and experience.

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Bibliographic Notes to References for Unit V

§[1] *Japanese Journal of Legal Medicine* (*Jpn. J. Leg. Med.*) has Japanese title *Nippon Hoigaku Zasshi*

§[2] *Vox Sanguinis* began publication in 1951 as *Bulletin van het Centraal Laboratorium van de Bloedtransfusiendienst van het Nederlandse Rode Kruis.* Title changed to *Vox Sanguinis* (*Vox Sang.*) in 1953 but volume numbering continued with issues of 1953 as vol. 3. In 1956, the present series (new series) began with volume 1, and continued to the present. Those references to volumes 3 through 5 for 1953–1955 are designated "O.S." for "old series".

§[3] *Ochanomizu Medical Journal* (*Ochanomizu Med. J.*) has Japanese title *Ochanomizu Igaku Zasshi*

§[4] *Sbornik Klinický* had alternate French title *Archives Bohème de Médécine Clinique*

§[5] *Journal of the Tokyo Medical College* (*J. Tokyo Med. College*) has Japanese title *Tokyo Ika Daigaku Zasshi*

§[6] *Acta Criminologiae et Medicinae Legalis Japonica* (*Acta Criminol. Med. Leg. Jpn.*) has Japanese title *Hanzaigaku Zasshi*

§[7] *Japanese Journal of Human Genetics* (*Jpn. J. Hum. Genet.*) has Japanese title *Jinrui Idengaku Zasshi*

§[8] *Forensic Serology News* (*Forensic Serol. News*)—see Note §[1] to the References for Unit VI

¶ References marked with this symbol have been translated and appear in Unit IX

UNIT VI.
ISOENZYMES

SECTION 26. INTRODUCTION TO ISOENZYMES

The isoenzymes are an important class of genetic markers in human blood and body fluids, and a number of them have come to be applied quite widely in medicolegal investigations in the past twenty years or so. The existence of multiple molecular forms of enzymes as a general and significant biological phenomenon was recognized about 25 years ago. A very large number of enzymes from many species occur in multiple molecular forms. Those in the human species which are under the control of polymorphic genetic loci have potential applicability to medicolegal problems.

In 1957, Hunter and Markert reported that zone electrophoresis on starch gels, perfected by Smithies in 1955 (section 2.3.4) for serum protein separations, was applicable to the separation of enzymes from crude extracts if histochemical localization techniques were used to locate the zones of activity. They found the technique applicable to esterases, tyrosinases and phosphatases, and said that it was probably applicable to any enzyme system which could be resolved on starch gels, and for which a suitable histochemical localization procedure could be devised. The "activity stained" gel was referred to as a "zymogram". This relatively simple technique was quickly adapted to the study of many different enzymes from many different sources.

There were indications prior to the 1950's that enzymes might exist in more than one form, but it was not clear whether the observed heterogeneity was real or apparent. Vesell and Bearn (1957 and 1958), for example, observed heterogeneity of lactate and malate dehydrogenase activities in human serum upon electrophoretic separation of the serum proteins, and the activity patterns could be different in certain disease states. That the heterogeneity was indeed a property of the biological system, and not an artifact of preparation or assay technique, became clear in 1959 as Markert and Møller applied the zymogram method to lactate, malate and isocitrate dehydrogenases, esterases, alkaline phosphatases, and peroxidases from a number of tissues. Multiple molecular forms of the enzymes could occur in the same species, in the same organism, and even in the same tissue. Markert and Møller (1959) proposed that these forms be called "isozymes", and this term is now in widespread use. Lactate dehydrogenase was studied intensively over the next several years in a number of laboratories. Five distinct isozymes of LDH could be detected in mammals, there being a characteristic distribution of these in various tissues (Plagemann *et al.*, 1960; Vesell and Bearn, 1961). Any one of the isozymic forms could be dissociated into subunits with reagents that disrupt hydrogen bonding, such as urea or guanidine-HCl (Appella and Markert, 1961). There appeared to be two types of subunits, and the intact isozyme molecule was a tetramer. Two different subunits, taken four at a time in all possible combinations, could explain the five isozymes quite easily (Appella and Markert, 1961; Cahn *et al.*, 1962). There was much indirect evidence for this idea, and Markert proved it in 1963. He had found conditions under which the tetrameric enzyme could be dissociated into its constituent subunits, and then allowed to reassociate with the retention of activity. If the subunits are designated "A" and "B", Markert allowed a mixture of what were thought to be A_4 LDH and B_4 LDH to dissociate, and then reassociate at random. He predicted that if the two subunit hypothesis were correct, all five isozymes should appear, and in a predictable ratio, as indeed they did. For LDH, therefore, two different subunits, A and B, are used to construct five different tetrameric isoenzymes, which might be represented A_4, A_3B, A_2B_2, AB_3 and B_4. A review of the pioneering LDH work was given by Kaplan (1964).

Molecular multiplicity in a functional enzyme may come about in a number of different ways (Markert, 1968 and 1975). An enzyme consisting of a single polypeptide chain, coded for by a single gene, might exist in various forms because the genetic locus is multiple allelic. Two or more different genetic loci may control the synthesis of different polypeptide chains, which may function independently, or may polymerize to form a functional enzyme molecule. The number of combinations of functional polymers can vary. Activity may be associated with only one polymeric form, such as the tetramer, or more than one might be active, such as the dimer and the tetramer. Post-synthetic modifications of various kinds can also give rise to molecular heterogeneity. Proteolytic cleavage of a portion of a polypeptide chain could give rise to a population of cleaved and uncleaved molecules. Minor modification of a few residues of the polypeptide chain is possible. Association by the peptide with other molecules, such as carbohydrate or sialic acid units, would yield different populations of molecules. There might also be conformational differences in a set of molecules with the same primary and secondary structures. These differences might be the result of association of the peptide with small molecules or not, depending upon the molecular size of the functional unit and the number of subunits. Larger, more complex molecules could be expected to have more degrees of freedom in forming conformationally stable isozymes. It is quite likely that examples of all these devices could be found among the great variety of isoenzymes in nature.

A good sense of the progress in the isoenzyme field that was made in the 1960's can be gained by looking at the proceedings of the two major conferences sponsored by the

New York Academy of Sciences (Furness, 1961; Vesell, 1968). An excellent treatment of the biochemical genetics of human isoenzymes is given by Harris (1975).

The remainder of Unit VI is devoted to discussions of individual enzyme systems. Emphasis has been given to those which have medicolegal applications as genetic markers in populations. Isoenzymes which can be useful in identifying the tissue origin of a sample, or occasionally in other ways, such as in diagnosing pregnancy, were discussed in Unit II. The isozymes to be discussed in the present unit are controlled by polymorphic genetic loci, and are more or less useful in making distinctions in populations, depending upon the gene frequencies in the population of interest. Some of the isozymes can be reliably determined in bloodstains or in body fluid stains, while for others, techniques have not yet been devised. General principles of the medicolegal applications of genetic markers in identification and disputed parentage problems were discussed in section 18. The distributions of phenotypic frequencies in U.S. populations that could be located in the literature are given in tabular form, following the criteria outlined in the Preface. The abbreviations used to designate the isozymes follow Harris and Hopkinson (1976).

SECTION 27. PHOSPHOGLUCOMUTASE

27.1 Recognition of PGM

Phosphoglucomutase (PGM; EC 2.7.5.1; α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase) catalyzes the reversible conversion of glucose-1-phosphate and glucose-6-phosphate, glucose-1,6-diphosphate being required as a cofactor.

In 1936, Carl and Gerti Cori recognized a new glucose-phosphate ester in preparations in which frog muscle had been incubated with AMP and phosphate. This ester was converted to glucose-6-phosphate over time. The new ester was soon proven to be glucose-1-phosphate (Cori *et al.*, 1937) and it became clear that muscle tissue and yeast extracts contained an enzymatic activity responsible for the conversion of glucose-1-phosphate to glucose-6-phosphate. The enzyme was named phosphoglucomutase (Cori *et al.*, 1938a and 1938b).

27.2 PGM Polymorphism

27.2.1 *PGM_1*

In 1964, Spencer *et al.* found that red cell PGM from different persons gave three distinct patterns of isoenzymes following starch gel electrophoresis and specific histochemical staining (Spencer *et al.*, 1964b). Similar patterns could be observed in leucocyte, liver, kidney, heart or uterine muscle, brain, skin and placental extracts. Seven bands of activity were seen, and designated "a" through "g", the "a" being most cathodal. Only the "a" through "d" bands showed differences, and the simplest explanation was based on two alleles PGM^1 and PGM^2, conditioning the a and c, and the b and d bands, respectively. Studies on 133 families with 262 children showed no exceptions to this explanation. Many family studies support the two allele hypothesis of inheritance, including those of Monn (1969b), Wille *et al.* (1969), Renninger and Spielmann (1969) and Lamm (1970a). In 1965, Hopkinson and Harris found a family "Atkinson" in which variation in the e, f and g bands of PGM were observed. These bands were under the control of a second PGM locus. A subscript was used to designate the locus, the superscripts to designate the alleles. Thus, the PGM locus responsible for the a through d bands is PGM_1 with two common alleles, PGM_1^1 and PGM_1^2, giving rise to the three common phenotypes. Bands e through g are controlled by PGM_2. The majority of people are PGM_2^1 homozygotes, but occasionally, as in the Atkinson family, PGM_2^2 may be observed. People who were $PGM_2^1PGM_2^2$, i.e. Atkinson phenotype, were called Atkinson-1 if they were PGM_1 1, and Atkinson 2-1 if they were PGM_1 2-1. In addition, Hopkinson and Harris (1965) found five uncommon PGM_1 phenotypes in different families, best accounted for by the presence of three rare alleles of PGM_1^1 and PGM_1^2. These were called PGM_1^3, PGM_1^4 and PGM_1^5. All were observed as heterozygotes, PGM_1 3-1, 3-2, 4-1, 4-2 and 5-2. In 1966, Hopkinson and Harris found some further unusual phenotypes which were explained on the basis of two additional alleles, PGM_1^6 and PGM_1^7. PGM_1 6-1, 6-2, 7-1 and 7-2 patterns were seen in these studies. Harris *et al.* (1968) described PGM_1 8-1 and 8-2, adding PGM_1^8 to the list of rare PGM_1 alleles. Turowska and Gawrzewski (1979) found the PGM_1^3 allele segregating in a Polish family.

The rare phenotypes present many problems in comparing results from different laboratories. The only way to compare two samples properly is side by side, in the same gel, and this is not always possible. There are a number of rare phenotypes of PGM_1, particularly involving PGM_1^6 and PGM_1^8, whose relationships are not fully clear. An excellent discussion of the matter, with all the references, is given by Blake and Omoto (1975). Omoto and Harada (1970) and Shinoda and Matsunaga (1970a and 1970b) have reported several examples of PGM_1 8-1 in Japanese. Blake and Omoto (1975) compared several samples from Chinese, which had been diagnosed as PGM_1 8-1 with a reference PGM_1 6-1, which had been typed by Dr. Lie-Injo and Dr. Hopkinson. The samples were identical. This reference PGM_1 6-1 was then compared with a fresh specimen from the "PGM_1 8-1" subject of Omoto and Harada (1970). The samples were not identical, but very similar. The Japanese sample was not PGM 8-1 either, and it was proposed to call it PGM_1^{6JAPAN}. Further examples of it were found in the large population sample studied by Blake and Omoto (1975). In our own studies in New York, we found a peculiar PGM_1 phenotype in an Hispanic person, which was not quite PGM 6-1 nor PGM 8-1. We sent a part of our sample to Mr. B.G.D. Wraxall in London, who ran it against his reference PGM 6-1 and PGM 8-1 samples, and confirmed that it was neither of these. It did, however, match a sample in his collection which had been termed a PGM_1 "8-1 fast". We did not know of Blake and Omoto's work until after ours was at the printer (Mondovano and Gaensslen, 1975), but it seems quite possible that the London and New York samples might have been PGM_1^{6JAPAN} PGM_1^1. Blake and Omoto's (1975) paper should be consulted for further unusual variants at PGM_1.

So-called "silent" alleles have been described at the PGM_1 locus. They are very rare, but, if encountered, could lead to serious errors in disputed parentage cases. Fiedler and Pettenkofer (1969) found an individual without detectable PGM_1 isozyme activity. The father was PGM_1 1 and the mother, PGM_1 2. Their PGM isoenzymes had only about half the activity of normal samples of the same phenotype, and the authors supposed that these parents

were heterozygous for a silent allele, PGM^0, which the propositus had inherited from each of them. Brinkmann *et al.* (1972a) found an apparent PGM_1 1 mother with an apparent PGM_1 2 daughter. If the samples were run on polyacrylamide gels, however, weak "a" and "c" band activity could be detected in the daughter's cells. Further studies showed reduced PGM_1 isoenzyme activity in both, and the allele responsible for the weak kind of PGM_1^1 was called PGM_1^{1r}. The mother was presumably $PGM_1^1PGM_1^{1r}$, and the daughter $PGM_1^2PGM_1^{1r}$. They said that Fiedler and Pettenkofer's subject might have been the same, since on starch gels, PGM^{1r} isozymes showed no activity. Ueno *et al.* (1976) reported a PGM^0 allele segregating in three generations of a Japanese family.

Until relatively recently, PGM_1 isoenzymes have been separated by electrophoresis on starch, agarose or cellulose acetate membranes, and occasionally on polyacrylamide gels. In 1976, Bark *et al.* examined red cell lysates for PGM_1 by isoelectric focusing on polyacrylamide gels over a pH 5–7 range, and observed 10 different electrofocusing patterns with samples from PGM_1 1, 2-1 and 2 sources. This was not a sulfhydryl effect. Four bands of activity, denoted "1−", "1+", "2−" and "2+" could be seen. PGM_1 1 types on starch could be 1+, 1− or 1+1− by isoelectric focusing, PGM_1 2 on starch could be 2+, 2− or 2+2−, and PGM_1 2-1 on starch could be 2+1+, 2+1−, 2−1+ or 2−1−. The ten phenotypes could be accounted for on the basis of four, rather than two, alleles at PGM_1. There was some family evidence presented in support of this view. The alleles were called PGM_1^{1+}, PGM_2^{1-}, PGM_1^{2+} and PGM_1^{2-}. Within starch phenotype PGM_1 1, the most frequent new phenotype was 1+1+ and the least frequent, 1−1−. Within starch phenotype PGM_1 2-1, 2+1+ was the most frequent, 2-1- the least frequent. Within PGM_1 2 on starch, 2+2− was observed in four people, 2+2+ in 2 others, the 2−2− not being seem in this sample of 123 persons.

Kühnl *et al.* (1977a) reported very similar results independently. Isoelectric focusing was carried out on commercially obtained ampholine-polyacrylamide gels with a pH range of 3.5 to 9.5, and agarose gel electophoresis could detect the differences as well. Leucocytes were the preferred material, though hemolysates and sperm extracts were also run. Isoelectric focusing was the preferred method. These results, like those of Bark *et al.* (1976) above, could be explained on the basis of four, rather than two, alleles at PGM_1, giving rise to ten phenotypes. Investigations on 15 families with 36 children confirmed that the four allele hypothesis correctly predicted the inheritance pattern. The alleles in this work were called PGM_1^{a1}, PGM_1^{a2}, PGM_1^{a3} and PGM_1^{a4}. Burdett and Whitehead (1977) carried out separations of PGM isoenzymes by isoelectric focusing, and these studies were extended by Sutton and Burgess (1978). The latter looked carefully at the PGM_1 isoenzymes in an isoelectric focusing system similar to that employed by Bark *et al.* (1976) in 101 unrelated people. The phenotypic patterns reported by Bark *et al.* (1976) were confirmed, and pedigree studies on 24 families with 52 children showed no exceptions to the pattern of inheritance assuming four alleles at PGM_1. Sutton and Burgess (1978) used the designations of Bark *et al.* (1976) for the genes.

Kühnl *et al.* (1977a) had used a somewhat different isoelectric focusing system in their studies, and they looked at leucolysates primarily where the other groups looked at hemolysates. It is difficult, therefore, to compare the phenotypic patterns. Kühnl *et al.* (1977a) reported gene frequencies of 0.6186 for PGM_1^{a1}, 0.1718 for PGM_1^{a2}, 0.1426 for PGM_1^{a3} and 0.0670 for PGM_1^{a4} in 291 persons from Hessen. Our calculations suggest frequencies of about 0.63, 0.11, 0.18 and 0.07 for PGM_1^{1+}, PGM_1^{1-}, PGM_1^{2+} *and* PGM_1^{2-}, respectively, for the 123 English people of Bark *et al.* (1976), and 0.62, 0.12, 0.14 and 0.12 for the 102 English people of Sutton and Burgess (1978). Bissbort *et al.* (1978) indicated that the 10 phenotypes of PGM_1 which could be seen by isoelectric focusing could be detected by acid starch gel electrophoresis. Tris-histidine buffers at pH 5.9 were employed with 18% starch gels. A new nomenclature system was introduced in this paper as well. The alleles were called PGM_1^{1F}, PGM_1^{1S}, PGM_1^{2F} and PGM_1^{2S}, and the phenotypes would be called 1F, 1FS, 1S, 1F2S, etc. This usage corresponds more closely to the nomenclature usually used for plasma protein polymorphic systems (Unit VII). "F" and "S" mean "fast" and "slow", and are meant to be descriptive of the electrophoretic mobilities. The correspondences between the original nomenclature of Bark *et al.* (1976), of Kühnl *et al.* (1977a) and the new system just described is: 1− = a3 = 1F; 1− = a1 = 1S; 2− = a4 = 2F; and 2+ = a2 = 2S. Guise (1979) examined the acid starch gel electrophoretic system described by Bisshort *et al.* (1978). He said that with certain modifications, the ten phenotypes could indeed be diagnosed, but that the method was quite cumbersome and that bloodstains could not be typed. The system was not regarded, therefore, as being a good one for PGM phenotyping in forensic serology.

27.2.2 PGM_2

As mentioned in the foregoing secton, 27.2.1, the Atkinson family revealed the fact that electrophoretic bands e through g were governed by a second locus, PGM_2 (Hopkinson and Harris, 1965 and 1966). Most people are homozygous for PGM_2^1, but this family had members who were $PGM_2^1PGM_2^2$, and who could be either PGM_1 1 ("Atkinson 1") or PGM_1 2-1 ("Atkinson 2-1"). A further phenotype. Palmer, was also reported, and appeared to disclose another allele at PGM_2, namely PGM_2^3. The propositus was PGM_1 1. Further alleles at PGM_2 have been described. PGM_2^4 was found by Monn (1968a) in a healthy Italian male who appeared to be PGM_1 1, PGM_2 4-1. PGM_2^4 and PGM_2^5 were described by Harris and Hopkinson in a personal communication to Giblett (1969) and published by Hopkinson and Harris (1968 and 1969a). Monn (1969a) found what he thought was a PGM_2^5 pattern in a Norwegian Lapp, but the specimen could not be compared with known standards. Further examples of the Atkinson phenotype were found in San Francisco by Lie-Injo (1966), in Michigan

by Brewer *et al.* (1967) and in Seattle by Giblett (1967). All these Atkinson phenotypes, including those of the original family, occurred in Black people. Atkinson phenotype can, however, occur in Caucasians. Gordon *et al.* (1968) reported such a person in Capetown. They also reported what they thought was a PGM_2 2 homozygote in a Black person in Capetown, and called the phenotype "PGM Capetown". Giblett (1969) described an apparent PGM_2^2 homozygote from Mozambique, who was PGM_1 2-1. The Giblett (1969) and Gordon *et al.* (1968) "PGM_2 2" patterns, Mozambique and Capetown, do not look quite alike in the photographs.

In 1969, Santachiara-Benerecetti and Modiano found a variant at the PGM_2 locus in pygmies, which was provisionally called PGM_2^{Pyg}, but which, they said, could become PGM_2^6 (Santachiara-Benerecetti and Modiano, 1969a). Meanwhile, Monn and Gjønnaess (1971) found a PGM_2 locus variant, and suggested that it be called PGM_2^6. Santachiara-Benerecetti *et al.* (1972b) found another example of their PGM_2^6 in an Indian population. It differed slightly in activity from the pygmy phenotype, and they said that these would be distinguished as PGM_2^{6Pyg} and PGM_2^{6Ind} for the time being. They said that the Norwegian variant of Monn and Gjønnaess (1971) should be promoted to PGM_2^7. In this same Indian population was found a further variant type, and the gene responsible for it was named PGM_2^8.

In 1973, Kirk *et al.* reported a number of "PGM_1 5-1" types in an Asian-Pacific population. Studies on other populations in the area, however, led them to conclude that this was, in reality, a second locus variant which should be designated PGM_2^9 (Woodfield *et al.*, 1974; Blake and Omoto, 1975). Blake and Omoto (1975) found a number of other people with yet another PGM_2 phenotype, accounted for by a new allele in the series, PGM_2^{10}.

27.2.3 *PGM*$_3$

In 1968, Hopkinson and Harris found a third group of isoenzymes controlled by yet another PGM locus. In the original studies, as mentioned above, the PGM_1 and PGM_2 isoenzymes could be found in a variety of tissues other than red cells. In placenta, the phenotype was found to be that of the baby rather than that of the mother, and placental tissue clearly reveals the third set of isozymes. They are detectable in other tissues as well, but not in red cells. Studies of dizygotic twins showed that two common alleles at the PGM_3 locus, PGM_3^1 and PGM_3^2, can give rise to three common genotypes (Hopkinson and Harris, 1968; Harris *et al.*, 1968). Lamm (1969) found that PGM_3 isozymes could be detected in leucocytes. The concentration of PGM_3 isoenzymes is low, however, representing but a small part of total PGM activity, and PGM_3 locus typing usually requires that more material be applied to the gel. Family studies by Lamm (1969) gave results consistent with the two allele hypothesis for PGM_3. The PGM_3 locus is not closely linked to either of the other two PGM loci. Where the gene frequencies for PGM_1^1 are of the order of 0.75 in British people, and only slightly greater in most Black populations studied, those for PGM_3^1 differ significantly between the two groups. Hopkinson and Harris (1968) found 0.74 for British populations and 0.34 for Nigerians. Lamm (1970a) found PGM_3^1 to be about 0.75 in 1,031 unrelated Danes.

Electrophoretic patterns of many of the phenotypes are indicated diagramatically in Fig. 27.1. According to the data of Harris *et al.* (1974), the incidences of PGM_1 alleles other than PGM_2^1 and PGM_1^2 are very low in European Caucasians, of the order of 1 or 2 in 10,000 at most.

27.2.4 Linkage relationships of the PGM loci

The data of Hopkinson and Harris (1966) and of Parrington *et al.* (1968) indicate that none of the PGM loci are closely linked to one another. The study of linkage relations between blood group, isoenzyme, serum protein and other marker characteristic loci is moving forward at a very rapid pace. A review of the literature of all established and suspected linkages would be too involved, and the interested reader is referred to the specialized literature. McKusick (1975) and McKusick and Ruddle (1977) contain considerable information, and Figure 1.36 may be consulted for a tabular summary.

Generally speaking, there are two major approaches to human chromosome mapping. Studies of clones of human-hamster or human-mouse cell hybrids may be used to localize a genetic locus on a particular chromosome or chromosome group. Provided that a suitable reference marker is available on that chromosome, linkage analysis can then be carried out using pedigree data from informative matings. For a review of the methods, which can become quite involved, see Renwick (1971b). There is considerable information, too, in Chapter 27 of Race and Sanger (1975).

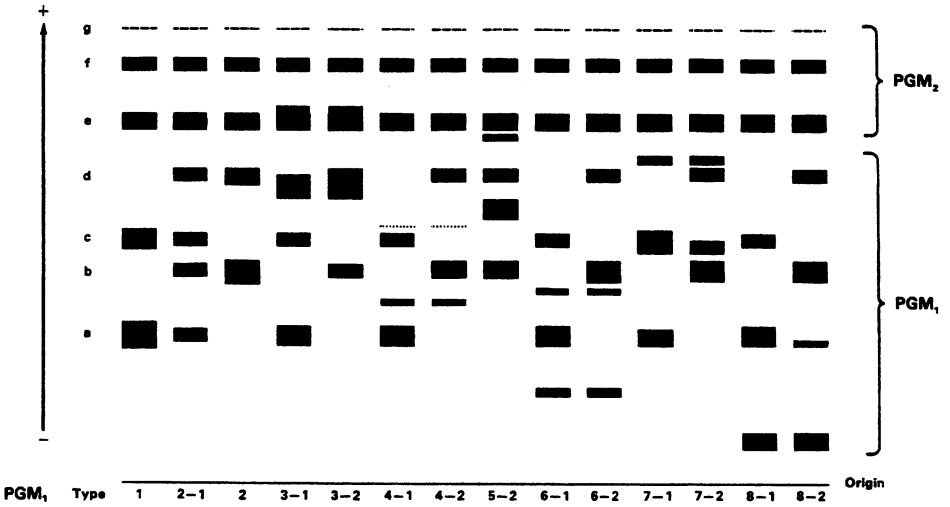
The *PGM* loci do not appear to be linked to one another nor to most other blood group, isoenzyme or serum protein marker loci (Parrington *et al.*, 1968; Lamm, 1970a; Lamm *et al.*, 1970). PGM_1 is linked to Rh and to PGD (Renwick, 1971a). Hybrid cell studies have shown that PGM_1 is on chromosome 6 (Jongsma *et al.*, 1973) and PGM_2 has been assigned to chromosome 4 (see McKusick, 1975, catalog no. 17200).

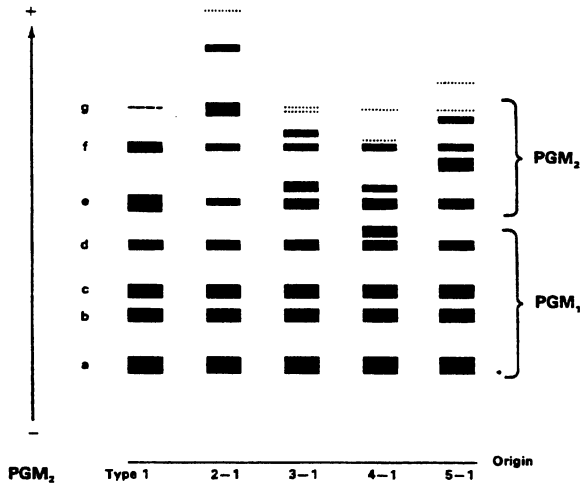
As has been discussed to some extent in section 1.2.4.3, linkage of loci means that they are located on the same chromosome, and have a crossover frequency of less than 50%. Renwick (1971b) used the term "synteny" to mean that loci are on the same chromosome, but are not "linked" because they are too far apart. The term is now widely used, and helps to make the term "linkage" more precise. Linkage is detected by studying crossover frequencies in the offspring of informative matings. The situation is complicated somewhat by the fact that the crossover frequencies between the same two loci may differ in the two sexes.

27.3 Biochemical Studies on PGM Isoenzymes

27.3.1 Properties of the PGM enzyme

PGM is ubiquitous, occurring in many cells of animals, plants and microorganisms. The rabbit muscle enzyme has





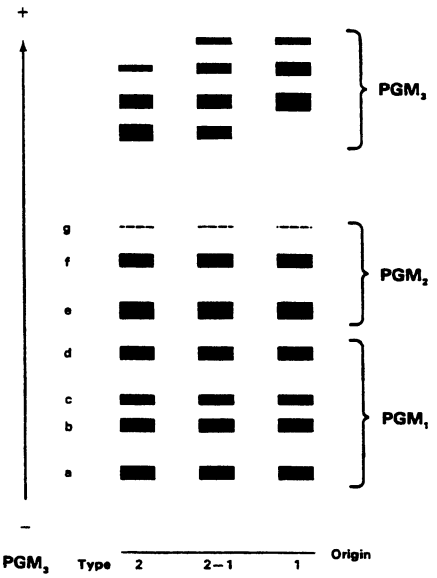


Figure 27.1
Electrophoretic Patterns of PGM Phenotypes

been particularly well studied. The natural substrate for PGM is glucose-1-phosphate (Glc–1–P), although the enzyme also catalyzes the reversible interconversion of C-1 and C-terminal phosphate groups of a number of sugars, including pentoses, though less efficiently. PGM exists in so-called "phospho" and "dephospho" forms, and these are separable. The enzyme requires Glc–1,6–diP and Mg^{++} for activity. It has been known for quite some time that serine is a component of the active site, and that phosphate is attached to the –OH group of serine in the phosphoenzyme (see in Handler *et al.*, 1965). Earlier studies on PGM, with documentation, were well reviewed by Najjar (1962).

PGM is activated by Mg^{++} and Glc–1,6–diP. Much of the data on activators and inhibitors of PGM is contradictory, and difficult to understand, particularly the work on "activation" by chelating compounds. Metal chelating agents, such as imidazole, appear to activate PGM (see Harshman *et al.*, 1965; Robinson *et al.*, 1965), though it now appears that the enzyme is very sensitive to inhibition by heavy metals, such as Zn^{++}, and that the imidazole works by removing these ions. In the absence of excess Mg^{++}, chelating agents can inhibit (Zwarstein and van der Schyff, 1967). The enzyme from rabbit muscle, at least, has six –SH groups, and these can be titrated with –SH reagents at a faster rate in the dephosphoenzyme than in the phosphoenzyme (Bocchim *et al.*, 1967). Oxidation or blocking of the –SH groups significantly reduces enzymatic activity. PGM is inhibited by a number of organic and inorganic anions. It has been known for quite some time that the phosphoenzyme reacts with glucose monophosphates, but not with Glc–1,6–diP, while the opposite is true of the dephosphoenzyme. Kinetic and ^{32}P transfer experiments have indicated that free Glc–1,6–diP is not formed in the reaction as an obligatory intermediate, and that dephosphoenzyme is not formed in every catalytic cycle (Ray and Roscelli, 1964; Goumaris *et al.*, 1967). These and other findings are consistent with a mechanism in which phosphoenzyme-glucose monophosphates are in dynamic equilibrium with dephosphoenzyme-Glc–1,6–diP. The dephosphoenzyme-Glc–1,6–diP may be regarded as the intermediate, capable of dead-end dissociation (Ray and Peck, 1972). A simplified scheme for the reaction, as given by Ray and Peck (1972) is shown in Fig. 27.2. The more recent biochemical work on PGM was well reviewed by these authors.

Purified examples of PGM exhibit molecular heterogeneity. The molecular species have been separated both by column chromatography and by electrophoresis (Joshi *et al.*, 1967; Dawson and Mitchell, 1969). Electrophoresis appears to give better separation. These forms are called "isoenzymes" by some workers, and this usage is certainly not incorrect. However, for those of us accustomed to thinking of isoenzymes as heterogeneous molecular species determined by different genes, a distinction is perhaps helpful. Dawson and Green (1975) have shown that the molecular heterogeneity appears to result from single unit charge changes in the same molecule, probably involving the sulfhydryl residues. The behavior is characteristic of PGM from many sources (Dawson and Jaeger, 1970). Dawson and Green (1975) found that successive alteration of the six free –SH groups in PGM results in systematic changes in the "isoenzyme" pattern. Blackburn *et al.* (1972) found that rabbit muscle phosphoglucose isomerase exists in different forms of this kind as well, and used the term "pseudoisoenzymes" to describe them.

27.3.2 Studies on the enzymes produced by the different PGM loci

The evidence suggests that the isoenzymic products of any particular locus are very similar in their properties. Modiano *et al.* (1970) found that the average PGM activity values of cells representing a number of people in each of the three common PGM_1 phenotypic classes were very close. Densitometric studies on the isoenzymes conditioned by PGM_1^1 and PGM_1^2 in all three phenotypes also indicated that there were no detectable differences (Terrenato *et al.*, 1970). This conclusion appears to be supported by the thermal denaturation studies of McAlpine *et al.* (1970a) who found no differences in the isoenzymic products of the alleles at any one of the loci. There were, however, differences in thermostability among products of the different loci, the isozymes of PGM_2 being most stable, those of PGM_3 least so, with PGM_1 products in between. The relative contribution of the isoenzymes governed by the three loci to the overall PGM activity in various tissues was examined by McAlpine *et al.* (1970b). In the majority, the PGM_1 isoenzymes accounted for more than 80% of the activity. In liver and cardiac muscle, the figure was 95%. PGM_1 isoenzymes in the red cell account for about 50% of the activity. PGM_2 enzymes represented the second largest fraction of total activity in most tissues. Fibroblasts in culture were an exception. Red cells were most active, PGM_2 isoenzymes accounting for about 50% of the activity. PGM_3 enzymes make a very small contribution to the total activity of all tissues studied, except cultured fibroblasts, where they contributed about 6.5% slightly more than did PGM_2 locus products. PGM_3 products are not detectable in red cells by the usual starch gel electrophoretic procedures.

Molecular weights of the PGM isoenzymes from human tissues were estimated by McAlpine *et al.* (1970c) by gel filtration chromatography to be about 51,000 for PGM_1, 61,000 for PGM_2 and 53,000 for PGM_3. The human muscle enzyme, purified many fold, has a molecular weight of about 60,000 as estimated by its sedimentation in the ultracentrifuge (Joshi and Handler, 1969). Santachiara Benerecetti and Modiano (1969b) estimated a MW of 71,000 for PGM_1 isoenzymes by sedimentation ultracentrifugation, and that data indicated that the PGM_2 isozymes might differ very slightly. The rabbit muscle enzyme MW has been estimated at 62,000–67,000 by different methods (Ray and Peck, 1972).

Fisher and Harris (1972) found that additional isoenzymes appeared in material from red cells, lymphocytoid cells or placenta, and seemed to be present in direct proportion to the *in vivo* age of the proteins. They could be found

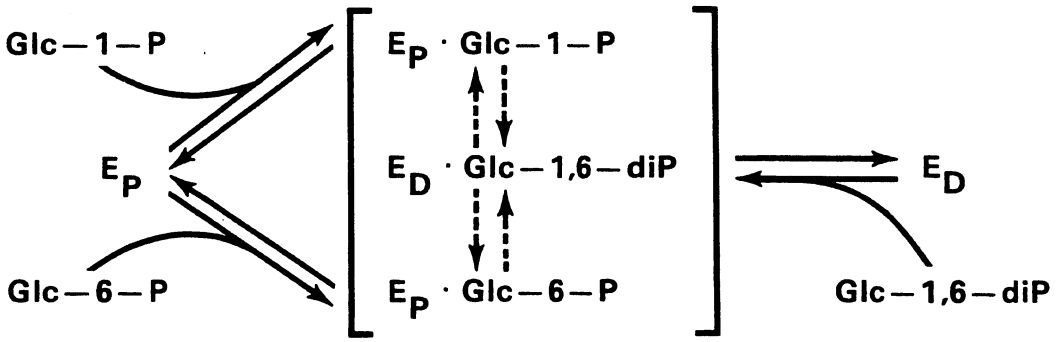


Figure 27.2 Scheme for the Phosphoglucomutase Reaction.

Abbreviations: Glc—1—P = glucose—1—phosphate; Glc—6—P = glucose—6—phosphate; Glc—1,6—diP = glucose—1,6—diphosphate; E_P = phosphoenzyme; E_D = dephosphoenzyme

associated with the products of all three *PGM* loci and represented molecules more negatively charged than the usual isoenzymes. These were called "secondary" isoenzymes. The phenomenon was not thought to be attributable to changes in sulfhydryl groups. Storage changes were noted in the PGM_3 isozymes from placenta. These changes affected both the primary and secondary isoenzymes. Such changes were not seen with PGM_1 and PGM_2 products, but could be produced by sulfhydryl reagents.

Turner *et al.* (1975) looked at PGM patterns in red cells of varying average age, and noted that there was a consistent increase in the c and d bands of the PGM_1 locus at the expense of the a and b isozymes as the cells aged. Similarly, the f and g bands of PGM_2 increased at the expense of band e. These observations are consistent with, but do not prove, the idea that the c and d isozymes of PGM_1 and the f and g isozymes of PGM_2 represent post-synthetic alterations of the primary gene products. In this view, PGM_1^1 codes for the a band protein, PGM_1^2 for the b, and PGM_2^1 for the e.

Quick *et al.* (1972) noted that the enzymes of the PGM_2 locus had considerable phosphopentomutase (PPM) activity, as defined by catalysis of the reversible conversion of deoxyribose-1-phosphate and deoxyribose-5-phosphate. PGM_1 and PGM_3 enzymes had PPM activity as well, but it was much lower. Quick *et al.* (1974) enlarged these studies. The K_m for Glc-1-P was dependent upon the Glc-1,6-diP concentration. PGM_1 isozymes were most efficient at [Glc-1,6-diP] of less than 10 μM. The K_m of PGM_2 enzymes for Glc-1-P decreased as a function of increasing [Glc-1,6-diP], especially over the 100–400 μM range. The K_m of PGM_1 or PGM_2 enzymes for ribose-1-P was unaffected by Glc-1,6-diP concentration. Ribose-1-P competitively inhibited the phosphoglucomutase activity of all the isozymes.

27.4 Medicolegal Applications

27.4.1 Disputed parentage

The PGM_1 isoenzymes have been used for some years in some laboratories in cases of disputed affilitation (Monn, 1969c; Herbich and Pesendorfer, 1969; Kneiphoff and Nagel, 1970; Halasa (1977); Boorman *et al.*, 1977). The chances of excluding a true nonfather by PGM_1 is of the order of 14% in western European populations. Polesky *et al.* (1976), said that the chances of excluding a falsely accused Caucasian father with the PGM_1 system is 14.21%, while for a falsely accused Black father, it is 11.86%. These figures are applicable to U.S. populations, and are similar to those quoted in Dykes and Polesky (1978) and in Chakraborty *et al.* (1974).

The possibility of encountering a silent allele of PGM_1 should not be overlooked. While rare, such alleles do exist (Fiedler and Pettenkofer, 1969; Brinkmann *et al.*, 1972; Ueno *et al.*, 1976), and would, if overlooked, lead to incorrect interpretations in parentage investigations. Silent alleles of PGM_1 were discussed in section 27.2.1.

Welsh *et al.* (1979) have shown that the expected increase in exclusion probability in parentage cases, using isoelectric focusing for PGM_1 phenotyping, is realized in practice. The chances of excluding a true nonfather are about 25% with the 10 phenotypes in western Europeans.

Chen *et al.* (1977) have recently shown that PGM_1 patterns are fully developed in fetal blood.

27.4.2 PGM grouping in bloodstains

27.4.2.1 Development of methods. In 1967, Culliford reported that dried bloodstains could be successfully typed for the PGM_1 isozymes. The method was the original one of Spencer *et al.* (1964b). Horizontal starch gel electrophoresis was performed for about 17 hours at 5° using 0.1M Tris, 0.1M maleic acid, 0.01M EDTA and 0.01M $MgCl_2$ adjusted to pH 7.4 with NaOH as bridge buffer, and a 1:10 dilution of this solution as gel buffer. Gels were sliced horizontally following electrophoresis, and overlaid with a 1% agar gel containing Glc-1-P, Glc-1,6-diP, $MgCl_2$, NADP, Glc-6-P dehydrogenase, PMS and MTT. The original workers had applied the "activity stain" to the gel on filter paper. The activity stain for PGM is based on the reaction sequence indicated in Fig. 27.3. NADPH generated in the reaction sequence will reduce the MTT-tetrazolium dye to an insoluble formazan in the presence of PGM. It may be noted that Glc-1,6-diP is required in catalytic quantities, and that most examples of less than highly purified Glc-1-P contain sufficient amounts of it as an impurity. Wraxall and Culliford (1968) soon found that the starch gels could be made "thin" to begin with (about 1 mm), and that enzyme typing on these was far more convenient because they did not have to be sliced prior to application of the detection mixture. The technique is fully described by Culliford (1971). The second locus phenotypes could be determined as well, but the isozymes determined by PGM_2 were found to be not as stable as those of PGM_1. The former were detectable in bloodstains a week to two old. PGM_1 isozymes were generally determinable in stains a month old, and sometimes to as much as three months. Occasionally, much fresher stains are found to be untypable too.

There have been many technical modifications of the original procedure, and a number of supporting media other than starch have been employed for the determination of PGM isoenzymes. Brinkmann and Fritz (1968) described a buffer system for PGM consisting essentially of the original buffer components, but adjusted to pH 7.2. The gel was made up in a 1:9 dilution of bridge buffer, adjusted to pH 7.5. This system was used to group PGM in bloodstains (Brinkmann, 1969). Oepen (1970) found the Culliford (1967) system entirely satisfactory for bloodstains, except that the buffer system of Radam and Strauch (1969) was used. These latter described a discontinuous buffer system for PGM consisting of Tris-acetic acid, pH 7.4, for bridge buffer, and a 2.9 mM phosphate buffer, pH 7.2, for the gel. This, they said, gave lower current flow and less heating during the electrophoretic run.

Horizontal polyacrylamide gels have been employed for typing PGM and other isoenzymes as well. Wrede *et al.* (1971) reported successful PGM_1 typing in such a system using the original buffer system of Spencer *et al.* (1964b) for the anodic tank and 0.33M phosphate buffer, pH 6.2 for the cathodic. Gel buffer was 10 mM phosphate, pH 6.9. Further details were given by Hoppe *et al.* (1972). Some polymerization initiators and catalysts (see in section 2.3.7.1) can inhibit some of the enzymes. Notably, TEMED inhibits red cell acid phosphatase (section 29.5.3), and it was found best to polymerize the gels with 3-dimethylaminopropionitrile (DMAPN), potassium ferricyanide and ammonium persulfate. The gels were soaked a long time in water, and then in gel buffer prior to use, this procedure serving to have gels ready when they were required as well as to wash out excess monomers and initiators which might inhibit enzymatic activity. This system was applied to red cell lysates, rather than to bloodstains.

Cellulose acetate foils have been used as well. Sonneborn (1972) reviewed his own work, as well as that of some others, in the development of cellulose acetate membrane (CAM) methods for a number of enzymes, including PGM. References to his earlier papers are cited. The methods were developed for lysates. Raszeja and Miscicka (1976), however, said that they had obtained very good results using a slightly modified Sonneborn method for bloodstains. Miscicka *et al.* (1977) reported good results with Cellogel CAM foils for PGM in bloodstains. Grunbaum (1974) developed a microprocedure for PGM on CAM using the Beckman microzone system. Eight samples could be run simultaneously. Zajac and Sprague (1975) said that this system gave good results with bloodstains.

Agar and agarose have been used as electrophoretic support media for the separation of PGM isozymes. Monn

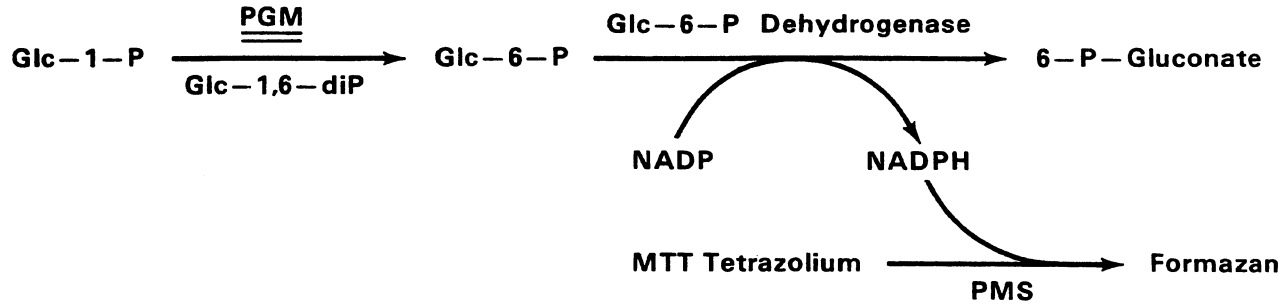


Figure 27.3 Detection Reaction Sequence for PGM

(1968b) looked into this type of system in a systematic way. He preferred Reinagar to the agarose and Bactoagar that were tried, and the gels were made to 1% concentration on microscope slides. The most desirable gel buffer was 2.7mM monobasic potassium phosphate and 1.3 mM dibasic sodium phosphate, ionic strength 0.01, pH 7.4. Twice this concentration was used for the bridge buffer, and the system was recommended for routine lysate typing. Kneiphoff and Nagel (1970) employed this system in their work. Agarose-acrylamide gels have been used on a preparative scale to separate the products of the three PGM loci (McAlpine *et al.*, 1970b).

The isoelectric focusing systems discussed in section 27.1.1, whose application to PGM_1 isozyme typing suggested the four allelic gene hypothesis, are applicable to bloodstains (Bark *et al.*, 1976; Sutton and Burgess, 1978). With 10 phenotypes, instead of the three seen on other support media, the discrimination index for PGM_1 is significantly increased. Application of procedures capable of detecting the ten (rather than three) phenotypes is often referred to as "PGM_1 subtyping".

There are a number of reports describing what may be called multiple systems, in which more than one polymorphic enzyme or protein is determined in one and the same electrophoretic run. Such systems may be devised in a number of ways. Buffer systems can be found in which the products of several independent isozyme system loci are separated on a gel, and can then be detected separately. If thick starch gels are used, and sliced before staining, there are two gel surfaces to work with. Some of the enzymes can be detected with substrates which yield a fluorescent product. Gels can sometimes be incubated first with these substrates, and examined for the phenotypes under long wave UV light, and then stained for other enzymes with filter paper or gel overlays. Hummel (1970) combined PGM with EAP on one gel, and ADA with AK on a second. Wrede *et al.* (1971) could detect PGM_1, ADA and AK isozymes on one 30 cm long acrylamide gel. Martin and Niebuhr (1971) did the same with PGM, ACP, AK and ADA. Gussmann and Rames (1972) combined PGM and GPT typing on one gel, and Neilson *et al.* (1976) reported a system for the simultaneous detection of PGM, AK and Peptidase A. Wraxall and Stolorow (1978) have devised a system for determining PGM, ESD and GLO on one plate, and the procedure is applicable to bloodstains as well as to lysates.

Weidinger and Schwarzfischer (1980) reported on a procedure for PGM_1 subtyping using agarose gel isoelectrofocusing (AGIF) for hemolysates. All ten types were readily determinable using this system.

Shaler and Dhawan (1980) recently reported that the PGM_1 subtypes could be determined by conventional electrophoresis on agarose gels (1%) using 5.7mM phosphate-2.5mM citrate gel buffers and 0.29M phosphate-0.1M citrate bridge buffers, both at pH 5.6. This system was suggested as an alternative for laboratories not set up for isoelectric focusing. Bloodstains could be typed using the system. They have also found (Dhawan and Shaler, 1980) that this electrophoretic system is suitable for the simultaneous determination of PGM_1 subtypes and Gc phenotypes (section 41).

Marbach (1980) carried out a series of experiments in which the concentration of PMS in the overlay detection mixture used for visualizing PGM isozymes was varied. It is well known that the gel "background" slowly darkens upon incubation with PMS- and MTT-containing overlays because of the light sensitivity of the system. Most authors have recommended PMS concentrations ranging from 0.16 to 0.65mM. Marbach (1980) said that PMS concentrations of 0.016mM gave clearer results in PGM typing, and allowed for longer incubation periods than were possible with the higher PMS concentrations.

Goetz and Baxter (1980) reported a most interesting case in which two blood specimens (including stains), which were identical in ABO, Rh, MN, Duffy, Kidd, Lutheran, Xg(a), Hp, AK, ADA, ACP, ESD, GLO and conventional PGM types, were discriminated by PGM_1 subtyping.

27.4.2.2 Survival of PGM isoenzymes in blood. Most systematic studies on the survival of PGM or other markers in dried blood are carried out on experimental bloodstains. Such experiments yield very useful data, but the fact remains that examiners seldom have any control over, or knowledge of, the conditions characterizing the history of a bloodstain submitted in a case. Many factors affect the retention of enzymatic activity, and little is known about the detailed way in which these influence the proteins. As a result, occasional examples of quite fresh stains may be found which are without activity, and some rather old stains may yield satisfactory results. A related problem is that of extractability. If the protein cannot be induced to enter the gel or cellulose acetate membrane, the sample is not groupable even though the enzyme might still be active. The presence of inhibitors as contaminants is equally possible. There is also some apparent variability in the survival characteristics of an enzyme depending upon the electrophoretic and activity-detection systems used.

Culliford (1971) said that PGM_1 isozymes were usually determinable in four week old stains, and in occasional stains up to 12 weeks old. Brinkmann (1969) said that bloodstains on absorbent materials were typable for up to 7 weeks, and dried blood on harder substrata, which could be scraped off, could be typed for PGM for up to 12 weeks. Oepen (1971) could group stains 8–10 weeks old in most cases, and occasionally a stain 20 weeks old. Herzog and Sobotka (1972) typed bloodstains 9 weeks old. Welch (1972b) found that PGM was determinable in 4 week old bloodstains, at which point the experiment ended. Rothwell (1970) found that PGM was typable in some stains up to 5 months old, while others gave unreadable results at 2 months age. About half the stains were typable at 3 months age, 16% at 5 months, and by six months, activity was virtually gone in all samples. Turowska (1971) said that 2 month old bloodstains could be typed reliably. Denault *et al.* (1978) looked at the detectability of PGM isozymes in bloodstains

on glass, cotton, nylon, wool, perma-press material and denim under high and low humidity storage conditions. In most cases, the stains could be phenotyped for up to 26 weeks. Under some conditions, the phenotypes could be determined at 13 weeks, but not at 26 weeks. High humidity tended to increase the rate at which the enzymes became undetectable, and PGM was detected less often in bloodstains on perma-press and denim at 26 weeks than on other substrata. Several reports indicate that PGM in fairly old stains is possible on CAM. Raszeja and Miscicka (1976) and Miscicka *et al.* (1977) said that six month old stains were routinely determinable, and a type was obtained from one stain that was 2-1/2 years old. Zajac and Sprague (1975) could type stains 20 months old in their studies.

Herzog and Sobotka (1972) found that 90% of samples standing at room temperature for 106 days could be grouped for PGM. With post-mortem samples, they said that results could sometimes be obtained with bloods taken more than 4 weeks after death. Rothwell (1970) found that frozen lysates were typable for PGM for about 18 months with rare exceptions, but that only about half the samples were still active after 2 years. Rees *et al.* (1975) examined the PGM types in blood specimens collected for blood alcohol determination after various amounts of time at room temperature. The containers have NaF and potassium oxalate in them so as to yield final concentrations, when filled, of about 1% and 0.25%, respectively. The containers were only filled to about 2/3 capacity in the studies. Some samples could be correctly typed for PGM when 72 days old, but some failed to give results at 44 days. With older samples, there were some apparent changes in phenotype, which will be discussed below. All the samples could be correctly typed for up to 32 days.

It may be noted that PGM appears to be stable almost indefinitely in lysates kept in liquid nitrogen (Culliford, 1971; and our own observations).

27.4.2.3 Problems in PGM grouping of blood. Most of the time, PGM_1 enzymes can be typed in relatively fresh stains without difficulty. In older stains, there is sometimes development of a diffuse band in the d band region, which can cause difficulty in typing (Culliford, 1971). Older specimens of whole blood, or post-mortem samples can show considerable enhancement of activity, leading to large diffuse bands. This effect is usually accompanied by bacterial infection of the sample (Culliford, 1971). We have observed such patterns in post-mortem samples on a number of occasions.

The only reports on actual misgrouping because of sample age have been concerned with stored whole blood, rather than with dried stains. Brinkmann (1974) noted that samples containing NaF, and standing for up to a year at 4°, could undergo apparent phenotypic changes. In eight samples, out of 58 studied, PGM "2-like" patterns were seen in samples that were PGM 1 or PGM 2-1 when fresh. Brinkmann thought that the fluoride might be affecting the enzyme. Culliford (1971) noted that the presence of fluoride in a sample can cause considerable distortion of the PGM band pattern, but this was primarily an ionic strength effect. Rees *et al.* (1975) found two alterations in their study of the blood alcohol samples, which had been kept at 4°, and contained NaF. In one case, a PGM 2-1 gave correct results for 32 days, no result at 44 days, and then a PGM 2 pattern at 51 days. In another, a PGM 1 sample, correctly grouped at 32 days, was negative at 44 days, and gave a PGM 2-1 result at 51 days. It would appear that considerable caution should prevail in interpreting PGM patterns from these kinds of samples.

27.4.3 PGM grouping of semen, vaginal secretions and other tissues.

Culliford first reported the detectability of PGM_1 isozymes in seminal plasma in 1969 at the 5th International Meeting of Forensic Sciences in Toronto. Culliford (1971) discussed the subject in his book as well. Around this same time, Renninger and Sina (1970) noted that PGM_1 enzymes may be determined in spermatozoa, and this observation has since been confirmed in a number of laboratories (Brinkmann and Koops, 1971; Erickson, 1974; Blake, 1976; Blake and Sensabaugh, 1976). The PGM_1 isozymes, as determined by thin starch gel electrophoresis, were the same in seminal plasma as in red cells lysates from the same individual, although the concentration of the enzyme was lower in seminal plasma, and more material was required (Culliford, 1971). It was found, too, that detectable PGM_1 enzymes may be seen in vaginal secretions as well. In sexual assault cases, therefore, the victim's PGM_1 type must be known before any typing result from a vaginal swab that contains semen can be interpreted. Red cell and seminal plasma PGM phenotypes in the same person were always the same, and this fact has been confirmed by Rees *et al.* (1974). Radam and Strauch (1971) confirmed that PGM_1 phenotypes can be detected in seminal stains, and that cervical mucus obtained post-mortem had considerable PGM_1 activity.

In 1975, Rees and Rothwell carried out a study on the determination of PGM_1 isoenzymes in seminal stains and in post-coital vaginal swabs. Seminal stains, made from semen samples of known age, were examined for PGM a number of times up to a stain age of 35 days. 30 stain specimens from 28 individuals were included, and seminal plasma type was the same as red cell lysate type in every case where the comparison could be done. Two seminal plasma specimens had no detectable PGM activity. Eight stain specimens were inactive or untypable at 2 days, although prepared from active semen. In one peculiar case, a PGM 2-1, the specimen was untypable at 2 days, inactive at 8 days, but correctly typed at 15 days. From the data, it appeared that loss of activity in seminal stains upon aging occurs at a faster rate than in bloodstains. There was one discrepant result. A PGM 1 stain grouped as a PGM 2-1 at 7 days, but was correctly grouped at 17 days. It should be noted that the seminal samples had in some cases been stored frozen after collection and prior to making stains. Sixty-two post-coital swabs from 37 persons were examined for PGM. The

amount of semen present was assessed by sperm density, and qualitative acid phosphatase tests. As many as three swabs were taken and tested but not with every subject. In 21 of 37 "first swabs", no PGM activity could be detected. In 12 of the remaining "first swabs", the PGM type was identical to that of the woman's red cells, although in the case of seven of the couples, the PGM types were the same in both people. Discrimination of PGM isoenzymes of seminal origin is possible only in cases where the woman is homozygous, and components representing the product of the other allele are found. Taking several swabs appeared to be useful, since in a few cases results were obtained with a second and/or third one, while the first swab was negative. In some cases, the swabs contained blood, which could account for the results obtained. There were four instances of discrepant results. In two, the swab type differed from that of either partner. In another, a swab from a PGM 1 woman with a PGM 1 partner grouped as PGM 2 on the first swab, but as PGM 1 on two further swabs. In one case, a pair of swabs was grouped as PGM 2-1 from a woman whose red cells may have been PGM 4-1, and who had a PGM 1 partner. Rees and Rothwell (1975) said that seminal stain PGM grouping appeared to be reasonably worthwhile, but that much difficulty was associated with PGM typing of vaginal swabs.

In 1976, Price *et al.* presented the results of another extensive study of PGM typing in seminal plasma, seminal stains and post-coital vaginal swabs. Semen could be grouped reliably, and loss of activity was temperature dependent, being greatest at 37°, least at −15°. Seminal stains were active for 12 days when stored at room temperature, but at 37°, activity was lost within 24 hours. Vaginal swabs were examined from two groups of subjects, one in which no semen was present in any of the swabs, and the other in which swabs were taken post-coitally. 471 semen-free swabs from seven subjects over the course of 33 menstrual cycles were examined for PGM activity. 27% of these had readable PGM isoenzymes. Between 25% and 30% of these showed activity only in the first few days of the menstrual cycle, and the activity was thought to be due to the presence of blood. Other donors showed sporadic activity around midcycle, or else more or less continuous activity with a gap around midcycle. In all, 17% of the swabs examined showed PGM activity attributable to vaginal secretions rather than blood. In the post-coital swab part of the study, 769 samples were examined, and 471 of these were semen-free. Of the remaining 298 swabs from subjects who were sexually active during the course of the study, about half (158) were found to have semen present. Of these 158, 59 had readable PGM activity. It can happen that swabs with high semen density, as judged by sperm cells or acid phosphatase activity, do not yield readable PGM. Experiments on the artificial mixing of semen of known PGM type with semen-free swabs, and incubation of these swabs at 37° in a moist environment, indicated that PGM activity was lost within about 6 hours. The length of time between the deposition of semen and collection of the swab was regarded as the most critical factor in determining whether or not a typable PGM would be observed. It was found that inexperienced readers of PGM plates can sometimes misinterpret the band patterns, but experienced readers did not make errors. It was concluded that, under favorable circumstances, there is no reason not to attempt PGM typing in swabs, provided an electrophoretic system is used which gives a clear b and c band separation, and that care is taken with the interpretation of the patterns obtained. Only experienced readers, it was said, should be allowed to read and interpret PGM plates for judicial presentation. The potential value of PGM typing in vaginal swabs in cases of sexual assault was briefly discussed by Willott (1975).

Eastwood (1977) reported some experiences with the PGM typing of vaginal swabs. In many samples, a "fast" band of PGM activity was seen with swabs, and in semen-free swabs this band was much clearer than the bands due to the first and second PGM loci. The band was seen in semen-positive swabs as well. PGM activity in vaginal swabs was greater in samples collected from sexually stimulated women than otherwise. Tanton (1979) was unable to confirm any correlation between vaginal PGM patterns and degree of sexual stimulation. White *et al.* (1978) recently noted that, in grouping 52 semen-positive vaginal swabs from sexual assault cases, the results were very frequently identical to the victim's PGM type. It is unreasonable to imagine that their results are entirely due to chance alone, and the explanation would appear to be that vaginal PGM is being observed in a significant number of case swabs examined. They emphasized the necessity of knowing the victim's PGM type if PGM typing in vaginal swabs is to be carried out in the investigation of sexual assault cases.

Linde and Molnar (1980) reported on a procedure in which they could simultaneously determine the PGM_1 types of seminal samples from sexual assault cases, and separate the seminal and vaginal acid phosphatases for identification purposes (section 10.3.5).

Renninger and Sina (1970) first noted minor differences in banding patterns between the red cell PGM_1 as against that in spermatozoa. There is a band in approximately the d–e position which is not seen in red cells. The presence of this band was noted by Brinkmann and Koops (1971), and called "d'". They observed two additional bands, called e' and f', running slightly faster than e and f, the latter being characteristic of PGM_1 2 types. They noted that the PGM_3 isoenzymes were present as well. These observations were confirmed and extended by Blake (1976) and Blake and Sensabaugh (1976). They noted that the "extra" band in sperm cell PGM may be the same, but slightly differently expressed in PGM_1 1 and PGM_1 2. The PGM_2 locus was said to be monomorphic in seminal plasma and sperm cells, i.e., the genetic variants seen in the red cells are not expressed. PGM_3 isoenzymes are present and can be grouped, but the concentration is low, and the PGM_1 enzymes have to be overstained to be able to read the third locus types. The differences in PGM_1 expression particularly must be appreciated in reading PGM types from seminal stains or from

semen-positive vaginal swabs. The differences in PGM_1 locus expression, Blake and Sensabaugh said, could account for some of the aberrant results reported by Rees and Rothwell (1975). Sensabaugh *et al.* (1979 and 1980) have found that seminal PGM_1 patterns are altered if the seminal samples are contaminated with saliva. These alterations could lead to typing errors if the presence of the contaminant was unrecognized. Blake (1976) did not find PGM_1 activity in vaginal secretions in the absence of blood, except in one case where the pattern in no way resembled that of human isoenzymes, and was attributed to microorganisms.

Blake and Sensabaugh (1978) have carried out an extensive series of studies on the concentrations of isoenzyme and serum protein (see Unit VII) genetic markers in human spermatozoa and seminal plasma as compared with that in the blood. Most of the PGM activity of semen is attributable to seminal plasma rather than to cells. Semen contains about half the PGM activity found in blood on a per mℓ basis. It was estimated that the minimum quantity of whole semen or seminal plasma needed for PGM detectability was about 1 μℓ.

Sutton (1979a and 1979b) established that the extended *PGM*$_1$ system phenotypes detected by isoelectric focusing, can be determined in semen, and further that the seminal type always matched the blood type from the same individual. It was also possible to diagnose the multiple phenotypes in human buccal cells (Sutton, 1979c).

The stability of PGM enzymes has been discussed above in connection with the phenotyping of this system in blood, bloodstains, semen, seminal stains, and vaginal swabs. Rothwell and Sayce (1974) found that PGM isoenzymes in a number of other human tissues, including adipose tissue, muscle, liver and brain, deteriorate very rapidly unless the material is kept at refrigerator or freezer temperatures.

PGM isoenzymes may be determined in the tissues of human teeth, particularly in dental pulp (Suyama and Imai, 1975; Turowska and Trela, 1977). PGM_1 can be phenotyped in hair roots as well (Oya *et al.*, 1978; Twibell and Whitehead, 1978; Yoshida *et al.*, 1979). The isoelectric focusing procedure is equally applicable to hair roots, and the ten phenotypes can be distinguished with this procedure (Burgess *et al.*, 1979).

Brinkmann (1971) gave a good review of the literature of PGM isoenzymes and the medicolegal applications of the system.

27.5 The Distribution of PGM Phenotypes in U.S. Populations

The data are presented in Table 27.1.

Table 27.1 Distribution of *PGM*, Phenotypes in U.S. Populations

Population	Total	Frequency — Number (Percent) PGM, 1	PGM, 2–1	PGM, 2	Other	*PGM*$_1^1$ ★	Reference
CAUCASIAN							
San Francisco, CA	271	169 (62.4)	83 (30.6)	19 (7.0)		0.777	Lie-Injo, 1966
Chicago, IL	101	68 (67.3)	30 (29.7)	3 (3.0)		0.824	Shih and Hsia, 1969
Seattle, WA	508					0.752	Giblett, 1969
New York, NY	164	102 (62.2)	51 (31.1)	11 (6.7)		0.777	Mondovano and Gaensslen, 1975
Philadelphia, PA	180	(55.9)	(35.1)	(7.9)	(1.1)	–	Polesky et al., 1976
Pittsburgh, PA	1,253	698 (55.7)	487 (38.9)	67 (5.3)	1 PGM, 6–2	0.751	Hagins et al., 1978
California	5,972	(58.9)	(35.6)	(5.4)	(0.1)	0.768	Grunbaum et al., 1978b
Bexar County, TX	200	(64.0)	(30.0)	(6.0)		0.790	Ganaway and Lux, 1978
Detroit, MI	503	288 (57.3)	179 (35.6)	36 (7.2)		0.751	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade Co., FL	367	218 (59.4)	123 (33.5)	26 (7.0)		0.762	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	386	220 (56.9)	149 (38.6)	17 (4.4)		0.763	Siglar, 1979 and see Shaler, (1978)
NEGRO							
San Francisco, CA	284	188 (66.2)	77 (27.1)	17 (6.7)	2 PGM_2 2–1	0.801	Lie-Injo, 1966
Ann Arbor, MI	202	144 (71.3)	52 (25.7)	6 (3.0)	1 PGM_2 2–1	0.84	Brewer et al., 1967a
Chicago, IL	101	62 (61.4)	31 (31.0)	8 (7.9)		0.77	Shih and Hsia, 1969
Seattle, WA	654				4 PGM_2 2–1, 1 PGM_2 3–1	0.809	Giblett, 1969
New York, NY	133	88 (66.2)	39 (29.3)	6 (4.5)		0.808	Mondovano and Gaensslen, 1975
Philadelphia, Pa	180	(59.1)	(35.1)	(3.9)	(1.9)	–	Polesky et al., 1976
Pittsburgh, PA	714	481 (67.4)	209 (29.3)	(3.4)		0.821	Hagins et al., 1978

Table 27.1 (Cont'd.)

Population	Total	Frequency — Number (Percent) PGM, 1	PGM, 2–1	PGM, 2	Other	PGM_1^1★	Reference
California	1,024	(66.2)	(29.5)	(4.0)	(0.3)	0.812	Grunbaum et al., 1978b
Bexar County, TX	200	(64.0)	(32.0)	(4.0)		0.800	Ganaway and Lux, 1978
Detroit, MI	504	310 (61.5)	176 (34.9)	18 (3.6)		0.790	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade Co., FL	344	215 (62.5)	113 (32.8)	16 (4.7)		0.789	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	171	118 (69.0)	46 (26.9)	7 (4.1)	1 PGM, 3–2	0.825	Siglar, 1979 and see Shaler, (1978)
CHINESE							
San Francisco, CA	110	64 (58.2)	36 (32.7)	6 (5.5)	3 PGM, 6–1, 1 PGM, 7–1	0.764	Lie-Injo et al., 1968
Seattle, WA	212					0.776	Giblett, 1969
New York, NY	156	98 (62.8)	51 (32.7)	7 (4.5)		0.792	Mondovano and Gaensslen, 1975
ASIAN							
California and Hawaii	3,044	(59.0)	(35.0)	(5.6)	(0.4)	0.769	Grunbaum et al., 1978b
HISPANIC							
New York, NY	129●	74 (57.4)	43 (33.3)	11 (8.5)	1 probable PGM, 6^{JAP}–1	0.7461	Mondovano and Gaensslen, 1975
California ☆	1,586	(58.7)	(34.7)	(6.2)	(0.4)	0.764	Grunbaum et al., 1978b
Bexar County, TX	200	(61.0)	(34.0)	(4.0)		0.780	Ganaway and Lux, 1978
Miami/Dade Co., FL	362	204 (56.4)	139 (38.4)	19 (5.2)		0.756	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	198◇	117 (59.1)	76 (38.4)	5 (2.5)		0.783	Siglar, 1979 and see Shaler, (1978)

★ Gene frequency ☆ "Chicano/Amerindian" ● Primarily Puerto Rican ◇ Primarily Mexican

SECTION 28. ADENYLATE KINASE

28.1 Recognition of Adenylate Kinase

Adenylate Kinase (AK; ATP:AMP phosphotransferase; E.C. 2.7.4.3; myokinase) catalyzes the reversible conversion of ATP and AMP to ADP, according to the stoichiometry ATP + AMP $\rightleftarrows$ 2 ADP. In 1943, Colowick and Kalckar were studying transphosphorylation reactions in various tissues, and noticed that hexoses could in some way become phosphorylated, apparently enzymatically, in the presence of ADP. The hexokinase reaction, in which ATP transfers its terminal phosphate group to hexose, was well known. At the suggestion of Dr. Marvin Johnson of the University of Wisconsin, they pursued a search for an enzyme that could interconvert ADP and ATP. The enzyme was soon found (Colowick and Kalckar, 1943; Kalckar, 1943), and at the time, it was called myokinase. The reaction was characterized as a "phosphate dismutation" since ADP could be both donor and acceptor of phosphate. In 1951, Colowick re-named the enzyme "adenylate kinase". The presence of the enzyme in red blood cells was noted by Kotel'nikova (1949). In 1958, Kashket and Denstedt in Canada, and Tatibana *et al.* in Japan, recognized the enzyme in human red blood cell lysates. The enzyme was remarkably stable, as noted by a number of these workers. Cerletti and Bucci (1960) looked at the red cell enzyme's properties, and found that it was Mg^{++} activated and optimally active at pH 7.5. It was found in the soluble material of the red cell, and did not appear to be bound to stroma.

28.2 AK Polymorphism

28.2.1 *AK*$_1$

In 1966, Fildes and Harris found that red cell adenylate kinase from different persons exhibited electrophoretic heterogeneity on starch gels. Lysates were run in a gel made up in 5 mM histidine, pH 7, and with 0.41M citric acid adjusted to pH 7 with NaOH as bridge buffer. Three phenotypes could be distinguished, and these could be accounted for on the basis of two alleles, AK^1 and AK^2, operating at an autosomal locus. Family studies on 54 matings with 136 children were consistent with the postulated mode of inheritance. In almost 1,000 unrelated English people, some 90% were AK 1, about 10% were AK 2-1, and AK 2 was quite rare. Two different activity detection systems could be used for AK. The reaction sequences for these are shown in Figure 28.1. In the first, ADP is furnished as substrate, and the ATP produced coupled to the Glc-6-phosphate dehydrogenase reaction through the hexokinase reaction to produce NADPH, which can reduce MTT tetrazolium. In the second, an equimolar mixture of ATP and AMP is provided, and the ADP produced allowed to react with PEP to form pyruvate (and ATP). The pyruvate is reduced to lactate by exogenously added LDH, the NADH cofactor which is fluorescent being oxidized to nonfluorescent NAD in the process. Bands of AK activity thus show up as dark zones on a fluorescent background. The first procedure is somewhat more satisfactory and is usually used for routine phenotyping.

The two allele hypothesis of inheritance has been widely confirmed by family studies (Rapley *et al.*, 1967; Bowman *et al.*, 1967; Berg, 1969; Lamm, 1971b; and others). Bowman *et al.* (1967) found another phenotype which was believed to reflect heterozygosity of AK^1 and a new allele, called AK^3. Another phenotype, believed to be the result of a fourth allele, was observed too, and the phenotype was tentatively called AK 4-1. Electrophoresis was carried out by these workers in starch gels using 0.5M phosphate buffer, pH 6.2, for the electrode vessels, and 1:5 dilutions of this buffer for the gel. AK 4-1 was reported in a French family by Rapley *et al.* (1967), and shown to be due to a fourth allele AK^4. Giblett observed another example of an AK 4-1 (personal communication to Harris *et al.*, 1968). In 1972, Santachiara Benerecetti and collaborators found a new phenotype among 600 subjects in southern India, and it was shown to be due to the presence of a fifth allele, AK^5. The phenotype

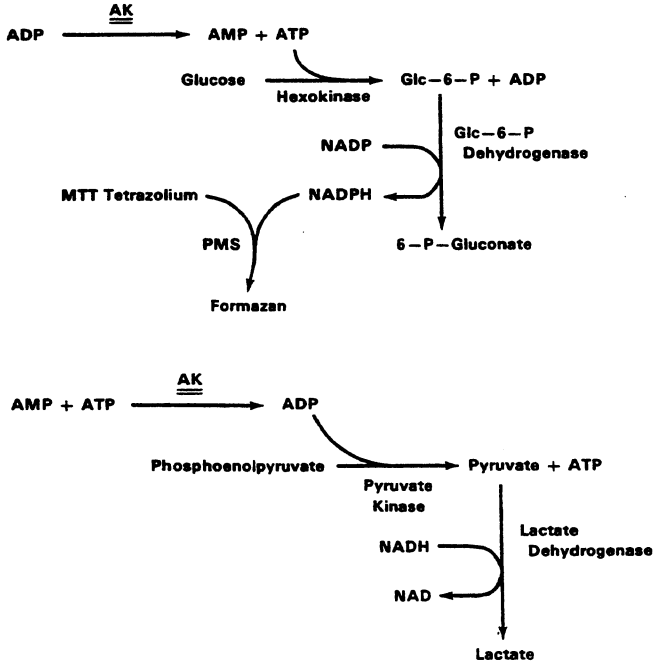


Figure 28.1 Detection Reaction Sequences for AK

observed was an AK 5-1 (Santachiara Benerecetti *et al.*, 1972a). The patterns for the AK phenotypes seen if electrophoresis is carried out at pH 7 are indicated diagrammatically in Figure 28.2.

There are a few reports of families having members lacking AK activity. Szeinberg *et al.* (1969a and 1969b) found an Arab boy and his sister who both had very low AK activity. The parents, who were related, and four other sibs, had AK activity varying from about 22% to 72% of normal. Some of the people had anemia, and the AK deficiency appeared to be transmitted autosomally, with partial expression in the heterozygotes. Singer and Brock (1971) found a patient with 50% normal activity, who was an AK 1. The half-normal activity was segregating in the patient's family, and was most easily accounted for by the presence of a silent allele. Weissmann and Pribeen (1979) formed an apparent AK° in a family in which two AK 2 children had been born to an AK 1 mother where the father was AK 2-1.

28.2.2 Additional AK loci—linkage relations

In 1970, Brock looked at the AK isozymes in a variety of human tissues (Brock, 1970a). Three sets of isozymes could be distinguished in the varous tissues, and were not expressed in the same way in different ones of them. The suggestion, however, was that the differences could be accounted for by differential expression of similar genotypes, and by the fact that there was complexation of hemoglobin by red cell AK. In 1972, Khoo and Russell found that the AK isozymes of both human and rabbit tissues could be distinguished not only on the basis of electrophoretic mobility, but also by differential inhibition of the enzymes with $AgNO_3$ and anti-rabbit muscle AK serum. In both species, the isozymes of the red cell, skeletal muscle and brain were similar, and inhibited by low concentrations of silver ion, while those of liver, kidney, spleen and heart were similar, and relatively insensitive to silver ion inhibition. Nguyen *et al.* (1972) thought that the "second set" of isozymes was the result of an additional AK locus, AK_2, and man-mouse hybrid cell studies indicated that AK_2 was syntenic with PGM_1 and Peptidase C loci on chromosome 1. In 1974, Russell *et al.* studied the AK isoenzymes of a number of human tissues, and said that on the basis of a number of different properties, there were at least six different tissue-specific AK isozymes. Some of these differed in MW as estimated by gel filtration chromatography.

In 1976, Wilson *et al.* found a set of isoenzymes active with GTP or ITP and AMP, but not with ATP and AMP. These enzymes were resistant to inhibition by Ag^+. Actually, this activity corresponds to the enzyme called GTP: AMP phosphotransferase (E.C. 2.7.4.10), but it was attributed to a third locus of AK, called AK_3. No genetic variation has yet been reported at AK_2 or AK_3, and the products of these loci are not expressed in red cells nor in skeletal muscle tissue. AK_1 has been assigned to chromosome 9, linked to the ABO and nail-patella syndrome loci (Westerveld *et al.*, 1970). Povey *et al.* (1976) confirmed the AK_1 assignment, and said that AK_3 was also assigned to chromosome 9, syntenic with the soluble aconitase locus.

28.3 Biochemical Studies on AK

The early studies by Kalckar and Colowick were mentioned in section 28.1. The older biochemical studies have

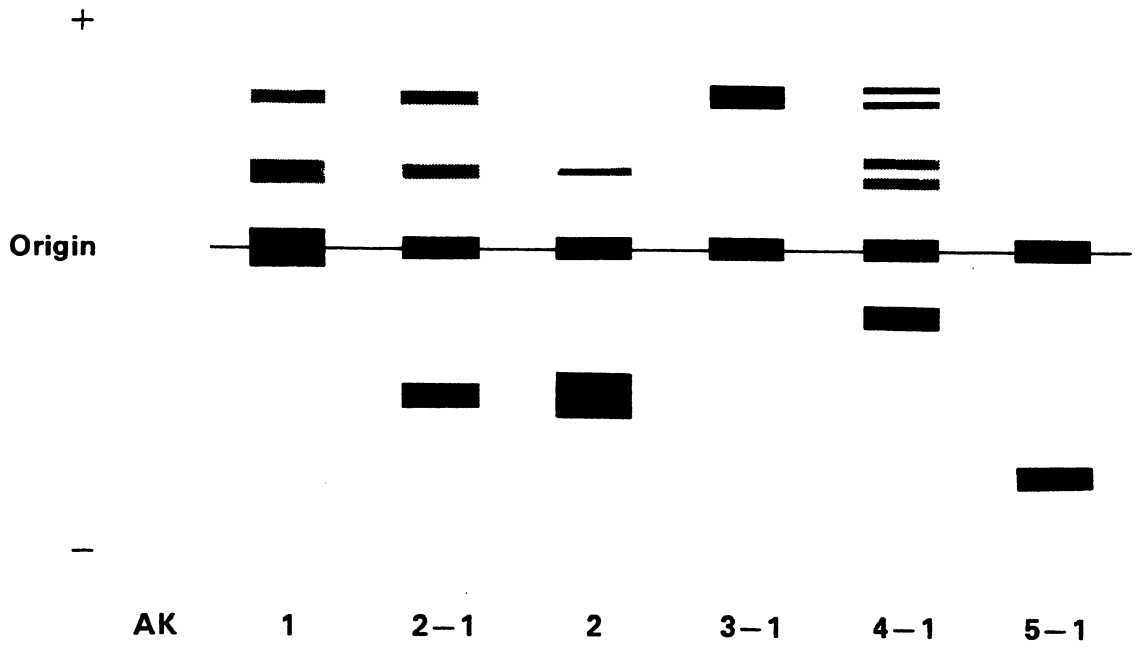


Figure 28.2 Electrophoretic Patterns of AK Phenotypes at pH 7.

been reviewed by Noda (1962), and the more recent work by the same author (Noda, 1973). The enzyme from muscle has 2 sulfhydryl groups per molecule, and although −SH reagents inhibit enzymatic activity, the −SH groups do not appear to be directly involved in substrate binding, nor in catalysis *per se*. There is good evidence that a histidine residue is involved in the catalytic mechanism. A divalent metal ion is required for AK activity, and Mg^{++} is the best one for the AK from most sources. In some cases, Ca^{++}, Mn^{++}, Co^{++} or Ba^{++} can substitute for Mg^{++}, but activity is lower. The $Mg^{++}$is required to bind the substrate, and the reaction can be written as: MgATP + AMP ⇄ MgADP + ADP. AK may be assayed in solution by coupling the ATP produced to the pyruvate kinase reaction, and then to the LDH reaction, and measuring the decrease in absorbancy at 340 nm (Levin and Beutler, 1967). This scheme may also be used for the detection of AK isozymes in gels (Figure 28.1).

AK from human muscle has been extensively purified and crystallized (Thuma *et al.*, 1972). The K_m for all three nucleotides was found to be about 0.3 mM, and the MW was estimated as 21,500 by sedimentation, and as 21,700 by calculation from the amino acid composition. These values are very similar to those reported for other mammalian muscle AK enzymes. Harris *et al.* (1968) estimated the MW of the red cell enzyme as 24,000 by gel filtration chromatography, in complete agreement with the estimate of Bowman *et al.* (1967) by the same technique. There are indications that the enzymes from different human tissues may differ in MW in some cases (Russell *et al.*, 1974)

Rapley and Harris (1970) found that the mean activity of lysates from AK 1 adults was significantly higher than the activity of those from AK 2-1 adults. The differences are not seen in lysates from newborns. At the same time, newborn lysates show only about 65% of the total AK activity seen in adult lysates.

There was a suggestion in the literature by Bockelmann *et al.* (1968) that pyruvic kinase (PK; E.C. 2.7.1.40) and AK share a subunit. The suggestion was based on evidence obtained in detecting the enzymes after electrophoresis. The detection reaction sequences overlap, as may be readily appreciated by looking at Figure 28.1. PK does not show genetic variation. However, Brock (1970b) said that the assay system could be adjusted so that AK is selectively inhibited while PK is detected, and that his results gave no indication of a common subunit.

28.4 Medicolegal Applications

28.4.1 Disputed parentage

AK phenotyping can be usefully applied to cases of disputed parentage, although the probability of excluding a falsely accused man by the AK system alone is not very high. Prokop and Göhler (1976) said that only 3.5 to 4% of true nonfathers would be excluded by AK alone in middle European populations. In England, the figure is about 4.5% (Boorman *et al.*, 1977). Polesky *et al.* (1976), said that the figure was 4.29% for Caucasians, and only 0.66% for Black people. Possible occurrence of the rare silent allele, AK_1^0 should be kept in mind in the interpretation of exclusions of the second order based on AK.

Chen *et al.* (1977) have shown that the AK isoenzymes are fully developed in fetal blood.

28.4.2 AK phenotyping in bloodstains

In 1968, Culliford and Wraxall reported that AK phenotypes could be reliably detected in bloodstains by starch gel electrophoresis. The original procedure of Fildes and Harris (1966) was first successfully employed, but it was soon found that the thin starch gel method (Wraxall and Culliford, 1968) was more satisfactory for small amounts of bloodstained material. The pH 7 system of Fildes and Harris (1966) was used. The results of additional investigations on phenotyping the enzyme in bloodstains were given by Culliford (1971). Phosphate reaction buffers should be avoided with AK since they were found to inhibit the enzyme. Absolute activity values were not given, but if the enzyme is taken to have full activity at 0.01M phosphate at pH 6.8, then 80 mM phosphate inhibited about 50% and 0.2M phosphate almost completely. The AK 2 isozymes appeared to be more sensitive to this inhibition than the AK 1 isozymes. Succinate was found to have an activating effect on AK, and a pH 5 buffer system consisting of Tris-succinate for the gel, and citric acid-NaOH for the electrode vessels was successfully employed.

Other electrophoretic media than starch have been employed for AK, as have a number of different buffer systems, although not all of these have necessarily been tested in the phenotyping of dried bloodstain material. Skude and Jakobsson (1970) used 1% agar gels prepared from Reinagar for electrophoresis, with 50 mM phosphate, pH 6.2, buffers. Thirty lysates could be run on a plate about 10 × 20 cm in about 45 minutes. Thin-layer agarose electrophoresis was employed by Tsuji and Weissman (1977). Hoppe *et al.* (1972) gave a procedure for phenotyping AK in lysates on horizontal polyacrylamide gels.

Rosalki (1970) reported successful typing of AK on cellulose acetate membranes, using either the Beckman Microzone system or the Gelman Sepraphore III system. Sonneborn and Renninger (1971) confirmed these results using different buffers. Sonneborn (1972) discussed the subject further in connection with a number of isoenzymes. Saenger and Yates (1975) applied a system similar to that of Sonneborn and Renninger (1971) to AK phenotyping in bloodstains. Phosphate buffer, pH 6.25, in a final concentration of about 1.55 mM was used, the electrophoresis being completed in about 70–90 minutes. Stombaugh and Kearney (1977) found the Beckman Microzone system applicable to bloodstain phenotyping, and sixteen samples could be typed simultaneously.

There are a number of procedures reported in which AK is simultaneously determined with other isoenzymes in the same electrophoretic operation: AK with ADA (Kirchberg and Wendt, 1970), AK with PGD (Brinkmann and Thoma, 1970), AK with ADA (Hummel, 1970), AK with ACP, PGM, and ADA (Martin and Niebuhr, 1971), AK with

ADA and PGD (Brinkmann and Dirks, 1971), AK with ADA and PGM on acrylamide (Wrede *et al.*, 1971), AK with GPT, PGD and PGM (Goedde and Benkmann, 1972), AK with PGM and PEPA (Neilson *et al.*, 1976) and AK with ACP and ADA (Wraxall and Stolorow, 1978). An effort to combine AK with PGD on a single plate for bloodstain grouping was made by Culliford (1970). Phosphate buffers were used, but the system was considered unsatisfactory because low activity stains from AK 2-1 people could give apparent weak AK 1 results. The Wraxall and Stolorow (1978) procedure was specifically devised for dried bloodstain phenotyping.

28.4.3 Survival of AK in blood and bloodstains

AK is one of the more stable enzymes. In the original bloodstain phenotyping work, it was noted that AK had been typed in stains up to 3 months old (Culliford and Wraxall, 1968). Culliford (1971) said that stains up to 6 months old had been typed, but that losses of activity in bloodstains after 3–4 weeks aging could occur commonly. He said further that the succinate buffer system (pH 5) was to be preferred over the histidine (pH 7) (section 28.4.1) since, in the latter, the 2 band weakened in older stains and the 1 band tended to become diffuse. Brinkmann and Dirks (1971) said that AK could be detected in stains up to 11 months old. Rothwell (1970) found that AK could sometimes be typed in stains up to 11 months old. In a group of bloodstains, about 2/3 were typable at 5 months aging, and about 15% after six months. Some stains were untypable after 2 months, however. Welch (1972b) found that AK was completely typable during the entire course of a 30 day aging study. Saenger and Yates (1975) found that AK could be typed in stains up to 6 months old, after which the limiting factor seemed to be the inextractability of the enzyme from the older stains. Stombaugh and Kearney (1977) found that bloodstains could be typed for up to 3 months if stored at 37°, while those kept at 4° or frozen could be typed up to 18 months. Denault *et al.* (1978) found that AK 1 bloodstains on a variety of substrata were typable up to 26 weeks, regardless of whether they had been stored at high or low humidity.

AK in lysates is stable for lengthy periods of time if the samples are kept frozen (Rothwell, 1970; Culliford, 1971), and for years if kept under liquid nitrogen. In samples collected for blood alcohol determinations, and kept at room temperature, Rees *et al.* (1975) found that AK determinations were reliable for up to 100 days. At 109 and 122 days of aging, AK 2-1 samples were read as AK 1, while AK 1 samples were read correctly. As noted previously for bloodstains by Culliford (1971), therefore, the 2 isoenzyme appears to be more labile than the 1 isoenzyme.

28.4.4 AK phenotyping in other tissues

AK isozymes of the AK_1 polymorphic locus may be detected in skeletal muscle and some other tissues without undue difficulty. Rothwell and Sayce (1974) found that deterioration of activity in tissues is very rapid unless the specimens are kept cold or frozen, in which case the samples were stable for months. Oepen (1974) found that AK typing from skeletal muscle was fully reliable in terms of being identical to the red blood cell type. Turowska and Trela (1977) reported phenotyping of AK from dental pulp.

AK is present in spermatozoa, but there is very little activity in seminal plasma (Blake, 1976; Blake and Sensabaugh, 1976 and 1978). Expressed in terms of Units/mℓ, seminal plasma has about 1/1,000 the AK activity of blood. A unit of AK activity was defined as 1 μmol NADP reduced/min at 37° and pH 7.5 in a hexokinase-G6PD coupled assay system. The AK 2 isoenzyme is extremely labile in sperm cells, 24 hours storage of a specimen at 4° being sufficient to reduce its activity enormously. For this reason, it cannot be regarded as a good genetic marker in spermatozoa.

28.5 Distribution of AK Phenotypes in U.S. Populations

The data are shown in Table 28.1. AK, though very stable, is not an especially good marker in populations, the probability of distinguishing between two random people on the basis of AK being about 18%. It is of interest to note that the AK^2 gene is virtually absent in Chinese, the sole AK 2-1 individual in all the literature having been reported by Shih *et al.* (1968a). World data for AK were given by Tills *et al.* (1970a), and a 10-fold error in the figures given in that paper was corrected in Tills *et al.* (1971a).

Table 28.1 Distribution of *AK* Phenotypes in U.S. Populations

Population	Total	Frequency — Number (Percent) AK 1	AK 2–1	AK 2	*AK'*★	Reference
CAUCASIAN						
Chicago, IL	1,315 ☆	1,193 (90.7)	118 (9.0)	3 (0.3)	0.9525	Bowman et al., 1967
Ann Arbor, MI	254	240 (94.5)	14 (5.5)	0	0.9724	Brewer et al., 1967
Seattle, WA	172	163 (94.8)	9 (5.2)	0	0.969	Giblett, 1969
New York, NY		127 (93.4)	9 (6.6)	0	0.9669	Mondovano and Gaensslen, 1975
Philadelphia, PA	180	(97.3)	(2.7)	(0)	–	Polesky et al., 1976
Washington, DC	364	338 (92.9)	25 (6.9)	1 (0.2)	0.9629	Stombaugh and Kearney, 1977
California	5,969 ⊙	(92.7)	(7.1)	(0.1)	0.963	Grunbaum et al., 1978b
Detroit, MI	503	474 (94.2)	29 (5.8)	0	0.971	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade County, FL	366	339 (92.6)	26 (7.1)	1 (0.3)	0.962	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	115	108 (93.9)	7 (6.1)	0	0.970	Siglar, 1979 and see Shaler, (1978)
NEGRO						
Chicago, IL	1,063 ☆	1,049 (98.7)	13 (1.3)	0	0.9934	Bowman et al., 1967
Ann Arbor, MI	139	135 (97.1)	4 (2.9)	0	0.9856	Brewer et al., 1967a
Seattle, WA	223	220 (98.7)	3 (1.3)	0	0.993	Giblett, 1969
Chicago, IL	101	99 (98.0)	2 (2.0)	0	0.991	Shih and Hsia, 1969
New York, NY	134	130 (97.0)	3 (2.2)	1	0.981	Mondovano and Gaensslen, 1975
Philadelphia, PA	180	(100)	(0)	(0)	1.000	Polesky et al., 1976
Washington, DC	76	75 (98.7)	1 (1.3)	0 (0.8)	0.9934	Stombaugh and Kearney, 1977
California	965	(98.4)	(1.6)	(0)	0.992	Grunbaum et al., 1978b
Detroit, MI	504	501 (99.4)	3 (0.6)	0	0.997	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade County, FL	346	339 (98.0)	7 (2.0)	0	0.990	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	54	53 (98.2)	1 (1.8)	0	0.991	Siglar, 1979 and see Shaler, (1978)
HISPANIC						
New York, NY	136 □	130 (95.6)	6 (4.4)	0	0.978	Mondovano and Gaensslen, 1975
California ●	1,344	(95.6)	(4.3)	(0.1)	0.978	Grunbaum et al., 1978b
Miami/Dade Co., FL	357	339 (95.6)	18 (4.4)	0	0.975	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	74 ■	69 (93.2)	5 (6.8)	0	0.966	Siglar, 1979 and see Shaler, (1978)
CHINESE						
New York, NY	156	156 (100)	0	0	1.000	Mondovano and Gaensslen, 1975
ASIAN						
Seattle, WA ◇	146	146 (100)	0	0	1.000	Giblett, 1969
California and Hawaii	2,304	(99.8)	(0.2)	(0)	0.999	Grunbaum et al., 1978b

★ Gene frequency ☆ One person was AK 3–1 ⊙ 0.1% were rarer phenotypes ● "Chicano-Amerindians" ◇ "Mixed Oriental" □ Primarily Puerto Rican ■ Primarily Mexican

SECTION 29. ERYTHROCYTE ACID PHOSPHATASE

29.1 Recognition of Acid Phosphatase in Blood

Erythrocyte acid phosphatase (ACP; EAP; E.C. 3.1.3.2) is systematically known as orthophosphoric monoester phosphohydrolase. The name of the enzyme is abbreviated as "ACP" in this book, following Harris and Hopkinson (1976), for the reasons noted in section 29.3.1.

In 1924, it was noticed independently in two different laboratories that drawn blood, left standing for a time, showed an increase in its inorganic phosphate content (Lawaczek, 1924; Martland and Robinson, 1924). The phosphate level increased more rapidly if the blood was left at 37° than at room temperature. It was correctly recognized by Martland *et al.* (1924) that this inicrease was the reflection of an enzymatic activity present in red cells, for the effect was pronounced only when the cells were lysed. The enzyme was called "phosphoric esterase", and could be shown to hydrolyze hexose phosphate, hexose diphosphate and glycerophosphates. Roche (1931) confirmed these findings. The red cell enzyme had a pH optimum of 5.8 with p-nitrophenyl phosphate, and hydrolyzed the α-isomer of glycerophosphate better than the β-isomer. Woodard (1942) noted the presence of "acid" phosphatase activity in a number of tissues. Earlier studies on the catalytic properties of red cell acid phosphatase were carried out by Tsuboi and Hudson (1953, 1954 and 1956).

29.2 ACP Polymorphism

Hopkinson *et al.* (1963) first reported genetic variation of ACP. Five phenotypes were seen by starch gel electrophoresis, using gels made in Tris-succinate, pH 6, with citric acid-NaOH, pH 6, bridge buffer. The zones of enzyme activity were best detected with 5mM phenolphthalein-diphosphate in a citrate buffer, at pH 6, followed by incubation with ammonia to make the gel basic, and the phenolthalein colored. More diffuse bands were said to be seen if p-nitrophenyl phosphate was used as substrate, and the enzyme showed no activity with naphthyl phosphates (but see section 29.3.2). Formalin inhibited activity, but tartrate did not. Acid phosphatase assay (detection) techniques with a variety of substrates were discussed in section 10.3.4, and the material in that section is equally applicable to acid phosphatase enzymes, regardless of the source.

Family studies were consistent with a genetic explanation for ACP polymorphism based on three allelic genes, called P^A, P^B, and P^C, determining 6 phenotypes called A, BA, B, CA, CB and C. Gene frequencies in 139 English people were about 0.35 for P^A, 0.6 for P^B and 0.05 for P^C. Homozygous ACP–C persons were to be expected only about once in 400 persons in this population. Additional family and population studies (Hopkinson *et al.*, 1964) confirmed the genetic hypothesis. Many other family studies (e.g. Prokop, 1967) have been in complete accord. In 1964, Lai *et al.* found an ACP–C individual in a Brazilian population, and studies on 80 families fully confirmed the three allele scheme of inheritance for the system. In 1965, Giblett and Scott found a new phenotype in a Black person in Seattle. They carried out electrophoresis according to the original method of Hopkinson *et al.* (1963), but with formic acid-NaOH, pH 5, buffers as well. The latter were best for detecting the new phenotype, which was believed to be the result of the heterozygosity of P^a with a new allele P^r. The phenotype was called RA. The symbols P^a and P^r are equivalent to the symbols P^A and P^R. Another phenotype was reported by Karp and Sutton (1967) which was the result of another allele, P^D, at the ACP locus in heterozygous combination with P^B. This BD phenotype was seen in two Black males. Karp and Sutton used citrate-phosphate buffers, pH 5.9, as well as phosphate, pH 6.2, buffers in their studies. Further examples of the RA phenotype of Giblett and Scott (1965) were seen, as well as several RB people and one RC. The P^R gene appeared to be restricted to Black populations. It has not been reported in Caucasians, but it has been seen in Chinese. Shih and Hsia (1969) found 2 RB in 100 Chinese from Taiwan whom they grouped for ACP. This fact is not mentioned in the original paper, but was ascertained from a personal communication to Mourant *et al.* (1976). P^R occurs more commonly in some southern African Black populations, particularly in the people called Khoisan, with a gene frequency in the neighborhood of 0.2 to 0.25 (Jenkins and Corfield, 1972). The results of Shih and Hsia suggest that P^R may have an appreciable frequency in Chinese, or perhaps that the selection of persons for grouping in this case was accompanied by a bit of luck. The P^D allele was found in a European by Lamm (1970b). One BD was seen in 209 unrelated Scandinavian adults. Lamm's family studies (1970b) were fully in accord with the three allele pattern of inheritance as well. A further allele, P^E, was found in a 55 year old Danish man. He was BE, and his only living near relative, a daughter, had not inherited it from him (Sørensen, 1975).

Silent alleles have been observed at the *ACP* locus. In 1969, Herbich encountered an apparently silent allele of ACP segregating in three generations. A BA mother and an "A" father had a "B" daughter, who, in turn, had a "C" son by a CA father. The best explanation for the observations was heterozygosity for a silent allele, P^o. This conclusion was supported by activity studies which showed that the suspected heterozygotes showed only about half the activity of normal controls. Studies on this interesting Viennese family were extended by Herbich *et al.* (1970). Turowska *et*

al. (1977) reported a silent allele in a south Polish family, this case also being supported by activity measurements. The family came to their attention because of an apparent mother-child incompatibility. A second Polish family with a silent allele segregating in three generations was described by Turowska and Bogusz (1978). Brinkmann *et al.* (1974) reported a number of German families in which P^o appeared to be segregating. They estimated that the allele seemed to occur in about 1 or 2 of every 2,000 people. Nezbeda (1979) found a silent allele of acid phosphatase segregating in three generations of a Czechoslovakian family.

In 1973, Gussmann encountered a peculiar kind of CB phenotype, which was electrophoretically identical to the usual CB, but was very weak. This was called a "BC^x". Smerling (1973) reported that he had encountered three new phenotypes in connection with a case. These were not understandable in terms of the P^R or P^D genes. White *et al.* (1979) described an apparently new phenotype of acid phosphatase in two unrelated samples. The patterns were similar to CA on starch gels in citrate-phosphate buffers (pH 5.5), but the b_2 band was absent.

The ACP system is thus governed by three relatively common alleles P^A, P^B and P^C; there are three rarer alleles, P^R, P^D and P^E, and there can be a rare silent allele P^o in an occasional person.

The ACP phenotypes as seen in citrate (or other tricarboxylic acid) containing buffers are indicated diagramatically in Figure 29.1. The mobility of the A bands is sensitive to buffer composition, and this effect should be appreciated when comparing patterns from different sources. The presence of citrate or other tricarboxylic acids results in what Hopkinson and Harris (1969a) called a "fast A" pattern. A "slow A" pattern is seen in phosphate buffers at about the same pH (near 6). "Intermediate" patterns can be seen with various dicarboxylic acids. Results with citrate bridge buffer and Tris-succinate gel buffer are of the "fast A" category. Further details of this effect have been discussed by Hopkinson and Harris (1969a).

Brinkmann *et al.* (1971) did an interesting analysis of the aggregate ACP phenotypic frequency data in the published literature for something more than 13,000 Europeans. When a X^2 test of significance was done on the data, there was a significant discrepancy between the expected and observed values, almost entirely accounted for by the CB and C frequencies. The simplest interpretation of this finding was that significant errors had been made in reading CB and C phenotypes. Correct phenotyping of ACP should not necessarily be considered simple, therefore, even with hemolysates.

Petersen Inman (1980) did a very similar analysis of the ACP frequency data reported by Grunbaum *et al.* (1978b), and suggested that the lack of goodness of fit between the "expected" and "observed" values might indicate misclassification of some phenotypes. Selvin (1980a) replied to these observations, saying that certain of the ACP phenotypes, particularly the "C" phenotypes, could be difficult to classify in some samples. He noted, however, that the gene frequencies would be changed very little by the level of misclassification that may have been present in the data; Selvin (1980b) has treated the problem of gene frequency estimates in the presence of specific patterns of misclassification in a more general paper as well.

Problems with the phenotyping of ACP, particularly with certain of the "C" phenotypes are discussed in section 29.5.2. There is no doubt that certain phenotypes can be misjudged in this system, and that certain types of samples can cause more problems than others. A modest level of misclassification will not appreciably change the gene frequency estimates in a large population study, as Dr. Selvin (1980b) has shown, and may not, therefore, affect the data obtained very much at all. No level of error or misclassification would be tolerable, however, in the typing of ACP (or any other system) in an individual medico-legal case; here, unlike in a population survey, the individual sample types could greatly influence the outcome of the case.

29.3 Additional Genetic Loci Determining Acid Phosphatase Enzymes—Tissue Acid Phosphatases

29.3.1 Human tissue acid phosphatase isoenzymes

Apart from the red cell ACP, the other medicolegally significant acid phosphatase is the prostatic one, which occurs in relatively high concentrations in most examples of human seminal plasma, and is the basis of the most widely employed non-morphological test for semen identification (section 10.3). The genetic relationships between the ACP of the red cell, the prostate and a variety of other body tissues have only recently become clear. Tissue acid phosphatases, and their relationship to the red cell enzyme, are discussed in this section.

Recognition of the ACP activity in red cells was discussed in section 29.1. Recognition of the prostatic enzyme was discussed in detail in secton 10.3.2. Kutscher (1935) found an acid phosphatase activity in urine, which prompted him to look further for the source of the activity, particularly in male reproductive tract secretions. A high concentration of the enzyme was present in seminal plasma, and was shown to be of prostatic origin (Kutscher and Wohlbergs, 1935; Kutscher and Wörner, 1936). The Gutmans recognized the potential clinical significance of this enzyme when it was noticed that prostatic tumors led to significant increases in the ACP levels in serum and in metastasizing tissue (Gutman *et al.*, 1936; Gutman and Gutman, 1938). This enzyme displays electrophoretic heterogeneity (Sur *et al.*, 1962; Smith and Whitby, 1968; and see in section 10.3.6).

Lundin and Allison (1966a and 1966b) found electrophoretic heterogeneity in the ACP in a variety of human and animal tissues. In 1967, Beckman and Beckman reported the results of their investigations on the ACP from a number of different human organs, and from placental tissues of 1,200 individuals. Four zones of activity could be present, each organ giving a characteristic isoenzyme pattern. The zones were called "A" through "D", "A" being the most anodal

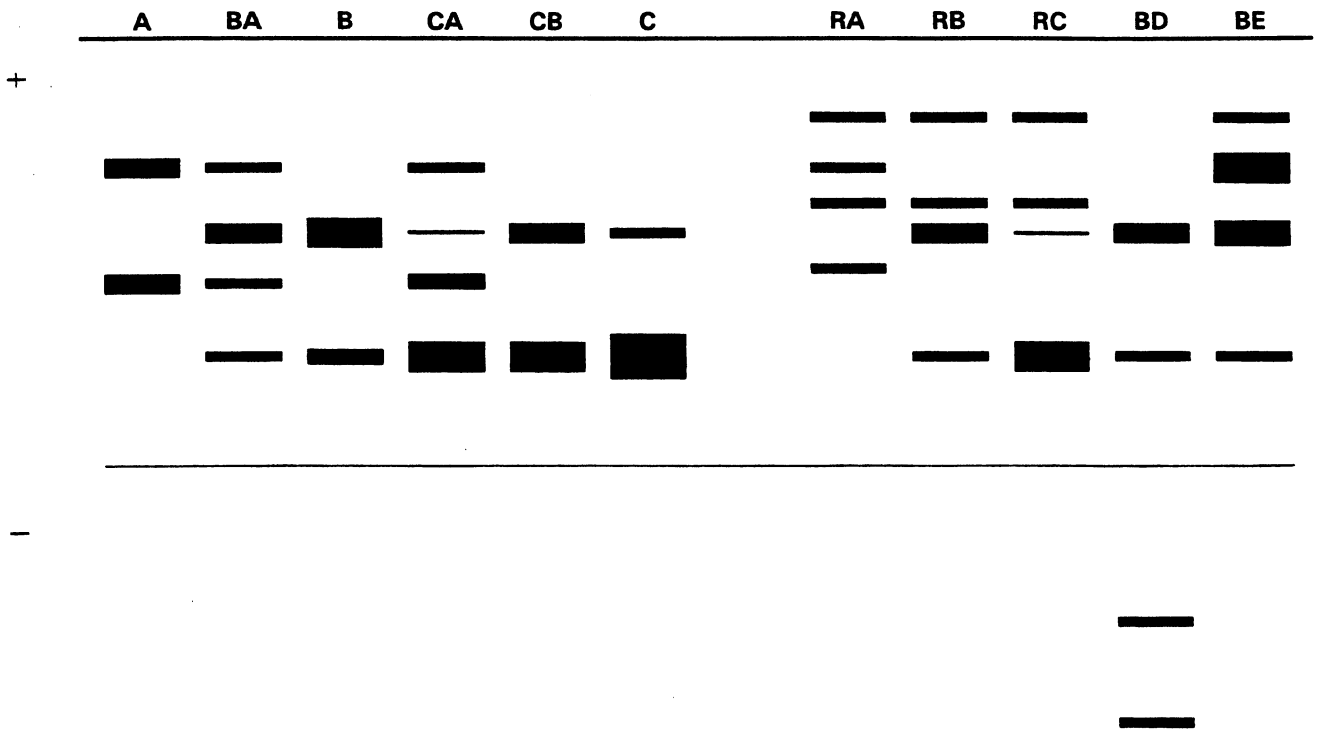


Figure 29.1 Electrophoretic Patterns of ACP_1 Phenotypes in pH 6 Buffers Containing Citrate

at pH 8.6. C zone was found only in placental tissue along with B and D. In three placentae, deviant patterns were seen. All the isozymes were inhibited by L-tartrate and reacted with a specific rabbit antiserum, and differed in pH optima, thermostability and sialic acid content. The four isoenzymes could be found in extracts of cultured human cells as well (Beckman *et al.*, 1968), C occurring regularly in fetal, but not in adult tissues. The variant seen in placental tissue (Beckman and Beckman, 1967) could be reflected in leucocytes which, however, do not have quite the same profile of isoenzymes, and mononuclear cells differ somewhat from polymorphonuclear ones (Beckman *et al.*, 1970b). It was suggested, therefore, that the placental and leucocyte enzymes might have a subunit in common. Others have looked at the ACP isozymes in leucocytes from normal subjects as well as from those with various diseases (Li *et al.*, 1970; Avila and Convit, 1973). In 1972, Swallow and Harris examined the ACP isozymes of 963 placentae and found an additional variant, which was shown to be inherited through family studies using leucocytes. It was found too that the A, B and C isoenzymes had molecular weights of about 95,000, 106,000 and 120,000, respectively, as estimated by gel filtration chromatography. A genetic explanation for the A, B and C isoenzymes, consistent with their own data and with Beckman's data, was proposed in which two independent loci were involved in the synthesis of the isoenzymes. Each locus coded for a different polypeptide chain, and the chains were called α and β. Assuming that the isozymes were dimers, the A, B and C isozymes would then have the compositions $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, respectively. Swallow *et al.* (1973) suggested that the structural loci determining the ACP enzymes be designated "*ACP*". Using methylumbelliferyl phosphate as substrate, it could be shown that the "red cell" enzymes are not restricted to the red cell, but can be identified in other tissues as well. In sum, they said, there are then three distinct *ACP* loci, designated ACP_1, ACP_2 and ACP_3. ACP_1 codes for the "red cell" isoenzymes, and is the only one of the three loci exhibiting genetic polymorphism. The other two loci code for enzymes that can be seen in placenta, white cells and other tissues. Although they are apparently not polymorphic, an occasional rare variant is seen, and it is on this basis that they may be distinguished. ACP_2 codes for the β peptide, and ACP_3 codes for the α peptide, which make up the A, B and C isozymes (Swallow and Harris, 1972). The variant described by Beckman and Beckman (1967) reflects a rare allele of ACP_2, while the variant described by Swallow and Harris (1972) reflects a rare allele of ACP_3. In these terms, it could be said that most people are ACP_2^1 and ACP_3^1 homozygotes. Beckman and Beckman's (1967) variant person could be designated $ACP_2^1ACP_2^2$, while Swallow and Harris' (1972) one would be $ACP_3^1ACP_3^2$. Similarly, the "red cell" locus alleles may

be designated ACP_1^A, ACP_1^B, ACP_1^C, ACP_1^R, ACP_1^D, ACP_1^E and ACP_1^o. This "red cell" acid phosphatase nomenclature is followed in this book, as it appears to reflect the genetic situation more accurately than the older usages.

29.3.2 Some comparisons of the various acid phosphatases

Molecular heterogeneity among acid phosphatases is a generalized biological phenomenon. In human tissues, it appears that there are at least three structural loci determining enzymes with acid phosphatase activity. In any given tissue, the multiplicity of enzymes present is, thus, a reflection of the expression of one or more of these loci. The isoenzymes observed result from differences in primary gene products, as well as from post-synthetic alterations in the primary gene products. The number of distinct activities observed also depends on the substrate used, since the different isoenzymes show varying substrate specificities. The relationships between the various isozymes from all the various different tissues are not yet fully clear.

Two major methods have been used to fractionate the isoenzymes of various tissues: electrophoresis and gel filtration chromatography. Electrophoresis separates the proteins on the basis of net charge primarily, while gel filtration does so on the basis of molecular size. Electrophoretic studies on the placental and white cell ACP enzymes particularly may give different results, depending upon the substrate employed in the detection scheme.

It is now clear that "red cell" ACP isozymes occur in placental tissue, the phenotype corresponding to the red cell type of the fetus. The isozymes are detectable in placental tissue using phenolphthalein diphosphate (Blake *et al.*, 1973a) or methylumbelliferyl phosphate (Swallow *et al.*, 1973) as substrates. Blake *et al.* (1973a) found that there are some differences in the expression of ACP_1 phenotypes in placentae as against in red cells. A minor fast band is seen in placental B and CB phenotypes, which is just slightly faster than the most anodal component of A, BA and CA phenotypes in red cells. In B phenotype samples, the slower band is the more intense in placenta, whereas in red cells, it is the other way around. The CB phenotype shows a more intense slow band in red cells, while in placenta, the intensity of the slow band is proportionately greater. In the original studies, Hopkinson *et al.* (1963) observed no activity with α- or β-naphthyl phosphate. Sørenson (1970) showed, however, that the red cell enzyme does in fact hydrolyze β-naphthyl phosphate, but not the α-isomer. A number of diazonium salts, with which the liberated β-naphthol is coupled to form a colored product (see in sections 10.3.2 and 10.3.4), were found to inhibit the ACP enzymes. If substrate and diazonium salt were added to the reaction mixture together, therefore, no activity was seen. But if substrate was added, and allowed to incubate and react before adding the diazonium salt, clear zones of activity were seen. Fast garnet GBC salt (C.I. 37210) was found to be the best coupling salt of the several tested in the experiments. The β-naphthyl phosphate gave more zones of activity than phenolphthalein diphosphate, and was more sensitive. The ACP isoenzymes were about 10% less active by quantitative assay with β-naphthyl phosphate than with p-nitrophenyl phosphate. Blake *et al.* (1973a) looked at placental tissue ACP using the naphthyl phosphates as substrates. The "red cell" acid phosphatases gave somewhat different patterns in placentae with β-naphthyl phosphate than with phenolphthalein diphosphate, and these were not as easy to identify. Further complicating the picture was the fact that placenta showed ACP activity patterns with α-naphthyl phosphate, which does not react with ACP_1 products. The ACP activity patterns in a number of other tissues were examined in these studies as well.

If MW is used as a basis for dividing the ACP enzymes into categories, certain patterns become apparent, just as is the case when electrophoresis is used. The correlations between the MW classes and the electrophoretic ones are not usually apparent. A very good over-all view of the ACP isoenzymes considered from the point of view of MW is given by Sensabaugh (1975). Generally, there are four MW classes of ACP enzymes in mammalian (including human) tissues. These may be partitioned by gel filtration chromatography, as has been done with bovine kidney and liver tissues (Heinrikson, 1969), human placenta (DiPietro and Zengerle, 1967) and a number of other human tissues (Sensabaugh, 1975). Sensabaugh (1975) found that a high MW enzyme (on the order of 200,000) was present in most tissues, but not in red cells nor in semen. A second class (MW 100,000 to 130,000) was present in all tissues except for red cells. Another class had MW in the range of 30,000 to 60,000, and is not present in all tissues. The fourth class is present in all tissues except semen and prostatic tissue extracts. This class is of low MW (about 13,000–20,000), includes the erythrocyte ACP enzymes, and is perhaps the best characterized of the four. These classes of enzymes show some other properties in common within a given class, apart from being of similar MW. The low MW enzymes, including the red cell ones, are inhibited by formaldehyde (Abul-Fadl and King, 1948) but not by tartrate, which strongly inhibits the prostatic (Abul-Fadl and King, 1949) and other high MW phosphatases. The 30,000–60,000 MW class is activated by Mg^{++}. The red cell enzymes show a preference for FMN (riboflavin-5′-phosphate) as substrate (Luffman and Harris, 1967; Sensabaugh, 1975), a property shared by the low MW (16,300–16,600) enzyme purified from beef liver by Heinrikson (1969). And the red cell enzyme is activated by L-methyl adenine (Sensabaugh, 1975), a property shared by the low MW enzyme purified from human placenta (DiPietro and Zengerle, 1967). The placental enzyme hydrolyzes 17-β-estradiol-3-phosphate (DiPietro, 1968). The low MW enzymes, generally, show significant activity with a restricted number of phosphate esters. Sensabaugh (1975) presented some correlations between the electrophoretically distinct ACP's and the MW categories. The highest MW fraction appeared to be associated with the "A" band of Beckman and Beckman (1967), while the 100,000–125,000 fraction contained B, D and

some A band material. The prostatic enzyme electrophoreses as an "A" band. This would mean that the seminal ACP would be composed of two α polypeptide chains, as defined by Swallow and Harris (1972), and would be coded for by the ACP_3 locus (Swallow *et al.*, 1973).

Seminal plasma and prostate contain only one of the MW classes of ACP, the MW of the enzyme being about 100,000 (see in section 10.3.6). The enzyme exhibits molecular heterogeneity upon being subjected to starch gel electrophoresis (Sur *et al.*, 1962), but this multiplicity of forms derives from the attachment of varying numbers of sialic acid residues to the same protein. The molecular heterogeneity is thus post-synthetic, and not genetic, in origin (Smith and Whitby, 1968; and see section 10.3.6).

Lysosomes contain acid phosphatase enzymes of the 100,000–130,000 MW class. The rat liver enzyme was purified and studied by Brightwell and Tappel (1968). There is no evidence for polymorphism in human lysosomal ACP, but a rare, autosomally inherited deficiency of the enzyme has led to significant pathological findings, and early death in one family (Nadler and Egan, 1970).

29.3.3 Linkage relations of the *ACP* loci

ACP_1, which determines the polymorphic red cell isozymes, is not closely linked to any of the principal blood group or serum protein marker system loci (Conneally *et al.*, 1965). ACP_1 was found to be syntenic to soluble isocitrate dehydrogenase by somatic cell hybrid studies (Povey *et al.*, 1974), thus assigning it to chromosome 2. A peculiar deletion in a family allowed further localization of the ACP_1 locus to the short arm of chromosome 2. The ACP_2 locus was found to be syntenic with LDH-A, and thus assigned to chromosome 11, by human-rodent somatic cell hybrid studies (Bruns and Gerald, 1974).

29.4 Studies on the ACP_1 Isoenzymes (EAP Isoenzymes)

The earlier studies on the red cell ACP (Abul-Fadl and King, 1948 and 1949; Tsuboi and Hudson, 1953, 1954 and 1956) were carried out on mixtures of isoenzymes since the polymorphism had not yet been recognized. Some of the findings concerning inhibitors and substrate specificities of the red cell enzymes are quite useful in distinguishing them from other kinds of acid phosphatases.

Hopkinson *et al.* (1964) and Spencer *et al.* (1964a) determined the mean enzymatic activity of a number of individuals of the five common phenotypes, and found significant differences. They used p-nitrophenyl phosphate as the substrate. The results indicated that the ACP_1^A allele gave a product with about 61 units of activity, ACP_1^B and ACP_1^C yielding products with 94 and 120 units, respectively, where a unit was defined as 1 μmol p-nitrophenol liberated/30 min/g hemoglobin at 37°. Scott (1966) studied the kinetic properties of the enzymes from A and B homozygotes, and found only slight differences. His finding that ACP-A cells exhibit about 65% of the activity of ACP-B cells is fully confirmatory of the findings of Hopkinson *et al.* (1964). The differences in kinetic constants were not alone sufficient to explain the activity differences. Luffman and Harris (1967) carried out further extensive biochemical studies on the red cell isozymes. Although each isoenzyme differed somewhat in its substrate specificity, there was a clear overall pattern showing that all the isozymes were most active with p-nitrophenyl phosphate and FMN. Phenyl phosphate was a poorer substrate, and 3-phosphoglycerate, α- and β-glycerophosphates, 6-phosphogluconate, glucose-6-phosphate and methyl phosphate were all less active. The α-glycerophosphate was a better substrate than the β-isomer. Glc-1-phosphate, 2,3-diphosphoglycerate, ATP and AMP were all inactive. Tsuboi and Hudson (1956) had obtained similar results concerning substrate specificity with a better purified preparation, except that p-nitrophenyl phosphate was not tested, and peculiarly, FMN was found to be only about 40% as active as phenyl phosphate. Luffman and Harris (1967) also confirmed the earlier findings of Tsuboi and Hudson (1953) that the red cell isozymes have considerable phosphotransferase activity, and that a number of alcohols can act as phosphate acceptors in place of water. Glycerol was the best of the alcohols, followed by methanol, propanol and ethanol. Sensabaugh and Golden (1976a) carried out studies on the phosphotransferase activity of ACP, and extended the range of alcohols that had been tested as acceptors. They showed that the overall rate of the reaction was limited by the transfer of the phosphate group from the phospho-enzyme to water, and that the transfer to alcohol proceeded along a parallel pathway, and was additive in terms of the rate of the over-all reaction. Glycerol, and molecules containing a glycerol moiety, were found to be the most effective acceptors of all the alcohols tested. These findings have been exploited in devising better detection procedures for ACP on gels following electrophoresis. This matter is discussed in section 29.5.2. Thermal denaturation studies by Luffman and Harris (1967) indicated that the ACP_1^A products were most stable to heating, followed by those of ACP_1^B and ACP_1^C, in that order. Urea or guanidine caused denaturation of all the isozymes equally effectively. Rogers *et al.* (1978) showed that activity of ACP A and ACP B in red cells decreased at different rates as the cells age *in vivo*, but no secondary isozyme formation was noted during the aging process.

Fisher and Harris (1969) reported on the purification and characterization of the red cell isoenzymes in some detail. An effort was made to devise a purification procedure which was adaptable to large scale isolations. The results of this effort, which had been successful, were given by Fisher *et al.* (1968), with special reference to the ACP-B isozymes. Fisher and Harris (1969) found that inorganic phosphate stabilized the enzyme, and the addition of inorganic phosphate at various stages of the purification procedure improved the yield considerably. In 1964, Bottini and Modiano reported that the electrophoretic mobility of red cell ACP isozymes was strongly affected by sulfhydryl reagents. The addition of oxidized glutathione caused significant increases in the anodal mobility of the isozymes. Fisher and

Harris (1969) further investigated this effect, and found that oxidized glutathione did indeed lead to significant increases in mobility for all the types. A similar but less drastic effect was seen with lysates that had been aged. The presence of mercaptoethanol caused the isoenzymes to retain the mobility seen in fresh samples, and the reagent was thus routinely added to preparations of the isozymes. The enzymes apparently undergo oxidation of the -SH groups upon aging, and the effect can be mimicked in part by the addition of oxidized glutathione, though the glutathione effect is more drastic.

Dissing *et al.* (1979) tested a series of phosphonic and arsonic acids as inhibitors for ACP isozymes. Those which bound the enzymes well could then be used as ligands to design affinity chromatographic purification steps for the isozymes. There is some information on the properties of the less common red cell ACP enzymes. Jenkins and Corfield (1972) found that the products of ACP_1^R have about the same activity as those of ACP_1^A, and that the ACP_1^R isozyme was somewhat more thermostable than the ACP-A or ACP-B enzymes. Sørenson (1975) found that the products of the ACP_1^E locus were somewhat more active than those of other known alleles, and the ACP-E isozyme was more heat stable than the ACP-B one.

There have been some studies comparing the different products of the same ACP_1 allele. The apparent production of more than one product by a single allele, while far from unprecedented, wants some explanation. Hopkinson and Harris (1967) found that two principal isozymes could be separated by DEAE-cellulose column chromatography from each of the homozygous phenotypes AA and BB. Fenton and Richardson (1967) said that three distinct activities could be separated from red cells of any of the phenotypes by DEAE-Sephadex chromatography. These differed in a number of their properties, as well as in electrophoretic mobility. The five common ACP phenotypes contained different percentages of the three activities. In 1971, White and Butterworth purified the isoenzymes from red cells of the B and BA phenotypes, and found that they could be resolved into 5 and 7 components, respectively, which had similar substrate specificities, but differed in K_m and pH optima (White and Butterworth, 1971a and 1971b). The MW for the B components was estimated to be 13,000 by gel filtration, a value in reasonable agreement with the estimate of Luffman and Harris (1967) of 7,000 to 10,000 by the same technique but on cruder material. Fisher and Harris (1971) purified the separate isozymes from particular loci, and carried out studies on their characteristics. Isozyme products of the ACP_1^A locus are designated a_1 (slower) and a_2 (faster), and likewise, those of ACP_1^B are b_1 and b_2. The a_2 isozyme had a lower K_m than a_1 for p-nitrophenyl phosphate, and b_2 had a lower K_m than b_1 with the same substrate. If pure preparations of b_1 or b_2 were kept at 4°, and examined subsequently by electrophoresis, a "storage band" had developed that was identical to the other isozyme. Put another way, pure preparations of either b_1 or b_2, stored for a time, gave a $b_1 + b_2$ pattern upon electrophoresis. This transition occurred more rapidly in the absence of inorganic phosphate. There were indications that a_1 and a_2 might be interconvertible as well. This evidence suggests that the a_1–a_2 and b_1–b_2 pairs might be conformational isomers of one another. If so, the apparent production of more than one isozyme by a single genetic locus can be readily understood.

Kaczmarek (1976) reported that red cell ACP isozymes could be resolved into some 21 zones of activity by isoelectric focusing, and further, that neuraminidase treatment abolished the multiple banding and reduced the activity to a single band of ACP activity with a pI of 5.8. This observation is not in accord with the data of White and Butterworth (1971a) nor with that of McWright *et al.* (1975). The molecular weight data on the isozymes are not readily understandable in terms of the values determined in a number of other laboratories either.

The properties of acid phosphatases, including those of the red cell and of tissues, were reviewed by Hollander (1971).

29.5 Medicolegal Applications

29.5.1 Disputed parentage

The ACP system has been employed in disputed parentage cases in a number of laboratories since about 1966. A number of investigators have established that the ACP_1 system is fully reliable in these cases (Fuhrmann and Lichte, 1966; Fiedler, 1967; Speiser and Pausch, 1967; Broman *et al.*, 1971; and others). The isoenzymes are fully expressed in the newborn (Reimann and Römisch, 1968) as well as in fetal blood (Chen *et al.*, 1977). Jarosch (1968) discussed the use of ACP in paternity cases. Speiser and Pausch (1967), Krüger *et al.* (1968) and Hummel *et al.* (1969) gave accounts of the calculations of the probabilty of excluding a falsely accused man.

The probability of excluding a falsely accused western European man with the ACP system alone is about 25%. Speiser and Pausch (1967) quoted a value of 24.98% for the Viennese population, Broman *et al.* (1971) said that the figure was 25.9% for Sweden, and Boorman *et al.* (1977) gave 21% for the British population. Polesky *et al.* (1976), gave figures of 25.08% for Caucasians, and 15.44% for Blacks, and these were calculated from U.S. population data.

The possibility of encountering a silent allele in a parentage case should be kept in mind. Heide *et al.* (1974) discussed this problem, and said that quantitative enzyme assays could be employed in cases of apparently contradictory homozygosity. The silent allele of ACP_1 is extremely rare (section 29.2).

It should be kept in mind, too, that problems with ACP_1 typing can occasionally be encountered in "fresh" blood samples, as in the case reported by Andrus (1980) and discussed in section 29.5.2.

29.5.2 ACP phenotyping in dried bloodstains

Heidel (1968) reported that ACP_1 phenotypes were reliably determinable in dried bloodstains up to 30 days old.

Smerling (1969) agreed that the system could be typed in dried bloodstains, but only if they were quite fresh. No results could be obtained with stains more than about 30 hours old. Nagata and Dotzauer (1970) could not obtain results with bloodstains older than about 30 hours either. They investigated dried stains on a number of different substrata. If the stains were kept at −40°, the enzyme was preserved better, and could be typed in stains up to 80 hours old. Brinkmann (1971) found that stains kept at room temperature could be typed for up to 3 weeks by polyacrylamide gel electrophoresis. Subsequent investigations showed that such stains could be phenotyped for up to 6 or 8 weeks (Brinkmann *et al.* (1972b). The polyacylamide gel method used in these studies was described by Hennig *et al.* (1968). There were differences in the time limits of detection of the isoenzymes in bloodstains in different phenotypes, and at different storage temperatures. There were differences, too, between stains absorbed into substrata and dried blood on polished surfaces, which could be scraped off. All types could be determined at 60 days with blood crusts, after which A, CA and BA became difficult to distinguish because of the lability of a_2. Types B, CB and C could be determined after 12 weeks. In absorbed stains, all the phenotypes could be determined at 6 weeks, and the B, CB and C types at 8–9 weeks. Stains of all kinds stored in the deep freeze were stable for considerably longer periods of time (7½ months). It should be noted that absorbed stains were extracted in 60 mM phosphate buffer, pH 6.2, containing 60 mM mercaptoethanol. The extracts were then lyophilized, and the lyophilizate redissolved in minimal 30 mM phosphate buffer, pH 6.2, containing 30 mM mercaptoethanol. Blood crusts were dissolved in the 60 mM phosphate buffer, pH 6.2, 60 mM mercaptoethanol solution for three hours time.

A major study on bloodstain grouping of ACP was carried out by McWright *et al.* (1975). Starch gel electrophoresis was employed using the citrate-phosphate buffer system of Hopkinson and Harris (1969a) modified to include 20 mM $MgCl_2$ and 10 mM EDTA, and prepared at pH 5.5. The detection substrate was 4-methylumbelliferyl phosphate. Consistently better patterns were obtained after 4 hours of electrophoresis than after 15 hours. "Storage" bands were observed with all the types. One of these, associated with B, BA and CB was always present; another, associated with A, was present in A types but virtually absent in BA and CA types. The intensity of such storage bands is lessened by treatment of the samples with sulfhydryl reagents (0.1M dithiothreitol was used in this case). All phenotypes could be determined in bloodstains for up to 4 months, after which certain phenotypes became difficult. Somewhat in contrast to the findings of Brinkmann *et al.* (1972b), the CB and CA were found to be more stable than the A, B and BA types. Occasionally, it was noted that the faster isozymes of B and CB types could become weaker upon aging, while the slower isozymes become slightly more intense. In at least one instance, a B would have been read as a weak C in a four day old stain. Densitometric tracings of the phenotypic patterns were employed to supplement visual judgments throughout the study. Neuraminidase treatment of lysates for lengthy periods destroyed ACP activity, but did not lead to any alteration in the phenotypic patterns.

In 1976, Wraxall and Emes reported a starch gel electrophoretic method for ACP typing in bloodstains using the citrate-phosphate buffer system of Hopkinson and Harris (1969). Electrophoresis was carried out for 4½ hours on cooling platens, and methylumbelliferyl phosphate was used as a detecting substrate. Samples were treated with 50 mM dithiothreitol prior to electrophoresis. A storage band was noted in hemolysates, but this was removed in most cases by the DTT treatment. The storage band was not normally seen in dried bloodstains. Blind trial studies indicated that the procedure was completely reliable. Some older stains (up to 6 weeks), particularly those on synthetic fabrics, gave weak or negative results. Heated lysates can yield incorrect results, because the a isozymes are least stable, followed by the b, and then the c ones. A type B pattern can thus be induced to take on the appearance of a CB, and ultimately of a C, but these changes were not observed in aging bloodstains. Similar kinds of effects were observed by Berg *et al.* (1974b) in their studies on the effect of heating on ACP phenotype determination. Wraxall and Emes (1976) emphasized that care must be taken to insure that excess heating does not occur during the electrohoretic run. Caution in the interpretation of bloodstain patterns was recommended as well, especially in cases of older stains. It appears that Figures 2 and 5 in this paper were inadvertently interchanged in the printing process.

Sensabaugh and Golden (1976a) suggested a modification of in the detection reaction procedure for ACP, based upon their studies of the phosphotransferase activity of the enzyme. The phosphotransferase activity of ACP was noted by Tsuboi and Hudson (1953), and confirmed by Luffman and Harris (1967), the latter of whom studied this activity with a number of alcohols. Sensabaugh and Golden (1976a) extended the studies to include a wider range of alcohols, and could show that glycerol, and structures containing a glycerol moiety, gave the best rates. Taking advantage of this effect for the detection of ACP phenotypes following electrophoresis, they found that the incorporation of glycerol into the starch gel enhanced the rate of isozyme development considerably, with methylumbelliferyl phosphate as substrate. The bands were more compact, and mobility was decreased presumably because of the increased viscosity of the gel in the presence of glycerol. The optimal concentration of glycerol was 15% (v/v) in gel buffer. This interesting approach is, however, not without its problems and pitfalls. Sensabaugh and Wraxall (1977) took up the subject again, and pointed out several other problems associated with reliable ACP phenotyping. Since the activity of the various isozymes is quite different (section 29.4), i.e., the different isozymes differ in their K_m for substrate, the glycerol enhancement effect is differential. Because the differentiation of some ACP phenotypes on electrophoretic

plates is a matter of band intensity differences, misinterpretation of phenotypes is possible if these effects are not fully appreciated. It is essential, therefore, to include appropriate controls of known phenotypes when using any phenotyping procedure, and particularly when attempting to diagnose B, CB and C phenotypes. They also noted the importance of using substrate concentrations high enough to insure that all the isozymes would be saturated, since the K_m values are not the same for all the isozymes, and misleading intensity differences could be produced by using substrate concentrations that are too low.

In 1978, Grunbaum and Zajac reported that ACP phenotypes could be determined in bloodstains up to 30 days old, as well as on hemolysates, by electrophoresis on cellulose acetate membranes. These investigators reported a blind trial study on ACP phenotyping on starch gels and on cellulose acetate foils (Zajac and Grunbaum, 1978), in which bloodstains, hemolysates, and hemolysates which had been kept at 37° for up to 48 hours, were all tested. Samples of BA, B and CB phenotypes were determined on both kinds of media, and those of A and CA types were determined on cellulose acetate membranes. Stain phenotypes were correctly determined, with the exception of one "inconclusive" result on a BA stain on cellulose acetate, and of one CB stain. The CB could be confused with type B on either support medium. The heated samples caused problems in interpretation on starch or cellulose acetate, except for CB phenotypes. While the heated samples were correctly typed in some instances, they were misinterpreted in others. B was mistyped as CB, BA as CA or CB, and some results on heated lysates were inconclusive. Caution was recommended in cases where the history of the sample was not very well known.

Welch (1972b) found that ACP could be determined in 22 days old bloodstains, but not in 30 days old ones. Denault *et al.* (1978) found ACP phenotypes to be detectable in bloodstains on a variety of substrata for up to 13 weeks. In one case, a stain on cotton did not give a result after 4 weeks aging. Stains aged at higher humidities were generally more active than those kept at lower humidity. One B stain on denim, a BA stain on a perma-press fabric, and another stain on cotton kept frozen could all be typed after 26 weeks. Only B and BA stains were included in the study.

Brinkmann and Bruns (1979) conducted phenotyping tests on two series of bloodstains, representing all the phenotypes, one on cotton and the other on glass. Members of each series were kept at 37°, 22°, 4° and frozen. Electrophoresis was carried out on thin layer agarose gels as well as on Cellogel. The stains on glass were typable longer than those on cotton. The stains at room temperature were typable for 3 to 6 weeks. Those at 37° lasted only a few days. In the refrigerator and freezer, stains could be typed for 10 to 13 weeks. The Cellogel was preferred over the agarose gel, and was said to give clear, reliable typing results.

Andrus (1980) reported an interesting case in which a typing problem was encountered not with the dried blood (which was on window glass), but with the "fresh" blood sample taken from the suspect. The blood had been drawn into EDTA 4 days prior to its receipt by the lab, and had been kept in a refrigerator. There was little observable hemolysis, and the red cells from the sample and the bloodstains were typed for ABO, PGM and PGM subtypes, ESD, AK, ADA, GLO, Hp and Gc. The bloodstain yielded a type B for ACP_1, however, while the cells yielded CB. The subject had not been transfused at all recently, if ever. A second sample of blood from the same person, collected in EDTA and examined within 24 hours, revealed ACP type B. The other systems typed as they had in the original sample. There was no indication that the original blood sample had been mishandled, heated or improperly stored.

29.5.3 Methods of phenotyping ACP isoenzymes

Most investigators have used starch gel electrophoresis for typing acid phosphatase isoenzymes, although a variety of different buffer systems have been described. The buffer system employed in the original paper on the ACP_1 polymorphism was described in section 29.2. A discussion of buffer effects on phenotypic patterns was given by Hopkinson and Harris (1969), and was reviewed in section 29.2 as well. Radam and Strauch (1966) proposed a discontinuous buffer system for starch gel electrophoresis consisting of 12 mM phosphate, pH 6, gel buffer, and 0.4M citric acid-NaOH, pH 6, bridge buffer. Methods have been devised for phenotyping ACP on agarose (Sørensen, 1974a), on polyacrylamide gels (Hennig *et al.*, 1968), and on cellulose acetate foils (Grunbaum and Zajac, 1978) and Cellogel (Brinkmann and Bruns, 1979). Brinkmann *et al.* (1972b) used the horizontal polyacrylamide gel system of Hennig *et al.* (1968). It should be noted that the gels were prepared with DMAPN, potassium ferricyanide and ammonium persulfate, and not with TEMED, and photopolymerized. They were then soaked in gel buffer for a number of days, with several changes of buffer, in order to rid the gel of any small molecule inhibitors. The process was called "diffusion washing". This matter was discussed in section 27.4.2.1 as well. In the case of ACP, 3% gels were used. Less material was required with the system than with starch. Brinkmann and Bruns (1979) have more recently tested Cellogel and thin-layer agarose gels, and said they preferred the Cellogel. Isoelectric focusing has been used for ACP typing. Sørensen (1974b) used the technique to measure the isoelectric points of the isoenzyme components. Burdett and Whitehead (1977) reported good phenotyping results by isoelectrofocusing in polyacrylamide gels in pH 5.0–8.5 gradients.

A number of systems have been devised in which ACP may be phenotyped simultaneously along with other isoenzyme systems. Hummel (1970) described a system for ACP with PGM. Martin and Niebuhr (1971) gave a procedure for typing ACP along with PGM, AK and ADA in the same electrophoretic operation. Wraxall and Stolorow (1978) have recently described a system designed for dried bloodstains in which ACP can be phenotyped along with AK and ADA in the same gel. They refer to these three iso-

zyme systems as "Group II", to distinguish them from other multi-system procedures they have devised. Their "Group I" consists of ESD, PGM and GLO, and is mentioned in connection with the discussions of those systems.

Acid phosphatase assays were discussed in section 10.3.4, and most of the substrates used for phosphatases were covered in that section. The red cell enzyme shows a narrower range of substrate specificity than is characteristic of acid phosphatases in general. Most investigators used phenolphthalein diphosphate as the substrate for detecting ACP in gels up until fairly recently. For enzymatic assay of the ACP, p-nitrophenyl phosphate is often preferred because the p-nitrophenylate anion is so easy to detect spectrophotometrically. Sørensen's (1970) finding that β-naphthyl phosphate was a suitable substrate, provided that the coupling dye was not added simultaneously (section 29.3.2), caused some workers to switch to this system. The availability of the fluorigenic substrate 4-methylumbelliferyl phosphate has resulted in its being adopted as the ACP substrate in a number of the more recent studies. Sparkes *et al.* (1975) said that phenolphthalein monophosphate was as sensitive and as satisfactory as the MUP for red cell ACP typing, and had the advantage that exposure to UV light was not required to visualize the zones of activity.

29.5.4 Red cell ACP typing in aged whole blood samples

There are conflicting reports in the literature on the survival of typable ACP isozymes in aging blood samples, and in postmortem specimens. Like the comparable reports on the survival of the enzyme in bloodstains, some of these differences can probably be accounted for by variations in electrophoretic technique and/or in the substrate used to detect the isoenzymes. Reimann and Willner (1968) said that ACP was typable for a number of months in samples collected for blood alcohol determinations. Smerling (1969) found the time limit to be at least 6 months. Gussmann (1970) found that, after a month or two, the C phenotypes were very difficult to determine accurately. Brinkmann *et al.* (1972b) found that samples kept at 4° could be determined for up to 15 months, but increasingly larger quantities of sample had to be used as the blood aged. Rose (1971) noted problems with CB type blood after only 11 days storage, and said that the formic acid system of Giblett and Scott (1965) gave better differentiation of CB and CA in these samples. McWright *et al.* (1975) found that hemolysates or clotted blood kept at 25° lost activity after about 5 days. Blood in citrate-phosphate-dextrose anticoagulant at 5°, however, could be typed after 10 months.

Herzog and Sobotka (1972) said that ACP had been typed in postmortem specimens up to 5 days after death. Heidel and Reimann (1968) could type such samples up to a month old on some occasions.

Krauland and Smerling (1971) reported a most peculiar differential inhibition of red cell ACP by a commercial chemical contained in blood-drawing containers. The chemical was called "polyanetholsulfonsaurem natrium", and was present at a concentration of 1% in physiological saline. The compound is probably a poly-anethol sulfonic acid (sodium salt) of some sort (anethol is p-methoxypropenylbenzene). In any event, the A isozyme was differentially inhibited, so that BA could be taken for B in the presence of this material.

29.6 Distribution of ACP_1 phenotypes in U.S. populations

The data are presented in Table 29.1. ACP is one of the more useful systems for making distinctions in the population. The discrimination index is about 0.65 for Caucasians, and about 0.54 for Negroes.

Table 29.1 Distribution of *ACP*, Phenotypes in U.S. Populations

Population	Total	Frequency – Number (Percent) A	BA	B	CA	CB	C	Rarer	ACP_1^A	ACP_1^B	ACP_1^C	Reference
CAUCASIAN												
Seattle, WA	193	33 (17.1)	76 (39.4)	61 (31.6)	10 (5.2)	13 (6.7)	0		0.394	0.547	0.059	Giblett and Scott, 1965
U.S. Navy Personnel in Japan	272	27 (9.9)	120 (44.1)	107 (39.3)	7 (2.6)	11 (4.0)	0		0.3327	0.6342	0.0331	Omoto and Harada, 1968
Chicago, IL	100	14 (14)	43 (43)	38 (38)	4 (4)	1 (1)	0		0.375	0.600	0.025	Shih and Hsia, 1969
Pittsburgh/Allegheny County, PA	1,239	145 (11.7)	526 (42.5)	491 (39.6)	20 (1.6)	57 (4.6)	0		0.337	0.632	0.031	Hagins et al., 1978
California	4,850	(10.8)	(42.1)	(39.3)	(3.3)	(4.3)	(0.2)		0.332	0.630	0.038	Grunbaum et al., 1978b
Bexar County, TX	200	(12.0)	(38.0)	(50.0)	(>1)	(1.0)	(0)		0.310	0.690	0.010	Ganaway and Lux, 1978
Detroit, MI	503	65 (12.9)	185 (36.8)	193 (38.4)	22 (4.4)	38 (7.6)	0		0.335	0.605	0.060	Stolorow et al., 1979 and see Shaler, (1978)
Miami/Dade Co., FL	366	44 (12.0)	145 (39.6)	156 (42.6)	10 (2.7)	10 (2.7)	1 (0.3)		0.330	0.640	0.030	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	357	34 (9.5)	150 (42.0)	158 (44.3)	8 (2.2)	7 (2.0)	0		0.317	0.662	0.021	Sigler, 1979 and see Shaler, (1978)
NEGRO												
Seattle, WA	164	12 (7.3)	48 (29.3)	99 (60.4)	2 (1.2)	3 (1.8)	0		0.226	0.759	0.015	Giblett and Scott, 1965
Ann Arbor, MI	224	12 (5.4)	50 (22.3)	160 (71.4)	0	2 (0.9)	0		0.1651	0.8303	0.0044	Brewer et al., 1967a
Austin, TX	63	3 (4.8)	16 (25.4)	32 (50.8)	1 (1.6)	8 (12.7)	0	★	0.19	0.71	0.07	Karp and Sutton, 1967
Dallas/Houston, TX Male patients	294	11 (3.7)	100 (34.0)	166 (56.5)	1 (0.3)	7 (2.4)	0	☆	0.21	0.76	0.015	Karp and Sutton, 1967
Seattle, WA	429 ○	30 (7.0)	150 (35.0)	222 (51.7)	2 (0.5)	10 (2.3)	0	●	0.25	0.72	0.014	Giblett, 1969 and Mourant et al., 1976
Chicago, IL	101	8 (7.9)	19 (18.8)	66 (65.3)	1 (1.0)	2 (2.0)	0	◇	0.181	0.761	0.015	Shih and Hsia, 1969 and Mourant et al., 1976
Pittsburgh/Allegheny County, PA	718	39 (5.4)	239 (33.3)	426 (59.3)	2 (0.3)	11 (1.5)	0	□	0.222	0.767	0.009	Hagins et al., 1978
California	875	(5.6)	(31.4)	(60.2)	(0.2)	(1.3)	(0.1)	(1.1)	0.217	0.776	0.008	Grunbaum et al., 1978b

Table 29.1 (Cont'd)

Population	Total	Frequency — Number (Percent) A	BA	B	CA	CB	C	Rarer	ACP_t^A	ACP_t^B	ACP_t^C	Reference
Bexar County, TX	200	(7.0)	(27.0)	(66.0)	(0)	(0)	(0)		0.205	0.795	–	Ganaway and Lux, 1978
Detroit, MI	504	30 (6.0)	171 (33.9)	280 (56.0)	4 (0.8)	6 (1.2)	0	○	0.234	0.742	0.011	Stolorow et al., 1978 and see Shaler, (1978)
Miami/Dade Co., FL	345	24 (7.0)	110 (31.9)	201 (58.3)	1 (0.3)	6 (1.7)	0	/	0.229	0.743	0.010	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	161	5 (3.1)	66 (41.0)	89 (55.3)	0	1 (0.6)	0		0.236	0.761	0.003	Sigler, 1979 and see Shaler, (1978)
MIXED NEGRO AND CAUCASIAN												
Washington, D.C.	137	14 (10.2)	52 (38.0)	61 (44.5)	4 (2.9)	6 (4.4)	0					McWright et al., 1975
ORIENTAL												
Seattle, WA ("mixed")	77	4 (5.2)	22 (28.6)	51 (66.2)	0	0	0		0.195	0.805		Giblett and Scott, 1965
	221 ◎	8 (3.6)	70 (31.7)	142 (64.3)	0	1 (0.5)	0		0.2	0.8	0.002	Giblett, 1969 and Mourant et al., 1976
ASIAN												
California and Hawaii	2,462	(5.2)	(35.6)	(59.2)	(0)	(0)	(0)		0.23	0.77	–	Grunbaum et al., 1978b
HISPANIC												
California ("Chicano/ Amerindian")	1,360	(6.7)	(35.8)	(53.5)	(1.8)	(2.2)	(0)	(0.2)	0.264	0.726	0.019	Grunbaum et al., 1978b
Bexar County, TX	200	(9.0)	(31.0)	(60.0)	(2.0)	(0)	(0)		0.236	0.755	0.010	Ganaway and Lux, 1978
Miami/Dade Co., FL	362	27 (7.5)	123 (34.0)	184 (50.8)	7 (1.9)	20 (5.5)	1 (0.3)		0.254	0.706	0.040	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA Case material	179 ■	6 (3.4)	69 (38.6)	99 (55.3)	2 (1.1)	3 (1.7)	0		0.232	0.754	0.014	Sigler, 1979 and see Shaler, (1978)

★ 1 RA and 2 RB ☆ 1 RA, 5 RB and 1 RC ◎ May include the data of Giblett and Scott, 1965 ● 3 RA and 11 RB ◇ 5 RB □ 1 RA ○ 1 RA, 11 RB and 1 RC / 1 DB, 1 RB, and 1 RA ■ Primarily Mexican

SECTION 30. ADENOSINE DEAMINASE

30.1 Recognition of Adenosine Deaminase

Adenosine deaminase (Adenosine aminohydrolase; E.C. 3.5.4.4; ADA) is an enzyme of nucleoside catabolism, and catalyzes the conversion of adenosine to inosine with the liberation of a mole of ammonia. The enzyme occurs in the tissues of a wide variety of invertebrates and vertebrates.

Schmidt (1928 and 1932) provided the first systematic evidence for purine deaminase activities in rabbit skeletal muscle and liver. These studies included adenylic acid deaminase and adenosine deaminase. Conway and Cooke (1939) found adenosine and adenylic acid deaminase activities in the blood and tissues of rabbits. Brady (1942) found that the superficial mucosa of calf intestine was a rich source of ADA, and this enzyme was found to deaminate deoxyadenosine about as well as it did adenosine. The human blood enzyme was said to be only about half as active with deoxyadenosine as with adenosine. The calf intestinal mucosa enzyme was extensively purified by Brady and O'Connell (1962). The enzyme behaved homogeneously in the ultracentrifuge, but showed molecular heterogeneity upon starch gel electrophoresis. This enzyme has been studied quite extensively (Zielke and Suelter, 1971 for a review).

30.2 ADA Polymorphism in Human Red Cells

In 1968, red cell ADA was found to exhibit regular starch gel electrophoretic patterns which were different in different individuals (Spencer *et al.*, 1968). Three phenotypes were recognizable, and family studies indicated that these could be accounted for by a pair of codominant alleles, ADA^1 and ADA^2, at an autosomal locus. The ADA phenotypes were called 1, 2-1 and 2. The ADA^2 frequency was low in European Caucasians and in Negroes, but somewhat higher in Asiatic Indians. In 1969, Hopkinson *et al.* carried out further population and family studies, which were fully consistent with the single allelic pair hypothesis. This genetic model has been confirmed by a number of other population and family studies (e.g. Tariverdian and Ritter, 1969; Renninger and Bimboese, 1970).

A new phenotype was seen by Hopkinson *et al.* (1969) which was similar to an ADA 2-1, but with considerably reduced activity. The phenotype was attributed to heterozygosity between ADA^1 and a new, rare allele, called ADA^3. The rare allele, ADA^4, was detected as an ADA 4-1 by Dissing and Knudsen in 1969. The main isozyme conditioned by ADA^4 is slower (less anodal) than those of the other three alleles. Detter *et al.* (1970b) found a "fast" ADA pattern in two unrelated Black Americans in Seattle, and provisionally named the phenotype ADA 5-1. The subjects were unavailable for further study to prove the segregation of the postulated ADA^5 allele. Renninger and Bimboese (1970) found an unusual ADA phenotype in an African Black individual who was a member of the tribe of Macua of Moçambique. They did not give the new phenotype a designation, but it looks very much like the ADA 5-1 described by Detter *et al.* (1970b) in Seattle, although the specimens were never directly compared. Radam *et al.* (1974) described a family with yet another phenotype, called ADA 6-1, and family studies showed that the rare allele ADA^6 was segregating in a number of members. A diagrammatic representation of the ADA phenotypes, as seen by starch gel electrophoresis, is given in Figure 30.1. The single allelic pair hypothesis of inheritance for the common ADA phenotypes has been widely confirmed (e.g. Dissing and Knudsen, 1970; Lamm, 1971a).

Silent alleles have been reported at the *ADA* locus. The situation is somewhat complicated because it appears that not every example of deficiency of ADA activity in red cells has the same genetic basis. In 1972, Giblett *et al.* made the extraordinary observation that two unrelated young girls, both suffering from immunodeficiency disease, had no detectable ADA in their red cells. The probability that two such extremely rare conditions could occur in two unrelated individuals by chance alone was too great to be regarded as reasonable. The parents of one of the girls had about half as much ADA activity as normal cells, and the parents of the other girl about two-thirds as much. This report was quickly followed by another from Dissing and Knudsen (1972) that a six month old girl had been observed with combined immunodeficiency disease (CID) and an absence of red cell ADA activity. She was very ill, and did not survive. A young boy with CID, however, showed normal red cell ADA activity, as did his parents. These investigators thought it might be significant that the three CID-ADA-deficient subjects seen thus far had been female. But in 1974, Chen *et al.* found a little boy with CID and ADA deficiency, and studies on the family indicated that an apparently silent allele for ADA was segregating in four generations. The pedigree of this interesting family is shown in Figure 30.2. In 1973, Brinkmann *et al.* encountered an apparent mother-child ADA incompatibility in the course of a disputed paternity investigation. The mother was ADA 2, and three of her six children were ADA 1. Enzyme assays indicated that the mother and the three children had about 60% of the ADA activity of normal red cells, and the simplest explanation was heterozygosity for a silent allele, ADA^o. Everyone in this family was apparently healthy. Jenkins (1973) reported that he had found an ADA-deficient African boy in his studies of the blood groups of the !Kung, a group of bushmen in the northeastern part of southwest Africa. The boy

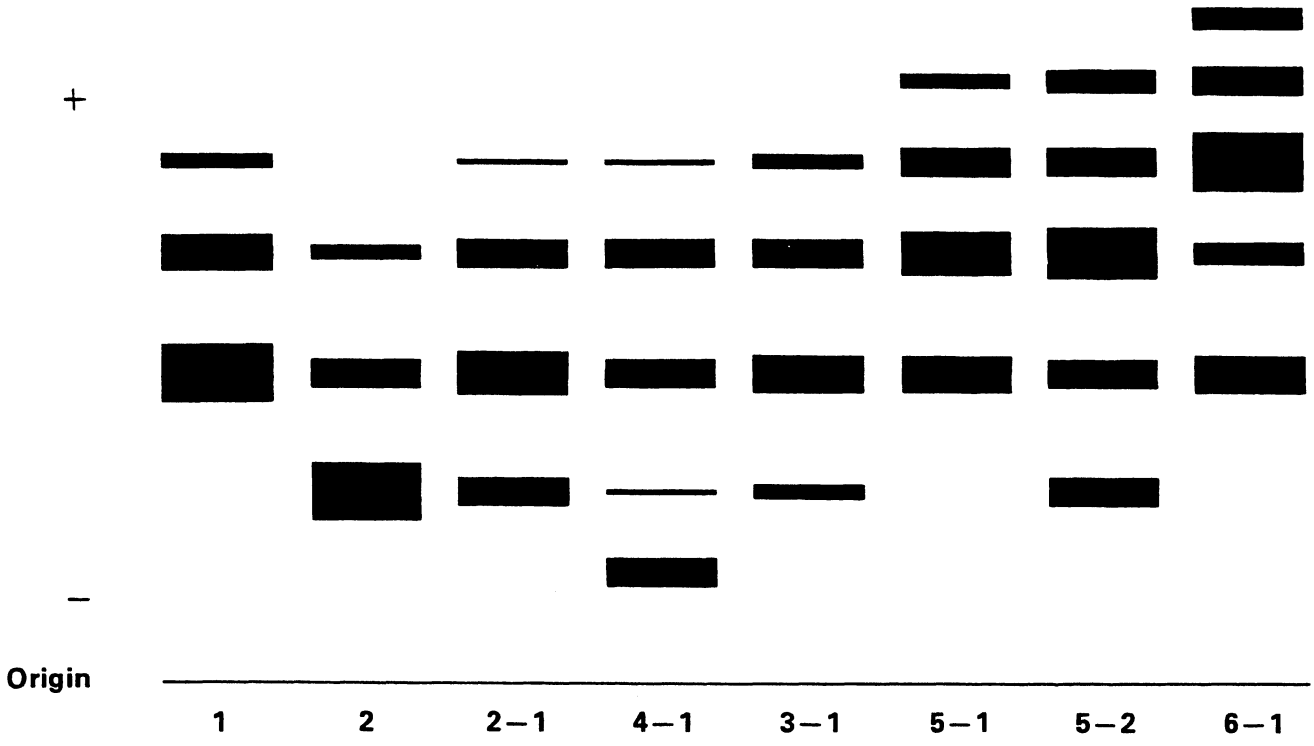


Figure 30.1 Electrophoretic Patterns of ADA Phenotypes

did not suffer from CID, and his father and sister showed reduced ADA activities. Additional studies on the !Kung have shown that an apparent silent allele for ADA reaches polymorphic frequency in these people (Jenkins *et al.*, 1979). In 1973, a conference was convened in Albany, NY, to bring together a group of interested experts for a discussion of the CID-ADA-deficiency relationship. A report may be found in Meuwissen *et al.* (1975). Fifty-five children with CID were known to the group, of whom 22 had had their red cell ADA tested. Thirteen of the 22 were ADA deficient. It appears, therefore, that there is more than one basis for ADA deficiency, as well as for CID. This subject is discussed further in section 30.3.

There is no pedigree evidence for close linkage of the *ADA* locus with the loci of other common blood groups, isozymes or serum proteins (Weitkamp *et al.*, 1970; Weitkamp, 1971). On the basis of human-rodent somatic cell hybrid studies, *ADA* has been assigned to chromosome 20 (Tischfield *et al.*, 1974).

30.3 ADA Isoenzymes in Other Tissues

Adenosine deaminase occurs in the tissues of a variety of mammals (Schmidt, 1928 and 1932; Conway and Cooke, 1939; Brady, 1942; Brady and O'Donovan, 1965). In 1969, Ressler found electrophoretically separable isozymes of ADA in a number of human tissues. Each tissue had a characteristic pattern, but changes in the patterns could be induced by heating the tissues *in vitro*. Ressler was inclined to the view that the isozymes might represent post-synthetically modified forms of a primary gene product. Akedo *et al.* (1970 and 1972) partially purified and characterized two molecular species of ADA from human tissues, distinguishable on the basis of molecular size, and thus separable by gel filtration. These forms were called "large" and "small" (MW about 230,000 and 47,000 respectively). Different tissues had different amounts of each, some tissues having primarily one or the other. There were no major differences in properties between the two, apart from heat stability. Denatured in guanidine, the large enzyme was converted into the small one; if this treated material was dialyzed, the large enzyme could be reconstituted. Therefore, either the large form was a polymer of the small form, or else it was constituted from the small form along with some other unidentified (and non-dialyzable) component.

In 1971, Edwards *et al.* investigated tissue adenosine deaminases. Most human tissues had ADA isoenzymes, and two sets of these could usually be distinguished. One of them was equivalent to "red cell" ADA, and showed the familiar polymorphism characteristic of erythrocyte ADA. The other isoenzymes, designated *a* through *e*, were heterogeneous, but showed a tissue-specific distribution pattern. The "red cell" isozymes in tissues resembled the isozymes from the actual red cells in MW as estimated by gel filtration (about 34,000 daltons) and in sulfhydryl reactivity

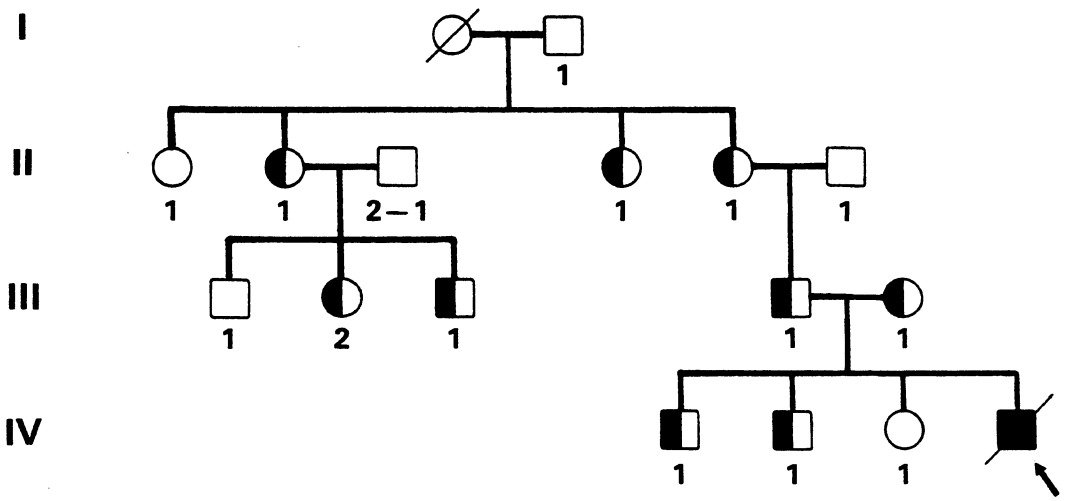


Figure 30.2 Segregation of Silent ADA Allele (after Chen et al., 1974)
ADA phenotypes indicated under each symbol: ◐ or ◧ are presumed heterozyotes for allele. ■ is homozygous for silent allele. Slash through symbol indicates deceased. Propositus indicated by arrow.

(see below). The tissue ADA isozymes differed from the "red cell" ones on both counts. The *b*, *c* and *d* isozymes were estimated to have MW of about 280,000, and the *e* isozyme, about 440,000;. the *a* isozyme was labile, and an estimate of its MW could not be obtained.

Van der Weyden and Kelley (1976) purified and examined ADA isoenzymes from human tissues. Four classes of enzyme could be distinguished on the basis of molecular size. One of these was "particulate" and had not been previously seen. The apparent MW was 20 million, and the activity was in some way associated with subcellular organelle membrane. This form was dissociable with nonionic detergent. The other three classes were soluble (i.e., not membrane-associated), and interconvertible, and had apparent MW's of 36,000 (small), 114,000 (intermediate) and 298,000 (large). The small form was converted to the large form in the presence of a protein with no ADA activity of its own and a MW of about 200,000. Conversion was best at 4°, and pH 5 to 8, and was associated with the loss of conversion activity. The small form of the enzyme predominated in tissues with little conversion activity, and conversely, the large form. Both small and large forms exhibited molecular heterogeneity upon isoelectric focusing. Apart from differences in pH optima, the small, intermediate and large forms were similar in their catalytic properties. Nishihara *et al.* (1973) isolated and purified the conversion factor about 600-fold, and characterized it further. It had a MW of about 139,000 as estimated by gel filtration, had no ADA activity of its own, and exhibited no sulfhydryl or metal requirements. The results of the study suggested that the large ADA molecule (MW 230,000) was some kind of a complex of the small ADA molecule (MW 47,000) and the conversion factor.

The interconvertibility of the "small" and "large" forms of the ADA isoenzymes, which was confirmed by Hirschhorn *et al.* (1974), suggests that they are all products of the same genetic locus. There is further evidence for this view as well. Hirschhorn *et al.* (1973) and Hirschhorn and Boratis (1973) examined the tissues, and cultured fibroblasts, of victims of the combined immunodeficiency disease-ADA deficiency syndrome which was discussed in section 30.2. Some of the tissues had been deep frozen at −70° for some time, but control tissues indicated that this treatment had no inhibitory effect upon ADA. No detectable ADA activity could be found in either the tissues or the cultured fibroblasts, suggesting that tissue as well red cell ADA isozymes were the products of the same genetic locus. In 1974, Hirschhorn *et al.* found that cultured fibroblasts from a patient suffering from CID–ADA deficiency syndrome had residual ADA activity. The residual enzyme was electrophoretically faster (more anodal) than control enzyme, but had a MW resembling that of the normal tissue enzyme (about 220,000). The enzyme from the patient differed somewhat in heat stability from the normal one as well, and these workers suggested that this residual enzyme was a "mutant" form of ADA. Chen *et al.* (1975) reported somewhat similar findings in their studies on the ADA from cultured fibroblasts of two unrelated patients with CID-ADA-deficiency syndrome. It is of some clinical interest that amniotic fluid cells in culture can be assayed for ADA

activity, and the "red cell" phenotype determined as well. This fact may be important in the application of amniocentesis to the prenatal diagnosis of CID-ADA-deficiency syndrome.

ADA activity in leucocytes was reported by Karker (1965), and was found to be some 30 times higher than in red cells on a "per cell" basis. The isoenzymes of white cells resemble those of heart, brain, muscle and spleen (Edwards *et al.*, 1971). Lymphocyte ADA isoenzymes were described by Wüst (1971a). The "red cell" isozymes are present in these cells, and the phenotype can be determined. There is an additional isozyme present, cathodal to the others. The pattern is similar to that reported by Edwards *et al.* (1971) for fibroblast ADA.

30.4 Purification and Properties of Red Cell ADA

30.4.1 Detection and assay of ADA

Assays for ADA in solution have been based upon following either the appearance of ammonia, or the disappearance of adenosine. The ammonia assay was used by the earlier workers (e.g. Brady, 1942). The disappearance of adenosine in nonturbid solutions can be followed at 265 nm (Kalckar, 1947), and ADA may be conveniently assayed in this way (Osborne and Spencer, 1973). More recent studies have employed radioactively-labelled adenosine for the ADA assay, since it is commercially available (van der Weyden and Kelley, 1976; Daddona and Kelley, 1977). ADA activity can be estimated in small amounts of dried blood by a micromethod that was recently described (Orfanos *et al.*, 1978).

The detection procedure for the ADA isozymes from crude tissue or hemolysate preparations involves a set of coupled enzymatic reactions. The original procedure, devised by Spencer *et al.* (1968), is widely employed. In it, the ADA reaction which brings about the conversion of adenosine to inosine is coupled through the nucleoside phosphorylase reaction to the xanthine oxidase reaction. MTT tetrazolium dye is reduced to formazan in the presence of PMS in the usual way as hypoxanthine is oxidized to xanthine. This detection reaction sequence is indicated in Figure 30.3.

30.4.2 Studies on red cell ADA

Hopkinson and Harris (1969b) noted that the isozyme pattern of ADA in hemolysates changed upon storage, and that these effects were accelerated by the addition of oxidized glutathione, but reversed by addition of mercaptoethanol or reduced glutathione. A detailed study of the sulfhydryl groups of ADA was carried out. Three different kinds of thiol reagents may be used to study reactive sulfhydryl groups in enzymes and proteins. Disulfides, such as oxidized glutathione, react to form mixed disulfides, i.e. $E-SH + GSSG \rightleftarrows E-SSG + GSH$. Oxidized glutathione has a net charge of -2, and the addition of what amounts to "half" an oxidized glutathione molecule to the enzyme alters the net charge on the latter by -1 per reactive $-SH$ group. Other parameters being equal, a change in electrophoretic mobility will be the result. Alkylating agents, such as iodoacetamide, iodoacetic acid, or NEM, constitute a second group of sulfhydryl reagents, and these form a stable adduct with the protein. Some of these reagents alter the electrophoretic mobility, e.g. iodoacetic acid (net charge = -1), while others do not (e.g. NEM). Organic mercurials, e.g. pCMB, react to form mercaptides, and pCMB does alter electrophoretic mobility (it has a net charge of -1). Through systematic studies with such reagents, data were obtained that indicated a single sulfhydryl group for each ADA enzyme. The "storage effects" are due to the formation of mixed disulfides between the enzyme and oxidized glutathione, which accumulates in stored hemolysates. Electrophoresis is, therefore, generally carried out on samples to which 10 mM mercaptoethanol has been added. This subject has been well discussed by Hopkinson (1975) in terms of a number of polymorphic red cell isoenzymes, including ADA.

Osborne and Spencer (1973) partially purified ADA from hemolysates of the three main phenotypes. The isoenzymes of each type were partially resolved by chromatography on DEAE Sephadex. The MW of the isozymes, as estimated by gel filtration, was 30,000 to 35,000. The four components of ADA 2-1 had isoelectric points of 4.7, 4.83, 4.94 and 5.06 by isoelectric focusing. The K_m of the isozymes for adenosine was 30μM for all isozymes. Some differences in heat inactivation profiles were noted among the isozymes, and the heat inactivation characteristics showed a marked dependence upon ionic strength.

Daddona and Kelley (1977) purified red cell ADA some 800,000-fold. The preparation was homogeneous by antibody affinity chromatography, using an antibody developed against purified calf intestinal enzyme, which cross-reacted with the red cell one. The purified preparation showed three bands of ADA activity on polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single band with an apparent MW of 41,700. The MW of the purified protein as estimated by ultracentrifugation studies was 38,000.

A broad pH optimum from 7 to 8 was observed. The K_m for adenosine was 52 μM, and the K_i for inosine was 700 μM. At 4°, the preparation retained activity for up to 3 weeks, but at $-70°$, glycerol or DMSO was necessary to preserve activity during freezing-thawing cycles. The purified enzyme consisted of a single polypeptide chain, and staining of polyacrylamide gels with periodic acid-Schiff reagent indicated the presence of carbohydrate. The differences in isoenzyme structure responsible for the molecular heterogentity were not established by this study.

A method for the purification of ADA from human erythrocytes was given by Agarwal and Parks (1978).

The metabolic role of ADA is not yet completely clear. Interest in blood ADA was stimulated by the suggestion of Berne (1964) that adenosine (and its phosphorylated derivatives) might play a regulatory role in coronary blood flow because of their vasodilator properties. Blood adenosine

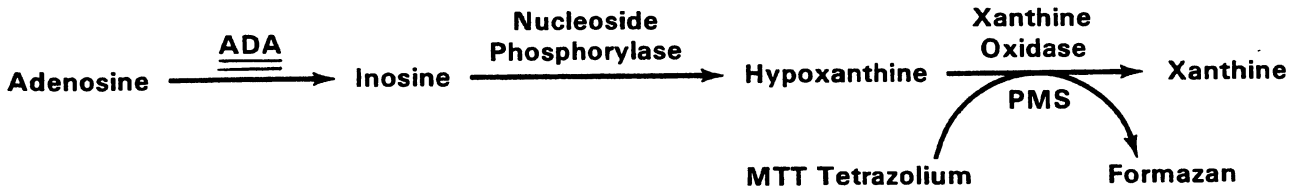


Figure 30.3 Detection Reaction Sequence for ADA

concentration is obviously influenced by the activity of ADA. Van Belle (1969) found that adenosine deamination in circulating blood was temperature dependent, but not affected by pH in the 6 to 8 range, nor by ionic strength. The situation was different in other animal species. More recently, interest in ADA has been prompted by the association between a deficiency of the enzyme and severe combined immuno-deficiency disease in certain patients (see section 30.2). Perrett and Dean (1977) have studied adenosine (and adenine) metabolism in human red cells over a range of substrate concentrations. At physiological adenosine concentrations, less than 1 μM, less than 10% of the adenosine was acted upon by ADA. Most of it was converted to adenine nucleotides by adenosine kinase. At much higher adenosine concentrations, in excess of 7 μM, deamination was the dominant pathway. If these findings accurately reflect the *in vivo* situation, then the role of ADA in purine metabolism is minimal.

30.5 Medicolegal Applications

30.5.1 Disputed parentage

ADA phenotyping is employed in a number of laboratories in cases of disputed parentage. Wüst (1971b) discussed the application of ADA typing to these cases, and said that about 5.5% of falsely accused men would be excluded by ADA phenotyping alone in the Viennese population. Bauer and Herbich (1972) recommended incorporation of the system into paternity investigations as well. They said that about 4% of falsely accused men would be excluded by the system in the Germanic population which they studied. Lefèvre *et al.* (1972) gave a detailed discussion of the probability calculations for paternity cases using the ADA system. Boorman *et al.* (1977) noted that 4.5% of falsely accused English men would be excluded by ADA phenotyping. Polesky *et al.* (1976) gave figures of 4.5% for Caucasians and 1.9% for Blacks for the overall probabilities of excluding a true nonfather in the U.S. population. ADA isozyme patterns are fully developed in fetal blood (Chen *et al.*, 1977). Caution should be exercised in the interpretation of second order exclusions based on ADA, since silent alleles are known to occur rarely.

30.5.2 ADA phenotyping in dried bloodstains

ADA phenotyping in bloodstains was described by Culliford in 1971. The buffer system originally described by Spencer *et al.* (1968), consisting of 0.1M phosphate, pH 6.5, bridge buffer, and a 1:10 dilution of that solution for gel buffer, was used. Electrophoresis was carried out on thin, 10% starch gels. Samples were treated with 1% mercaptoethanol in gel buffer prior to electrophoresis to insure that sulfhydryl effects (see in section 30.4.2) would not interfere with the interpretation of phenotypic patterns. ADA could be typed in 3 to 4 week old stains without difficulty, and occasionally in stains up to 3 months old. In samples infected with bacteria, activity could be lost, but in some samples of this kind, a single band occurred at a position cathodal to (slower than) the ADA^2 band.

Brinkmann and Dirks (1971) described ADA typing in bloodstains as well. Electrophoresis was carried out a system which allowed simultaneous determination of ADA, AK and PGD. ADA 1 and ADA 2-1 phenotypes could be distinguished in bloodstains up to 5 months old, but ADA 2-1 and ADA 2 were difficult to distinguish in the older stains.

Welch (1972b) found that ADA was detectable in 15 day old stains but not in 22 day old ones. Denault *et al.* (1978) reported that ADA was detectable in 13 week old stains on a variety of substrata, regardless of whether the phenotype was 1 or 2-1. Bloodstains kept at higher humidity gave better results than those kept at low humidity. At 26 weeks of age, only one of 14 stains was able to be phenotyped, and this stain had been kept frozen.

A number of variations in electrophoretic procedure have been applied to ADA phenotyping. Bauer and Herbich (1972) described a high voltage procedure for hemolysates on starch gels which was said to reduce running time and give sharper patterns. Sonneborn and Renninger (1970) described a cellulose acetate membrane procedure for ADA typing in hemolysates. The technique was further discussed by Sonneborn in 1972. Hoppe *et al.* (1972) gave a horizontal polyacrylamide gel technique for ADA.

A number of techniques have been described in which ADA is simultaneously determined with other isozyme systems in the same electrophoretic operation: ADA with AK on starch gels (Hummel, 1970; Kirchberg and Wendt, 1970); ADA with AK, ACP and PGM on one thick starch gel (Martin and Niebuhr, 1971); ADA with PGM and AK on horizontal polyacrylamide gels (Wrede *et al.*, 1971); and ADA with ACP and AK on starch gels in a system designed for bloodstain phenotyping (Wraxall and Stolorow, 1978).

30.5.3 ADA phenotyping in other tissues

ADA occurs in a variety of tissues apart from red blood cells, as indicated in the discussion in section 30.3. The evidence also suggests that a single genetic locus is responsible for all the red cell and tissue isozymes observed. Oepen (1974) found that ADA could be determined in fresh, dried or refrigerated skeletal muscle tissues, but that it was considerably more labile than the AK enzymes in this material. Turowska and Trela (1977) described ADA detection in the tissues of human teeth. ADA does not occur in detectable amounts in either seminal plasma or spermatozoa (Blake, 1976; Blake and Sensabaugh, 1976).

30.6 Distribution of ADA Phenotypes in U.S. Populations

There are only a few published studies of ADA phenotype distributions in this country, and the data are shown in Table 30.1.

The data of van den Branden *et al.* (1971) covered a number of different populations in other countries. The *ADA*[1] frequency in Europeans varies from about 0.91 to 0.95. In Black African peoples, it is somewhat higher. The only populations studied in which *ADA*[2] gene frequencies are relatively high are Asians and the Kurds of Iran, where the frequency can exceed 0.1 (van den Branden *et al.*, 1971; Mourant *et al.*, 1976).

Table 30.1 Distribution of ADA Phenotypes in U.S. Populations

		Frequency — Number (Percent)					
Population	Total	1	2-1	2	Other	*ADA*[1]★	Reference
CAUCASIAN							
Seattle, WA	168	152 (90.5)	16 (9.5)	0		0.950	Detter et al., 1970
Philadelphia, PA	180	(88.1)	(11.9)	(0)		–	Polesky et al., 1976
California and Hawaii	5,883	(90.0)	(9.8)	(0.2)	(0)	0.949	Grunbaum et al., 1978b
Miami/Dade County, FL	360	323 (89.7)	36 (10.0)	1 (0.3)		0.947	Stuver, 1979 and see Shaler, (1978)
Detroit, MI	503	446 (88.7)	56 (11.1)	1 (0.2)		0.942	Stolorow et al., 1979 and see Shaler, (1978)
Los Angeles, CA	135	123 (91.1)	12 (8.9)	0		0.956	Siglar, 1979 and see Shaler, (1978)
NEGRO							
Seattle, WA	186	178 (95.7)	6 (3.2)	0	two "5-1"	0.980	Detter et al., 1970
Philadelphia, PA	180	(97.2)	(2.8)	(0)		–	Polesky et al., 1976
California and Hawaii	927	(97.8)	(2.2)	(0)	(0)	0.989	Grunbaum et al., 1978b
Miami/Dade County, FL	344	333 (96.8)	11 (3.2)	0		0.984	Stuver, 1979 and see Shaler, (1978)
Detroit, MI	504	496 (98.4)	8 (1.6)	0		0.992	Stolorow et al., 1979 and see Shaler, (1978)
Los Angeles, CA	56	54 (96.4)	2 (3.6)	0		0.982	Siglar, 1979 and see Shaler, (1978)
HISPANIC							
California and Hawaii	1,260 ☆	(93.8)	(5.9)	(0.3)	(0)	0.964	Grunbaum et al., 1978b
Miami/Dade County, FL	355	329 (92.7)	24 (6.7)	2 (0.6)		0.961	Stuver, 1979 and see Shaler, (1978)
Los Angeles, CA	81 ⊙	77 (95.1)	4 (4.9)	0		0.975	Siglar, 1979 and see Shaler, (1978)
ASIAN/ORIENTAL							
Seattle, WA "Mixed Oriental"	118	113 (95.8)	5 (4.2)	0		0.980	Detter et al., 1970
California and Hawaii "Asian"	1,821	(95.2)	(4.6)	(0.2)	(0)	0.975	Grunbaum et al., 1978b

★ Gene frequency ☆ "Chicano/Amerindian" ⊙ Primarily Mexican

SECTION 31. ESTERASES

31.1 Introduction to Esterases

There are a number of carboxylic ester hydrolases in blood and in tissues, each group of enzymes possessing its own properties and peculiarities. Not all of these groups show genetic variation. The ones which do not are discussed only briefly in the following sections for the sake of completeness.

Generally, enzymes which exhibit esterase activity may be divided into three major categories: carbonic anhydrases; cholinesterases; and carboxylesterases. Carbonic anhydrases, which exhibit esterase activity, will be discussed in section 32. This section is concerned with cholinesterases and carboxylesterases. Many esterases exhibit very broad substrate specificities. In addition, there are many different esterase isoenzymes, and the physiological significance of many of them is not clear. For these reasons, attempts to classify the esterases, and to understand and account for them from a genetic point of view, have met with difficulty.

31.2 Cholinesterases

31.2.1 Recognition and classification of cholinesterases

In 1914, Dale suggested that a mechanism for the very rapid hydrolysis of acetylcholine might exist in the body, in order to remove this substance after it had served its purpose as a neurohumoral transmitter. Indeed, it is now known that acetylcholinesterase occurs in the nervous tissue of all animals. In 1932, Stedman *et al.* established that horse serum contained a cholinesterase activity. Glick (1937) carried out studies of the enzyme in human serum. In 1940, Alles and Hawes established that human blood contains two different sorts of cholinesterase activity, one being found in serum, while the other occurred primarily in red cells. These two activities differed significantly in their properties. Numerous studies have been conducted on both enzymes over the years.

The cholinesterases of human blood are thus of two kinds: (1) the cholinesterase found in red cells, which is called acetylcholinesterase (AChE; E.C. 3.1.1.7; true cholinesterase; acetylcholine hydrolase); and (2) the cholinesterases of plasma (or serum), which do not hydrolyze acetylcholine preferentially, and have been given a variety of different names, including pseudocholinesterases (E.C. 3.1.1.8). Cholinesterases are characterized simply by the fact that they hydrolyze choline esters. In general, they may be distinguished from other kinds of esterases by virtue of the fact that they are inhibited by 10^{-5}M eserine (physostigmine). The cholinesterases are, however, a family of enzymes with many divergent properties (Augustinsson, 1957). Studies on the purification and properties of acetylcholinesterases from a number of sources have been reviewed by Wilson (1960), Koelle (1963) and Froede and Wilson (1971).

31.2.2 Red cell acetylcholinesterase (E.C. 3.1.1.7)

In 1962, Johns described a patient who had only about one-third the normal red cell AChE activity. The man was healthy. It was found that his mother and sister shared this reduced red cell AChE activity, strongly suggesting a genetic basis for the condition. The red cell enzyme is bound to, or perhaps an integral part of, the erythrocyte membrane, and, as with other membrane-associated proteins, special procedures must be employed to solubilize it. As a result, this enzyme does not readily lend itself to electrophoretic analysis. In 1972, Coates and Simpson reported that they had detected genetic variation in the enzyme by disc electrophoretic analysis of Triton-X-100-extracted red cell stroma. There were three apparent phenotypes, called 1, 2-1 and 2, and limited family studies indicated that these phenotypes were consistent with control of the enzyme by an allelic pair, called $AChE^1$ and $AChE^2$. In 1976, Das and Lo obtained somewhat similar results using a somewhat different polyacrylamide gel electrophoresis procedure. Additionally, their material was obtained exclusively from Chinese men. They could distinguish three patterns as well, and called them 1, 2 and 3. Type 2 looked quite similar to the Type 1 of Coates and Simpson (1972). They did not do family studies, however, and did not say explicitly that the observed variation had a genetic basis.

A number of studies have been carried out on the purification and characterization of red cell AChE, and it is not very clear from these investigations whether this enzyme does or does not really show genetic variation. In 1971, Shafai and Cortner purified red cell AChE to a certain extent, and could resolve two components on DEAE-Sephadex which differed in charge, but were similar in size (Shafai and Cortner, 1971a). Further studies on DEAE ion exchange media suggested that the enzyme consisted of subunits, which could dissociate and reassociate on the column itself, during the separation (Shafai and Cortner, 1971b). Ciliv and Özand (1972) purified the enzyme, and found it to be a glycoprotein which formed easily reversible aggregates. Paniker *et al.* (1973) studied the effects of using a number of non-ionic detergents in the purification of the enzyme, and there were no differences, suggesting that membrane dissolution was the key requirement in releasing the enzyme from the stroma. Wright and Plummer (1973), Das and Lo (1976) and Das *et al.* (1977) have all reported purification of the enzyme, and all have observed apparent multiple forms of it on ion-exchange chromatographic media and/or disc electrophoresis. There is not much doubt that some of the different forms represent different states of aggregation

of the same subunit(s). The purest preparations have been obtained by affinity chromatography of Triton-X-100-solubilized membrane fractions (Grossmann and Liefländer, 1975; Ott *et al.*, 1975; Ott and Brodbeck, 1978). Studies on the pure preparations indicate that the enzyme behaves as a single molecule with MW 80,000 in the presence of solubilizing detergent, but that the detergent-free enzyme shows multiple forms upon ion exchange media, polyacrylamide gels, isoelectric focusing, or density gradient centrifugation. The multiple forms are presumably different aggregates of the 80,000 MW subunit. Grossmann and Liefländer (1975) said that the MW of the purified preparation, as estimated on detergent (SDS)-containing polyacrylamide gels was 80,000 in the presence of mercaptoethanol but 154,000 in its absence, suggesting a disulfide bond in the molecule.

31.2.3 Plasma (Serum) cholinesterase (ChE; Pseudocholinesterase; PCE; E.C. 3.1.1.8; acylcholine acyl-hydrolase)

31.2.3.1 Early studies on plasma ChE. The studies leading to the recognition of plasma ChE enzymes, with an identity separate from the red cell cholinesterase, were mentioned in section 31.2.1. The earlier studies on plasma ChE have been reviewed by Augustinsson (1948 and 1961) and the same author has written an extensive review of the assay methods employed for ChE (Augustinsson, 1957).

31.2.3.2 Genetically controlled variation in plasma ChE. Interest in plasma ChE was stimulated in part because of the role of this enzyme in hydrolyzing the muscle relaxant succinyldicholine (also called suxamethonium; Fig. 31.1). The drug was apparently quite commonly used in patients undergoing anesthesia. It was known to be short-acting, because of its rapid hydrolysis by serum ChE. In 1956, Kalow established with certainty, both *in vitro* and *in vivo*, that plasma ChE hydrolyzes succinyldicholine, and he carried out measurements on the reaction (Kalow, 1956a). Three people were described, however, whose serum showed a very low succinyldicholinesterase activity. They had been detected because of adverse reactions to the usual dosages of the drug. Profound muscle relaxation followed administration of the substance in these people, along with a period of apnea (cessation of respiration). Many people with unusual kinds of plasma ChE have been detected through their response to the routine administration of succinyldicholine in clinical settings. Goedde *et al.* (1968) suggested that pro-

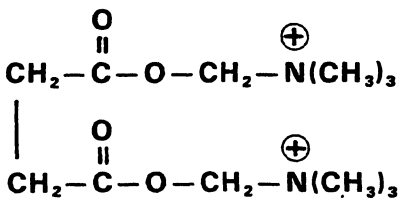


Figure 31.1 Succinyldicholine

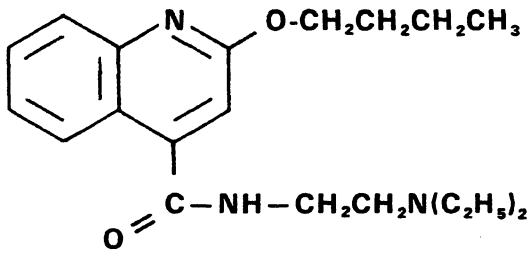


Figure 31.2 Dibucaine

longed apnea following treatment with succinyldicholine be treated by administration of purified plasma ChE.

Kalow and Genest (1957) soon showed that the unusual ChE present in the sera of the suxamethonium-sensitive people could be detected in another way, namely on the basis of inhibition of the enzyme by dibucaine (Fig. 31.2). Dibucaine is a local anaesthetic, and is also called Nupercaine, Percaine and cinchocaine. Using benzoyl choline as substrate for the enzyme, assays were conducted with and without 10^{-5}M dibucaine. Under a standard set of conditions, 5×10^{-5}M substrate in 66.67 mM phosphate buffer, pH 7.4, and 25°, serum was assayed at a 1:100 dilution by following the decrease in absorbance at 240 nm. Normal (usual) sera were inhibited about 79%, but the "atypical" sera only about 16%. The % inhibition with dibucaine seen under these standard conditions was defined as the "Dibucaine Number" (or DN). In 1957, Kalow and Staron reported extensive cholinesterase assay studies on almost 1,700 sera, many from members of families. Using the DN, three different groups could be distinguished, and these were called "usual", "intermediate" and "atypical". Family studies indicated that these phenotypes could be accounted for on the basis of a pair of codominant alleles operating at an autosomal locus. The "usual" allele and the "atypical" allele were presumably responsible for different cholinesterase enzyme molecules, which were found in approximately equal mixture in the plasma of heterozygotes ("intermediate" types). The frequency of the atypical gene in the Toronto area was about 0.015. Kalow and Davies, in 1959, tested a series of esterase inhibitors for their ability to discriminate the three phenotypes. Most showed a differential effect on the usual and atypical forms, like dibucaine does. The organophosphates (such as DFP), however, which are classical inhibitors of ChE, inhibited both forms equally. These inhibitor studies were consistent with the genetic hypothesis. Harris and Whittaker (1962b) carried out family studies on the serum ChE polymorphism, and the results were fully consistent with the two allele hypothesis.

Other ChE inhibitors, as noted above, will differentially inhibit the usual and atypical forms of ChE, and one interesting inhibitor was first found in potatoes. In 1956, Pokrovskiy observed that watery extracts of potatoes would inhibit plasma ChE. Orgell *et al.* (1958) confirmed this finding, and showed further that many plants which were

members of the *Solanaceae* contained the inhibitor. The potato (*Solanum tuberosum* L.), which has the inhibitor in the tuber and the tuber peel, was especially well studied. The results indicated that the inhibitor was not a macromolecule. Harris and Whittaker (1959) showed that the potato extract inhibitor differentially affected the usual and atypical serum ChE enzymes, and could therefore be used to discern the phenotypes. Harris and Whittaker (1962a) carried the studies further. They gave credit to Pokrovskiy (1956), whose work they had not known about, for establishing that the potato inhibitor was solanine, and they showed that solanine, and its alkaloid moiety, solanidine (Fig. 31.3), both give the differential inhibition of plasma ChE types.

31.2.3.3 Further genetic variation in plasma ChE. In 1961, Harris and Whittaker examined the plasma ChE of all three "dibucaine" types for inhibition by 5×10^{-5}M NaF. In general, sera fell into three categories based upon inhibition by NaF, and these corresponded to the division seen with dibucaine as inhibitor. Fluoride Number (FN) was defined in the same way as Dibucaine Number, except for the difference in inhibitor concentration. DN values for the three phenotypes are about 80, 62 and 20 for usual, intermediate and atypical forms, respectively, and the corresponding FN were 61, 48 and 23. Some discrepancies were found, however, in which the FN was about 26–34 (with DN about 52–54), and in which FN was 50–55 (with DN 73–78). The values were consistent over time, and were attributed to the expression of another allelic gene at the ChE locus, in heterozygous combination with one of the known alleles. Family studies by Harris and Whittaker (1962b) supported this notion.

In 1968, Whittaker found that aliphatic alcohols would differentially inhibit the usual and atypical ChE enzymes. The effects were rather complicated, but alcohol concentrations and other reaction conditions could be found where the differential inhibition was maximal (Whittaker, 1968a). Further studies (Whittaker, 1968b) were carried out with n-butanol. An "Alcohol Number" was defined in the same way as had been the dibucaine and fluoride numbers (i.e., as % inhibition). The numbers were different from the DN or FN for similar types, but generally followed the same pattern. There was a suggestion that alcohol inhibition studies might define still more phenotypes of ChE. Whittaker (1968c) also found that NaCl (0.5M) would differentially inhibit the usual and atypical enzymes, and these values of inhibition were called "Chloride Numbers". The work of Clark *et al.* (1968), discussed in section 31.2.3.6, might be relevant to these NaCl inhibition studies.

In 1966, Neitlich described a man with consistently elevated plasma ChE, and studies on his family indicated that the condition had a genetic basis. Yoshida and Motulsky (1969) studied the ChE from this individual by immunological and biochemical techniques, and concluded that a structurally abnormal enzyme was present, and was being "overproduced". This variant type was called "Cynthiana".

31.2.3.4 Electrophoretically detectable genetic variation in plasma ChE—a second plasma ChE locus. In 1962, Harris *et al.* carried out an electrophoretic study of plasma ChE, using two dimensional electrophoresis. Serum was run first on paper, and the paper was then inserted into a starch gel, and electrophoresis carried out at right angles to the direction of the first separation. Electrophoretic steps were done in pH 7.1 phosphate buffers, and the ChE detected with α-naphthyl acetate and Fast Red TR salt. In most sera, four zones could be distinguished. These were designated C_1

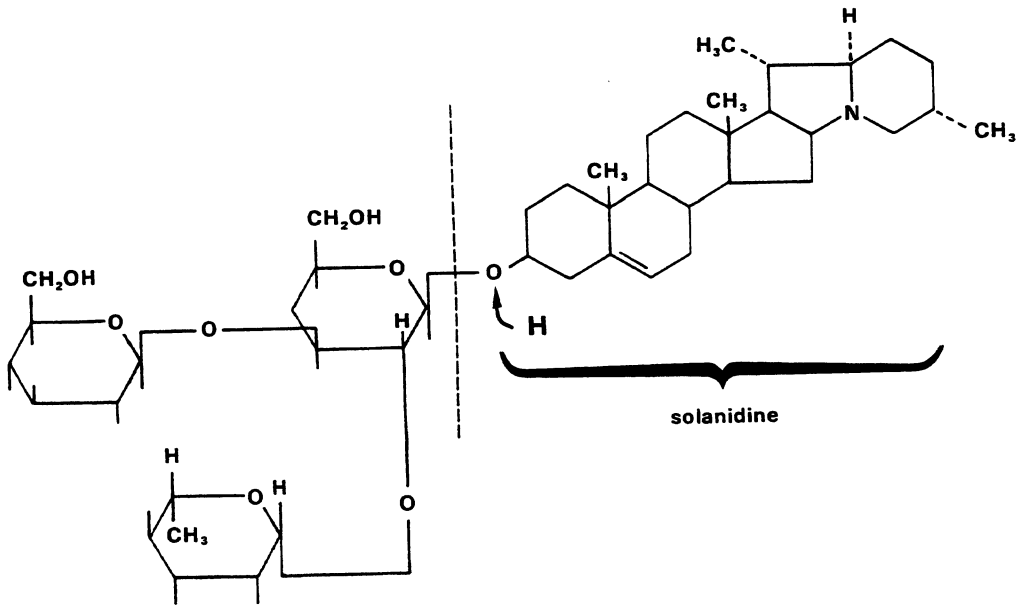


Figure 31.3 Solanine

through C_4, the last representing most of the enzyme activity. Occasionally, a fifth zone was seen. It was less anodal (slower), and closer to the origin, than C_4, the slowest of the other four common isozymes, and it was called C_5. Further studies by Harris *et al.* (1963a) supported a genetic basis for the presence of C_5 in some people's sera. About 5% of 248 British people were C_5^+. It was shown, too, that one dimensional starch gel electrophoresis at pH 6 would serve to distinguish between C_5^+ and C_5^- people. The C_5^+ phenotype was regarded as the expression of a gene whose allele did not code for the enzyme. Accordingly, no C_5^+ offspring should result from $C_5^- \times C_5^-$ matings, and, in a few cases, C_5^- parents with a C_5^+ child were seen in the study. Electrophoretic typing of C_5^+, however, is not always completely unambiguous. Difficulty in phenotyping and/or variability in the expression of C_5^+ in C_5^+ individuals could explain the apparent discrepancies in the family material. Ashton and Simpson (1966) raised this problem as well, noting that there had been several discrepancies in the typing results carried out by different laboratories on the same sera. They noted that caution was necessary in the interpretation of the C_5 phenotypes. There were some significant deviations from the expected segregation patterns in 1,000 Brazilian families in these studies.

Harris *et al.* (1963b) studied the expression of the "dibucaine sensitivity" and of C_5^+ simultaneously, and the data indicated quite conclusively that the loci for the two types of plasma cholinesterases were not identical. Further studies by Robson and Harris (1966) supported the notion of a separate locus for the gene controlling the C_5^+ polymorphism, and they noted that the C_5 isoenzyme might not always be detected in heterozygotes by standard electrophoretic typing procedures.

By 1964, some general agreement had been reached among workers in the field on plasma ChE nomenclature. The recommended scheme was summarized by Motulsky (1964), and is given in Table 31.1. There are two known plasma ChE loci, called E_1 and E_2. Four alleles are known to occur at E_1, and they are denoted by superscript lower case letters: E_1^u, E_1^a, E_1^f, and E_1^s. The first three of these alleles control the usual, atypical and fluoride-resistant forms of plasma ChE, and E_1^s is silent (or almost silent; see section 31.2.3.5). There are two alleles at E_2, simply denoted E_2^+ and E_2^-, which give rise to three genotypes, $E_2^+E_2^+$, $E_2^+E_2^-$ and $E_2^-E_2^-$. The first two of these are indistinguishable, both being C_5^+.

31.2.3.5 Silent alleles at E_1. In 1956, Kalow and Lindsay reported that four people out of about 1,000 they had studied in Canada showed very low plasma ChE activity, almost too low to be measured. Independently, Lehmann and Ryan (1956) described an English family, a number of whose members had very low plasma ChE activity. Kalow (1956b) said that these observations were essentially in agreement with his own. The people were detected because

Table 31.1 Nomenclature and Properties of the Plasma Cholinesterase Variants

Genotype		Phenotype				Typical	
Standard Designation ★	Other Designation ☆	Standard Designation ★	Other Designation ☆	Type of Esterase Present	Amount of Esterase Present (Relative %)	DN ●	FN ◇
$E_1^u E_1^u$	N–N	U	usual	u(sual)	100	80	64
$E_1^u E_1^a$	N–D	I	intermediate	u + a(typical)	78	62	48
$E_1^a E_1^a$	D–D	A	atypical	a	25	20	23
$E_1^s E_1^u$	S–N	U	usual	u	65	80	64
$E_1^s E_1^s$	S–S	S	silent; zero	none	0	–	–
$E_1^s E_1^a$	S–D	A	atypical	a	20	20	23
$E_1^f E_1^u$	F–N	UF	U_1	f(luoride) resistant + u	80	76	52
$E_1^f E_1^f$	F–F	F		f	50	67	34
$E_1^f E_1^a$	F–D	IF	I_1	f + a	60	50	30
$E_1^f E_1^s$	F–S	F		f	never observed		
$E_2^+ E_2^+$, $E_2^+ E_2^-$		C_5^+		u + C_5	130	80	64
$E_2^- E_2^-$		C_5^-		u	100	80	64

★ After Motulsky (1964) ☆ After Lehmann and Liddell (1964) ● Dibucaine Number ◇ Fluoride Number

they were extraordinarily sensitive to succinyldicholine administration. In 1962, Liddell *et al.* studied a number of individuals who had shown unusual succinyldicholine sensitivity. They found subjects in whom the DN corresponded to homozygosity for E_1^a, but where family studies indicated heterozygosity for E_1^a. The best explanation of the results, they said, was heterozygosity of E_1^a with a silent allele at E_1. They described a Greek woman who had no plasma ChE, and thought that she was probably a homozygote for the silent gene.

Doenicke *et al.* (1963) reported on an individual with no detectable plasma ChE, and they confirmed the absence of activity in a biopsied liver specimen. In 1964, Simpson and Kalow agreed with Liddell *et al.* (1962) that absence of serum ChE was attributable to homozygosity for a silent allele at E_1, and they called it E_1^s. Although a suppressor gene could not be excluded, it has generally been accepted since that time that the $E_1^sE_1^s$ genotype is the correct explanation for an absence of plasma ChE activity. The frequency of E_1^s was estimated to be about 10^{-5}. Other cases of apparent homozygotes of E_1^s have been reported by Dietz *et al.* (1965), Hodgkin *et al.* (1965) in an American Irish family, Szeinberg *et al.* (1966) in a Morrocan Jewish family, and Jenkins *et al.* (1967) in an African Bantu girl.

There is considerable evidence that there is more to the "silent allele" story than was first realized. It appears now that not every "silent" serum is the same, and that there may be more than one genetic basis for the absence, or near absence, of plasma ChE activity. Goedde *et al.* (1965a and 1965b) examined sera from two people who were presumed to be homozygous for the silent allele. Upon close examination, the sera revealed low activity, 2–3% of normal. These sera also reacted with a precipitating antibody against normal plasma ChE prepared in rabbits, suggesting that, at least in these people, the "silent" gene coded for a structurally altered protein. These studies have been pursued by Goedde and Altland (1968) and Altland and Goedde (1970). On the basis of activity with different substrates, electrophoretic and chromatographic studies, and of the behavior of the "silent" sera toward anti-plasma ChE, it is clear that not every example of ChE-deficient serum has the same properties. These findings have been confirmed by other workers (Gaffney and Lehmann, 1969; Rubinstein *et al.*, 1970). There are cases in which the plasma has no activity whatsoever, apparently the result of a truly silent allele. Hodgkin *et al.* (1965) showed that the sera of their propositi did not react with an anti-normal ChE antibody, suggesting that no gene product was present. In general, ChE deficiency is extremely rare. If the estimate of Simpson and Kalow (1964) of 10^{-5} for the E_1^s frequency were correct, one would expect to find homozygosity for this allele in only about 1 in every 10 billion people. From the published results to date, more people than that have been found, but the frequency is still very low. The Eskimos of Western Alaska have been shown to have an inordinately high frequency of plasma ChE deficiency (Gutsche *et al.*, 1967; Scott, 1973). 37 people out of 5,000 have been found to be deficient. There are some people with complete deficiency, while others have a trace of activity, and it was suspected but not proven that the two conditions have a different genetic basis.

31.2.3.6 Molecular heterogeneity of plasma ChE—Biochemical studies. Over the years, a considerable number of studies have indicated that plasma ChE shows molecular heterogeneity, and that a number of isozymes can be identified, apart from the C_5 polymorphism. There was early kinetic evidence that serum contained more than one ChE activity (Heilbronn, 1958; Berry, 1960). Chromatography of plasma on calcium phosphate gels gave similar indications (Malmström *et al.*, 1956). Many investigators have examined plasma ChE isozymes by electrophoresis (Dubbs *et al.*, 1960; Bernsohn *et al.*, 1961; Hess *et al.*, 1963; Brody *et al.*, 1965; Juul, 1968). Depending upon the technique used, anywhere from 2 to 12 zones of ChE activity have been detected. On starch gel electrophoresis, it is common to observe 4 zones of activity, usually called C_1 through C_4. C_4 accounts for the majority of the total activity. These electrophoretically distinguishable forms can also be separated by gel filtration chromatography (Harris and Robson, 1963b), and the C_5 isozyme has been fractionated on calcium phosphate gels (Reys and Yoshida, 1971).

Svensmark (1961a and 1961b) established that plasma ChE was a sialoprotein, and the change in electrophoretic mobility following treatment with neuraminidase suggested that the various forms contain a different number of sialic acid residues per molecule. Removal of the sialic acid with neuraminidase did not affect enzyme activity though. A number of workers have purified the enzyme to a greater or lesser degree, and examined the properties of the multiple molecular forms. Svensmark (1965) partially purified plasma ChE, and estimated that the MW of the major fraction was 300,000. There was no indication of a prosthetic group, and no metal requirement. The atypical enzyme was found to differ from the usual one in substrate and inhibition characteristics. LaMotta *et al.* (1965, 1968 and 1970) purified the enzyme, and found that the isoenzymic forms were interconvertible. In some purified preparations, up to 7 isozymes could be detected. Based upon MW estimates for the major isozyme, they suggested that the different forms might simply be polymers of a subunit with MW about 30,000. Gaffney (1970), who observed six zones of activity in purified preparations by polyacrylamide gel electrophoresis, agreed that the forms differed in size (as well as in charge). He did not think, however, that the simple polymerization hypothesis of LaMotta *et al.* (1970) sufficed to explain his data. The interconvertibility of ChE forms might help to explain the observation by Dubbs (1966) that sonication in serum for about 15 min. greatly enhanced the intensity of the C_3 zone. Haupt *et al.* (1966) obtained a very pure preparation of plasma ChE, and the MW was said to be 348,000. They also noted that serum ordinarily contains about 0.9 mg of the enzyme per 100 mℓ of serum. Muensch *et al.* (1976) prepared a very pure C_4 (about 8000-fold). It had a MW of 345,000 and was found to be a tetramer of C_1.

These studies did not shed light on the nature of C_2 or C_3, nor on the role of the sialic acid residues in the isozymes.

In connection with the studies on C_5 (E_2) phenotyping, there have been several reports of additional bands less anodal than C_5 in fresh serum samples. Ashton and Simpson (1966) observed an additional band, a kind of "C_6". Van Ros and Druet (1966) observed a C_6 and two additional bands, C_{7a} and C_{7b}, in two Black African individuals, and suggested that these were genetically determined, although family studies could not be done. Ogita (1975) came up with a rather different interpretation of these patterns based on his studies of several Japanese subjects with unusual plasma ChE. The C_6 and C_7 forms of plasma ChE appeared in the serum of an old man who had a benign peritoneal tumor. His plasma ChE level had dropped to very low levels during the course of the disease, but later increased somewhat. Incubation of his serum with ordinary C_4 ChE induced the formation of the C_6 and C_7 components, and this transformation was shown to be due to a neuraminidase-like activity in his serum. There were indications from the family study that the neuraminidase-like activity was under genetic control, and Ogita suggested that some of the ChE components occasionally observed in plasma may have nothing to do with the E_1 or E_2 loci directly, but might represent epigenetic modifications.

Clark *et al.* (1968) carried out extensive kinetic studies on plasma ChE and on partially purified C_4 component. These experiments compared the properties of the usual and the atypical enzyme. Tris buffer was found to have complex stimulatory and inhibitory effects on both types of enzymes. At low concentrations of Tris, about 0.67 mM, NaCl in increasing concentrations enhanced the activity of the usual enzyme but not of the atypical one. Other salts had similar effects. Tris itself, at higher concentrations, stimulated both forms of the enzyme. The usual one was stimulated more, and the kinetics were complex. These effects were seen with benzoylcholine as substrate, but not when acetyl- or butyrylthiocholine were employed. These data, together with that from other series of experiments, led to the suggestion that the alteration in the atypical, as compared with the usual, enzyme affected both the esteratic and anionic sites in the molecule. Cholinesterases are thought to have both kinds of sites. The anionic site is a locus of negative charge, and attracts the quaternary ammonium group of the choline. This site determines the specificity of the enzyme with respect to the alcohol moiety of the ester. The esteratic site is the actual catalytic site, and determines the specificity of the enzyme toward the acid moiety of the ester (see Froede and Wilson, 1971, for review of the catalytic mechanism of AChE). The studies of Lockridge and La Du (1978) on highly purified usual and atypical ChE enzymes with a fluorescent probe substrate indicate that the two differ only in the structure of their anionic site. Lockridge *et al.* (1979) have extensively purified the usual ChE enzyme and studied its subunit organization. The native molecule is a tetramer of MW 340,000, and appears to be arranged as a dimer of dimers. Each dimer contained one interchain (and several intrachain) disulfide bonds. The subunits are apparently held together by noncovalent forces.

Reviews covering the plasma ChE variants may be found in Lehmann and Liddell (1964) and Simpson (1968).

31.2.3.7 Assay methods for ChE—Detection of E_1 phenotype and screening techniques. There are many different assay techniques for serum ChE. If phenotyping is the objective of the measurements, however, then standard assay techniques must be employed, since the E_1 phenotypes represent quantitative differences in enzyme activity in the presence and absence of specific concentrations of specific inhibitors. Assay methods have to be standardized and uniform so that results from different laboratories will be comparable. The original, and standard, method for the determination of Dibucaine Number (DN) was given by Kalow and Genest (1957). The method for the determination of Fluoride Number (FN) is the same, except that 5×10^{-5}M NaF is used instead of 10^{-5}M dibucaine (Harris and Whittaker, 1961). Any other procedure would have to be carefully standardized with established examples of sera of known phenotype.

If a large number of sera are to be tested, then spectrophotometric assay procedures are time consuming and cumbersome, and investigators have sought more rapid tests for the initial screening of sera. The point of such tests is the detection of possible variants, which may then be subjected to the more rigorous assay. In 1962, Kalow described a screening procedure which was relatively quick,, but which was spectrophotometric. Kalow and Davies (1959) had discovered that an inhibitor, called RO2-0683, at 10^{-7}M, completely inhibited the usual enzyme, but inhibited the atypical enzyme by about 30%, and "intermediate" serum activity by about 60%. This inhibitor was employed in the screening test at 2.26×10^{-6}M.

RO2-0683 was an experimental substance from Roche, and is chemically the dimethyl carbamate of 2-hydroxy-5-phenyl-benzyl trimethylammonium bromide (Fig. 31.4). It is first referred to as "RO2-0683" by Kalow and Davies (1959). The compound was previously called "Nu 683", and was introduced by Hawkins and Gunter (1946), who found that it was a selective inhibitor of plasma ChE. Myers (1952) did extensive studies on the characteristics of the inhibition of plasma ChE by the compound. Most screening tests have employed RO2-0683 as inhibitor.

In 1963, Harris and Robson described two types of screening tests for atypical and intermediate ChE phenotypes (Harris and Robson, 1963a). One of these was carried out in 1.5% agar gel, prepared in 0.1M Tris HCl, pH 7.4. Two identical gel plates were prepared, one containing 10^{-7}M RO2-0683, and the other not containing it. Identical sets of wells were punched in the gels, and the same serum was added to the corresponding wells of each plate. Serum dilutions were 1:32 for the control gel, and 1:8 for the inhibitor gel. After 17 hours of diffusion, the gels were flooded with α-naphthyl acetate in phosphate buffer, pH 7.1, containing Fast Red TR salt. Sera of the usual type showed activity in the control gel well, but no activity in the inhibi-

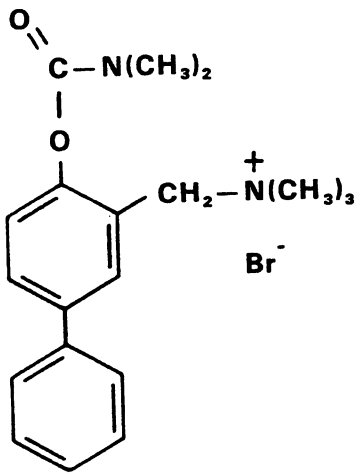


Figure 31.4 RO2 - 0683

tor gel well. Sera which showed activity in both were put aside as presumptive intermediate or atypical types, and subjected to spectrophotometric assay. The other procedure was done in a similar way, but in solution in test tubes. Drops of the solution were then applied to filter paper sheets containing substrate and coupling azo dye. Harris and Robson (1963a) tried the screening test on a number of sera of known type and it performed accurately. Goedde and Fuss (1964) described an agar gel screening test that was quite similar. Radam (1966) offered an improved filter paper screening test that was said to be more sensitive, primarily because a different azo coupling dye was employed. Simpson and Kalow (1965) used the agar gel screening test of Harris and Robson (1963a) to screen the sera of 6,500 people. There were some cases in which they said the screening test failed (0.1% of the sera). It also gave evidence of "atypical" sera in a number of cases where subsequent assay showed the serum to be of the "usual" phenotype.

The C_5^+ phenotype is detectable by electrophoresis, as noted above. A procedure is described by Harris and Hopkinson (1976) in addition to those given in the references cited in section 31.2.3.4. Scott and Weaver (1970) mentioned that electrophoresis could be used to detect simultaneously the C_5^+ variants and the low or zero activity ones. They noted that thiocholine, produced by the hydrolysis of butyrylthiocholine by ChE, will reduce MTT tetrazolium in the presence of PMS and DCPIP, yielding the familiar formazan bands of enzymatic activity.

31.3 Carboxylesterases

31.3.1 Classification of carboxylesterases

Carboxylesterases occur in a wide variety of tissues. Over the years, classification schemes have been proposed to bring some order to this diverse group of enzymes. The classifications are based primarily on substrate specificity of the enzymes, and on differential inhibition by various inhibitors, regardless of tissue of origin. In 1953, Aldridge designated two different forms of esterases in a number of animal sera as "A" and "B". A-esterases hydrolyzed p-nitrophenyl acetate better than p-nitrophenyl butyrate, and were not inhibited by diethyl p-nitrophenyl phosphate (called E-600); B-esterases hydrolyzed the butyrate ester as well as or better than the acetate ester, and were inhibited by about 10^{-7}M E-600. He found too that the A-esterase hydrolyzed the E-600 itself. In 1957, Bergmann *et al.* isolated an esterase from hog kidney whose properties were unlike either the A- or B-esterases, and they designated it a C-type esterase. The substrate specificity and inhibitor sensitivity characteristics of A and B esterases were discussed by Augustinsson (1961). Red cell esterases belonging to these three classes have been studied extensively (section 31.3.2) and the different classes of esterases can be separated electrophoretically, in addition to being distinguishable on the basis of substrate specificity and inhibitor properties. The same can be said for tissue esterases (section 31.3.6). The older carboxylesterase classification is shown in Table 31.2, and a more recent scheme appears in Table 31.3.

31.3.2 Red cell carboxylesterases

In 1958, Wachstein and Wolf showed by histochemical staining techniques that human red cells contained esterase activity with naphthol-AS-acetate. These results were confirmed by Davis (1959) using α-naphthyl acetate as substrate. As noted in section 26, esterases were among the first enzymes demonstrated to consist of multiple molecular forms by the combination of electrophoresis followed by histochemical staining of the separated enzymes. The esterase isozymes of mouse blood and tissues were extensively studied (Markert and Hunter, 1959; Hunter and Strachan, 1961). There was evidence for multiple molecular forms of human red cell esterases from the studies of Micheli and Grabar (1961). They separated hemolysate proteins in an immunoelectrophoretic system, and characterized some of the precipitin arcs thus obtained by specific histochemical staining procedures.

In 1961, Tashian first applied starch gel electrophoresis to the study of human red cell carboxylesterase isoenzymes, and a great deal of the pioneering work was done in his laboratory. Nine bands of esterase activity could be resolved on starch gels by electrophoresis at pH 8.6 using boric acid-NaOH buffers, and α-naphthyl acetate, propionate and butyrate as substrates. The α-naphthol was detected with Fast Blue RR salt. Those esterases that were not inhibited by 10 mM eserine, nor by 1 mM DFP, were designated "A", while those that were inhibited by the DFP but not by the eserine were designated "B", keeping the terminology that had been applied to the serum esterases (see in section 31.2.1). Altogether, eight A-esterase bands could be distinguished on the gel, along with one B-esterase band. One pair of altered A-esterase bands was found in a set of adult identical twins, and the pattern was shared by their mother. This genetic variation was studied further by Tashian and Shaw

Table 31.2 Classification of Carboxylesterases

Esterase Type	Synonyms	Substrates	Inhibitors	Activators
A esterases				
general	aromatic esterases, arylesterases	Ar-OAc > Ar-OBu not aliphatic or choline esters	pCMB, pHMB EDTA, La^{3+}	Ca^{2+}
red cell	acetylesterases	α-NOAc > β-NOAc > αNOPr > αN-OBu	pCMB, IA	
B esterases				
general	simple esterases, alliesterases	Al-esters > Ar-esters	organophosphates	
red cell	butyrylesterases	α-NOBu > β-NOAc > α-NOPr > α-NOAc	DFP	pCMB, IA
C esterases				
red cell	acetylesterases	α-NOAc > β-NOAc > α-NOPr > α-NOBu		pCMB, IA
D esterases	—	MU-OAc > MU-OBu	Hg^{2+}	
Cholinesterases				
red cell	acetylcholinesterase, AChE	choline-OAc > choline-OBu	organophosphates, carbamates, eserine	
plasma	nonspecific cholinesterases, pseudocholinesterases	choline-esters > Al-esters > Ar-esters	organophosphates, eserine	
Carbonic Anhydrases	—	CA_1 MU-OAc > β-NOAc > α-NOAc = MU-OPr CA_2 Fl-diOAc > β-NOAc > α-NOAc = MU-OAc = Fl-OPr	acetazolamide	

Abbreviations: Ar-OAc = aromatic acetate esters; Ar-OBu = aromatic butyrate esters; α-NOAc and β-NOAc = α- and β-naphthyl acetates; α-NOPr = α-naphthyl propionate; α-NOBu = α-naphthyl butyrate; Al = aliphatic; Ar = aromatic; MU-OAc, MU-OPr and MU-OBu = 4-methylumbelliferyl acetate, propionate and butyrate; Fl-diOAc = fluorescein diacetate; Fl-OPr = fluorescein propionate; pCMB = p-chloromercuribenzoate; pHMB = p-hydroxymercuribenzoate; EDTA = ethylene diamine tetraacetic acid; IA = iodoacetamide; DFP = diisopropyl fluorophosphate

(1962). The variant esterase was segregating in three generations of this family, and pedigree data were consistent with its inheritance as an autosomal allele. Somewhat better resolution of the esterase bands was achieved by Tashian and Shaw (1962), and the isoenzymes were characterized further. Accordingly, the classification scheme was changed. Old A1 and A2 were designated "C", A_3–A_6 were re-designated A_1a–A_1d, A_7 was resolved into four bands called A_2a–A_2d, B remained B, and A_8 was identified as carbonic anhydrase I (CA I). In 1965, Tashian described further studies on the separation and characterization of the esterase isozymes, as well as several genetic variants of them. The classification was again changed to reflect the newer findings. The A_1a and A_1b bands were designated "A_1", while the old A_1c and A_1d bands were now called "A_2". The old A_2 region now became "A_3". The most recent scheme is found in the Tashian (1969) review, and is presented in Fig. 31.5. It should be mentioned that Shaw *et al.* (1962) first characterized the carbonic anhydrases (CA I and CA II), which have esterase activity, as "esterases Da_1 and Db". It was very soon clear, however, that these enzymes were carbonic anhydrases (section 32), and they have no relationship to what we now call "esterase D" (see in section 31.3.4).

31.3.3 Genetic variation of the red cell A-esterases

Only a few rare variants of the A-esterases have been reported. The locus controlling the expression of these enzymes in red cells should not, therefore, be regarded as polymorphic. The variant pattern described by Tashian (1961) and Tashian and Shaw (1962) occurred in a White family, and was later called an "AB" phenotype (Tashian, 1965 and 1969). In these terms, an "A" phenotype is the normal pattern, and the variant pattern was thought to represent heterozygosity. A further variant pattern has been identified in a Black person (Tashian, 1965) and was called "AC". An additional example of "AB" was found in a Black individual as well. These variants were identified in a survey of 2638 Caucasian, 623 Black, 366 American Indian and 490 Micronesian bloods. Tashian (1969) has reviewed this material.

31.3.4 Esterase D and its polymorphism

In 1973, Hopkinson *et al.* described a new esterase in human red cells, which had been detected using fluorogenic substrates, 4-methylumbelliferyl acetate and 4-methylumbelliferyl butyrate (MUA and MUB). They called this enzyme Esterase D, and the locus controlling its expression exhibited genetic polymorphism in all populations studied. Esterase D (ESD) could be resolved by starch gel electrophoresis in a number of different buffer systems, but the most satisfactory was found to be Tris-citrate-borate-LiOH, pH 7.2. Electrophoresis was generally carried out at 1.5V/cm for 17 hrs at room temperature. ESD is not detected using α-naphthyl acetate as substrate, but MUA detects A_1 and B esterases in addition to ESD (see in section 31.5). MUB detects ESD and B esterases, while α-naphthyl butyrate detects the B esterase but not ESD.

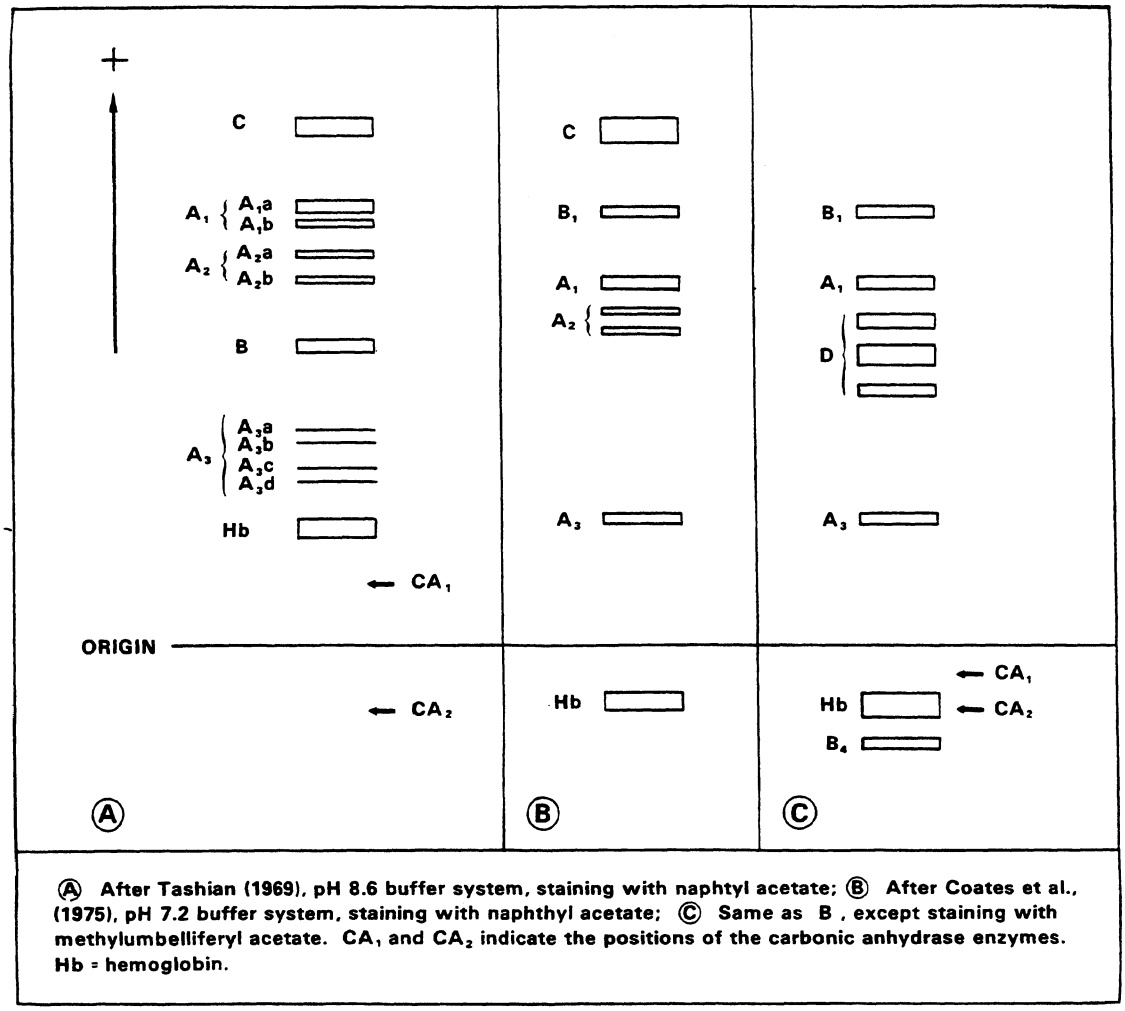
Three principal patterns of ESD were seen in red cell lysates, and were designated ESD 1, 2-1 and 2. The patterns are shown diagramatially in Fig. 31.6. Family studies indicated that these phenotypes represented the expression of a pair of codominant alleles, ESD^1 and ESD^2, at an autosomal locus.The ESD polymorphism was investigated in Europeans, Blacks and Asiatic Indians. ESD^1 was found to be about 0.9 in White and Black people, and about 0.77 in the Indian people. Thus, in White and Black populations, about 80% of people are ESD 1, about 20% are 2-1 and ESD 2 is relatively rare (<1%). In the Asiatic Indians, there were 63% ESD 1, 28% ESD 2-1 and 9% ESD 2 people. A number of workers have confirmed the single, autosomal allelic pair hypothesis of inheritance for ESD (Benkmann and Goedde, 1974; Ishimoto *et al.*, 1974; Kühnl *et al.*, 1974a; Bender and Frank, 1974). ESD phenotypes have been determined in a number of other populations as well (Welch and Lee, 1974; Kühnl *et al.*, 1974a; Ishimoto *et al.*, 1974; Bender and Frank, 1974; Sensabaugh and Golden, 1976b). A rare allele at the ESD locus, which was called ESD^3, was reported by Bender and Frank (1974). It was detected as an ESD 3-1 (see in Fig. 31.6). An additional allele, ESD^4, was detected by Berg *et al.* (1976) in two members of a family. One was an ESD 4-1, and the other an ESD 4-2. The ESD^4 had been inherited by a daughter from her father. These workers obtained a specimen of the ESD 3-1 blood from Drs. Bender and Frank, and ran all the phenotypes side by side using the original PGM buffer system of Spencer *et al.* (1964b). The patterns are indicated in Fig. 31.6. Grüner and Simeoni (1978) found an ESD 4-1 father with an ESD 4-2 son in a German population. Eriksen and Dissing (1979) reported an apparently new variant which was similar to, but not quite the same as a 3-1. It was not named (or numbered). Sparkes *et al.* (1979) have documented a probable silent allele of ESD, ESD^o, in a family in this country. Patscheider and Dirnhofer (1979) described an extraordinary child who appeared to be heterozygous for an ESD^o allele, and for a silent allele of Gc (section 41.2) as well. Her mother was thought to be ESD 2-0, Gc 2-0, and she had inherited the ESD^o from her mother and the Gc^o from her father.

The *ESD* locus has been tentatively assigned to chromosome 13 (Van Heyningen *et al.*, 1975).

31.3.5 Biochemical studies on esterases

The best studied esterase preparations are not from red cells, nor even from human sources. Krisch (1971) reviewed the biochemical studies on the most highly purified carboxylesterases. There are very few studies on the esterases from human tissues.

It may be noted that Hopkinson *et al.* (1973) characterized ESD and some of the other red cell esterases with respect to substrate specificity using sixteen different esters. ESD was most active with MUA, and hydrolyzed the butyrate and heptanoate esters relatively well. ESD was poorly active with the methylumbelliferyl esters of longer chain fatty acids, and it did not hydrolyze naphthyl esters,



Ⓐ After Tashian (1969), pH 8.6 buffer system, staining with naphtyl acetate; Ⓑ After Coates et al., (1975), pH 7.2 buffer system, staining with naphthyl acetate; Ⓒ Same as B , except staining with methylumbelliferyl acetate. CA_1 and CA_2 indicate the positions of the carbonic anhydrase enzymes. Hb = hemoglobin.

Figure 31.5 Relative Electrophoretic Mobilities of the Red Cell Carboxylesterases

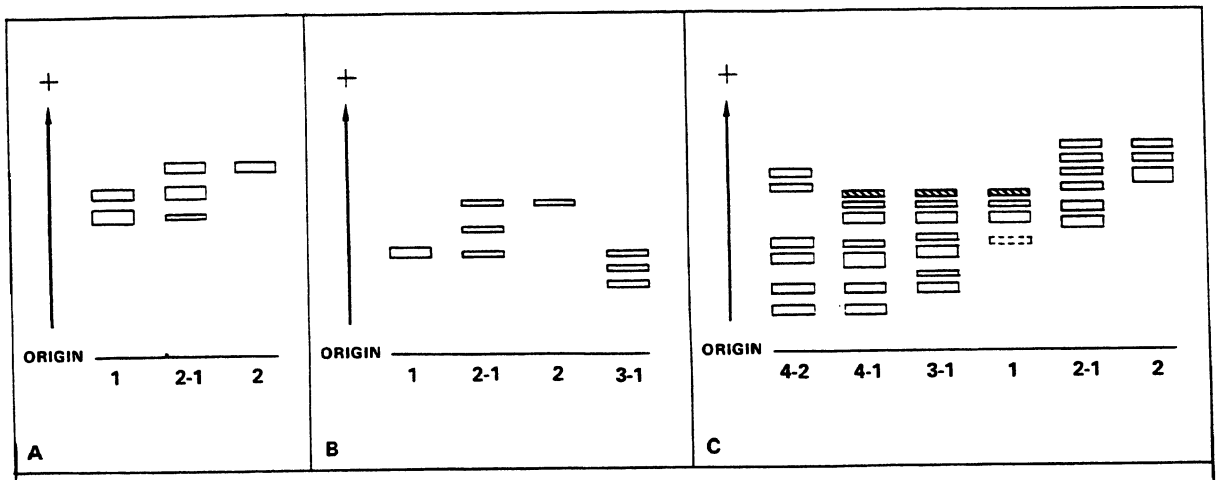
indoxyl acetate, 5-bromoindoxyl acetate, or thiocholine esters. ESD was optimally active at pH 5.0-5.5 with methylumbelliferyl esters, in contrast to the A, B and C esterases, which had pH optima of 7.5-8.0. Stored bloods or hemolysates could sometimes develop a "storage band". This band appeared less frequently in the presence of 20 mM mercaptoethanol. Finally, they noted that the electrophoretic patterns of the phenotypes suggested a dimeric structure for the enzyme, and that the MW of ESD as estimated by gel filtration was about 60,000.

Scott and Wright (1978) carried out a study on the substrate specificity of partially purified isozymes of ESD, and their results showed that methylumbelliferyl esters were the best substrates.

31.3.6 Tissue esterases—Carboxyl esterase classification and a genetic interpretation

Carboxyl esterases occur in a variety of tissues. Most esterases are apparently not very specific about their substrate preferences, and it is primarily this property which has made them difficult to classify. It turns out, too, that there are quite a large number of esterase isozymes in various tissues, and this fact adds to the difficulty.

In 1975, Coates *et al.* conducted an extensive study of tissue carboxylesterases, and tried to formulate a genetic interpretation of them based upon the results. The paper is quite involved, and not easily summarized. Many tissues were examined for esterase activity by electrophoresis, and with a variety of substrates and inhibitors. At least 13 different sets of human esterases, in addition to carbonic anhydrase and cholinesterase, could be identified, and the data indicated that there are at least 9 separate structural gene loci involved in the expression of these isoenzymes. On the basis of the data, Coates *et al.* (1975) suggested a modified nomenclature system for these esterases, in which the historical designations were retained where possible, and in which an effort was made to organize all the available information. The designation "ES" was used for "esterase", and the "A", "B", "C", etc., designation of the types of esterases was retained. Subscripts are used to distinguish sets



A: After Hopkinson et al. (1973), pH 7.2 buffer system. B: After Bender and Frank (1974), pH 7.4 buffer system, showing the 3-1 pattern. C: After Berg et al., (1976), showing the patterns of all known phenotypes, run in PGM, pH 7.4, buffer system. The mobilities of the isozymes with respect to the origin in A, B and C are not comparable, because these representations were taken from different sources.

Figure 31.6 Electrophoretic Patterns of ESD Phenotypes

of isozymes, e.g. ESA_1, ESA_2, etc. The information is summarized in Table 31.3.

There have been a number of reports on esterases in seminal plasma and spermatozoa, but Coates *et al.* (1975) did not examine seminal plasma or sperm cells. As a result, the relationship of the seminal and sperm esterases to the other tissue esterases is not clear (except for ESD). Sperm and seminal esterases were discussed in section 10.9. Blake and Sensabaugh (1976) found that ESD is expressed in sperm cells primarily, and only weakly in seminal plasma. The ESA_4 locus has been shown to be linked to LDH-A, thus assigning it to chromosome 11 (Shows, 1972).

31.4 Medicolegal Application of the Esterases

31.4.1 Disputed parentage

No references were found on the application of the E_1 or the E_2 locus polymorphisms in resolving disputed parentage cases. Chakraborty *et al.* (1974) said that about 1 or 2% of White, and about 0.5% of Black falsely accused fathers would be excluded by E_1 locus phenotyping, according to their calculations.

ESD is employed in a number of laboratories. Prokop and Göhler (1976) noted that 9–10% of falsely accused fathers would be excluded by ESD alone. The value for the British population is 9% as well (Boorman *et al.*, 1977). Polesky *et al.* (1976) said that 8.06% was the figure for this country, and it applied equally to White or Black populations. They recommended the system for parentage cases (Dykes and Polesky, 1977), and said that five exclusions had been obtained in 206 of their cases on the basis of the ESD system alone. The ESD isozyme patterns are fully developed in fetal blood (Chen *et al.*, 1977). The rare silent allele of ESD should give rise to caution in interpreting second order exclusions.

31.4.2 Plasma cholinesterase phenotyping in bloodstains

In 1962, Lehmann and Davies reported that the E_1 locus variants could be determined in bloodstains using 5×10^{-5}M benzoylcholine as substrate and 10^{-5}M dibucaine. They said that bloodstains on cloth and filter paper could be typed. Results were obtained on stains up to 6½ years old, but confirmed for accuracy with the serum of the donor only in stains up to 9 months old. The only other coverage of this subject that was found appeared in Culliford (1971). A procedure was given for the determination of the DN in 1:100 dilute serum, and in bloodstain extracts adjusted to be equivalent to about 1:50 dilute whole blood. Development of this procedure was attributed by Culliford (1971) to Parkin (M.Sc. Dissertation, 1968). Two rapid screening procedures were described as well. The difficulty with the screening procedures (section 31.2.3.7) in bloodstain extracts or hemolyzed specimens is that hemoglobin is present in these samples, and it interferes with the reading of the results because the reaction products are a reddish-brown color. Hemoglobin can be removed from the sample by treatment with CM-Sephadex in a phosphate buffer, pH 6.5, for about 30 min. After centrifugation, the supernatant fluid can be used in the agar gel screening test, essentially the same as the one described by Harris and Robson (1963a).

Another screening procedure relied upon the electrophoretic removal of Hb from the well of the agar gel plate. This procedure was said to have been devised by Wraxall. Here, "inhibited" and "control" plates of agar gel were prepared in Tris-HCl, pH 7, buffer, using RO2-0683 as inhibitor. Wells were punched in the gels, and appropriately diluted test material added. Electrophoresis for about 45 min at about 6.5 V/cm sufficed to allow the Hb to migrate into the gel, and away from the esterase reaction area. These plates were then developed for ChE activity as usual. All samples

Table 31.3 Nomenclature and Properties of Various Tissue Esterases (after Coates et al., 1975)

Esterase Isoenzyme	Genetic Locus	Optimal Substrate(s)	Occurrence in Tissues	Inhibitors
A_1	*ESA*,	MU-OAc, α-N-OAc, β-N-OAc	Pl, Leu, Er	Hg^+
A_2		α-N-OPr, ϕ-SAc, Ph-OAc		
A_3				
A_4	*ESA*,	α-N-OAc, β-N-OAc	All except Er	–
A_5	*ESA*,	α-N-OAc	All except Er	Hg^+
A_6	*ESA*,	MU-OAc, α-N-OAc	All except Er and Leu	Hg^+
A_7	*ESA*,	–	Foetal Br	–
B_1	*ESB*,	MU-OBu, α-N-OBu, β-N-OBu	All	Hg^+
B_2	*ESB*,	MU-OBu, Fl-OBu, α-N-OBu, α-N-OVa	All except Er, Leu, Ly, Fi, Re and Ch	E-600, NaF
B_3		β-N-OBu	All except Er	
B_4	*ESB*,	MU-OBu, Fl-OBu	All	Hg^+
C	*ESC*	α-N-OAc, β-N-OAc	Only Er	E-600, eserine
D	*ESD*	MU-OAc, MU-OPr	All	Hg^+
CA_1	*CA*,	MU-OAc, β-N-OAc, Ph-OAc	Er, Li, Ki, Re, Ch	acetozolamide
CA_2	*CA*,	Fl-diOAc, β-N-OAc	Er, Li, Ki, Re, Ch	acetozolamide

Abbreviations: Substrates: MU-OAc, MU-OPr, MU-OBu = 4-methylumbelliferyl acetate, propionate, butyrate; α-N-OAc, α-N-OPr, α-N-OBu, α-N-OVa = α-naphthyl acetate, propionate, butyrate, valerate; β-N-OAc, β-N-OBu = β-naphthyl acetate, butyrate; ϕ-SAc = phenylthioacetate; Ph-OAc = phenolphthalein acetate; Fl-diOAc = fluorescein diacetate; Fl-OBu = fluorescein butyrate

Tissues: Pl - placenta; Leu - leucocytes; Er - erythrocytes; Br - brain; Ly - lymphoid cell lines; Fl - fibroblasts; Re - retina; Ch - choroid; Li - liver; Ki - kidney

giving indications of "intermediate" or "atypical" behavior in either screening test were then subjected to DN determination by the standard method.

Culliford (1971) said that considerable ChE activity is lost when blood dries, but that satisfactory results could be obtained with stains up to 3 months old. After that, activity tended to be so low that differentiation of the inhibition variants was no longer possible. There were no reports found in the literature having to do with the detection of fluoride-inhibition variants in dried blood. The latest material from the London laboratory (MPFSL, 1978) does not mention plasma ChE phenotyping, and this procedure may have been eliminated from routine casework.

The typing of the C_5^+ variant (E_2 locus variant) in bloodstains was also described by Culliford (1971). The best procedure was found to be one devised by Parkin (M.Sc. Dissertation, 1968). Horizontal polyacrylamide gels with a starch insert were employed (see description in section 2.3.7.2), using Tris-succinate, pH 4.8, gel buffers and citric acid-NaOH, pH 4.8, bridge buffer. Threads from bloodstains were inserted directly into the gel after treatment with 1% mercaptoethanol. The mercaptoethanol was necessary to remove the "storage bands", which when present made the diagnosis of C_5^+ difficult. Examination of Figure 3–43 in Culliford (1971) shows this effect. The C_5^+ variant could be typed in bloodstains for a matter of months. The latest material from this laboratory, however, does not mention C_5^+ typing in bloodstains (MPFSL, 1978). Joshi *et al.* (1979) carried out studies on the typability of C_5^+ in bloodstains kept under a number of different conditions, stains from postmortem bloods and case bloodstains. Stains in hot, humid conditions lasted only about a day, but stains at room temperature at lower humidity could be typed for 12 days. The enzyme could be detected for a much longer time in stains kept at 4° or frozen. Stains from postmortem bloods retained activity for at least a month at room temperature. About 50% of the case stains were typable at 20 days age, but less than 2% of them at 40 days age or older.

31.4.3 ESD phenotyping in bloodstains

ESD phenotyping in bloodstains was first described by Blake and Sensabaugh in 1974, and much of this work appeared in 1975 in a separate publication. At least two buffer systems were found to be suitable for ESD typing. One was the original ACP buffer system of Hopkinson *et al.* (1963) (section 29.2), and the other was the original PGM buffer system of Spencer *et al.* (1964b) (section 27.2.1). The PGM buffer system was regarded as the preferable one. It was noted that, because ESD phenotypes are detected by fluorescence of methylumbelliferone under UV light at the sites of activity, that a gel run for ESD could be rinsed off and stained for PGM. It was also pointed out that the methylumbelliferyl esters are exceedingly unstable in basic solution, and that spontaneous hydrolysis of them is favored by higher Tris concentration and by imidazole, even at pH 7. As a result, phosphate or acetate buffers at pH 5.3 were recommended for the reaction buffer.

Parkin and Adams (1975) described a procedure for ESD typing in bloodstains, using a boric acid-LiOH bridge buffer and a Tris-citrate-boric acid-LiOH gel buffer, at pH 7.2. Hemolysates were diluted 1:1 with 50 mM DTT before electrophoresis, and bloodstained threads were inserted directly into the gel after soaking in 50 mM DTT. Stains up to 3 weeks old could be typed. Hayward and Bosworth (1975) described another procedure. They employed citrate-phosphate buffers at pH 5.9 for electrophoresis, and they recommended the substitution of MUB for MUA as substrate. The patterns obtained were less bright, they said, but other esterases were not stained, thus simplifying interpretation.

Grunbaum *et al.* (1978a) described a procedure for ESD phenotyping on cellulose acetate membranes which was applicable to hemolysates and to bloodstains up to 2 months old.

Jay and Philp (1979) found that bloodstains of type 1 or 2-1 could be typed for up to 4 weeks. Whole blood was typable for a few days if kept at 37°, but for 6 weeks if kept at 3°. As has been noted above, Blake and Sensabaugh (1974) recommended the simultaneous phenotyping of ESD and PGM in the same gel. Wraxall and Stolorow (1978) recently described a procedure, using the original PGM buffer system, for the simultaneous typing of ESD, PGM and GLO.

31.4.4 ESD phenotyping in other tissues

ESD occurred in significant concentrations in all tissues tested by Coates *et al.* (1975) (see in Table 31.3). Under favorable conditions, ESD phenotypes could be expected to be determinable in any of these tissues, which included muscle, kidney, heart, liver and many others. The enzyme was found in testicular tissue but seminal plasma and spermatozoa were not tested. Blake (1976) and Blake and Sensabaugh (1974 and 1976) have shown that ESD occurs primarily in spermatozoa, and is found in only trace amounts in seminal plasma. Oepen *et al.* (1980) said that they could not detect ESD in sperm, even in lysates. Blake and Sensabaugh (1978) have estimated, however, that ESD should be determinable in 10 $\mu\ell$ or more of whole semen. The estimate was based on a sperm count of 8×10^7 spermatozoa per mℓ semen. Yoshida *et al.* (1979) showed that ESD can be typed in hair roots.

31.5 The Distribution of Cholinesterase and ESD Phenotypes in U.S. Populations

The data are shown in Table 31.4. Cholinesterase polymorphisms have not been widely applied to bloodstain individualization. Neither of them is very powerful in making distinctions in the population. The discrimination index would be of the order of 0.05 for Caucasians and Hispanics with E_1 locus variants, and lower for Negroes and Orientals. For the E_2 locus, only about 5% of Caucasians are C_5^+, and even fewer Blacks. The DI for the ESD polymorphism is of the order of 0.35 for Whites, 0.29 for Blacks, 0.42 for Hispanics and 0.6 for Orientals. Steegmüller (1975) reviewed world population distributions for cholinesterase polymorphisms, and gave an analysis of the data.

Table 31.4 Distribution of Cholinesterase and ESD Phenotypes in U.S. Populations

Population	Total	E_1 Phenotype – Number (Percent)			E_2 Phenotype – Number (Percent)		ESD Phenotype – Number (Percent)				Reference
		U	I	A	C_s^+	C_s^-	1	2-1	2	*ESD'*★	
CAUCASIAN											
Seattle, WA	246	238 (96.7)	8 (3.3)	0							Motulsky and Morrow, 1968
U.S. Naval Personnel in Japan	137				14 (5.25)	123 (94.75)					Omoto and Harada, 1968 [1]
National Sample [2]	1,494	1,446 (96.8)	49 (3.3)	2 (0.1)							Lubin et al., 1971
Mississippi [2]	142	134 (94.4)	8 (5.6)	0							Lubin et al., 1971
Orange County, CA [3]	181						130 (72)	44 (24)	7 (4)	0.840	Fitzpatrick et al., 1976
Minnesota	506						422 (83.4)	78 (15.4)	6 (1.2)	0.911	Dykes and Polesky, 1977
Pittsburgh/Allegheny County, PA	545						(78.7)	(19.8)	(1.5)	–	Mortimer et al., 1978
California	5,377						(79.5)	(19.3)	(1.2)	0.892	Grunbaum et al., 1978b
Detroit, MI	503						392 (77.9)	106 (21.1)	5 (1.0)	0.885	Stolorow et al., 1979 [18]
Miami/Dade County, FL	367						286 (77.9)	76 (20.7)	5 (1.4)	0.883	Stuver, 1979 [19]
Los Angeles, CA	335						252 (75.2)	76 (22.7)	7 (2.1)	0.866	Siglar, 1979 [19]
NEGRO											
Seattle, WA	100				2 (2)	98 (98)					Robson and Harris, 1966
Seattle, WA	317				16 (2.6)	301 (97.4)					Ashton and Simpson, 1966
Seattle, WA	115	112 (97.4)	3 (2.6)	0							Whittaker, 1968d
Seattle, WA	666	659 (98.9)	7 (1.1)	0							Motulsky and Morrow, 1968
National Sample [2]	347	346 (99.7)	1 (0.3)	0							Lubin et al., 1971
Mississippi [2]	118	118 (100)	0	0							Lubin et al., 1971
Pittsburgh/Allegheny County, PA	152						(76.3)	(23.7)	(0)	–	Mortimer et al., 1978

Table 31.4 (Cont'd.)

Population	Total	E_1 Phenotype — Number (Percent) U	I	A	E_2 Phenotype — Number (Percent) C_2^+	C_2^-	ESD Phenotype — Number (Percent) 1	2-1	2	*ESD'*★	Reference
California	973						(83.6)	(16.0)	(0.4)	0.916	Grunbaum et al., 1978b
Detroit, MI	504						424 (84.1)	76 (15.1)	4 (0.8)	0.917	Stolorow et al., 1979 [10]
Miami/Dade County, FL	343						286 (83.4)	54 (15.7)	3 (0.9)	0.913	Stuver, 1979 [10]
Los Angeles, CA	146						121 (82.9)	23 (15.8)	2 (1.4)	0.908	Siglar, 1979 [10]
HISPANIC											
San Ysidro, CA [4]	105	99 (94.3)	6 (5.7)	0							Lubin et al., 1971
California [5]	1,580						(73.9)	(23.8)	(2.3)	0.858	Grunbaum et al., 1978b
Miami/Dade County, FL	360						259 (71.9)	89 (24.7)	12 (3.3)	0.843	Stuver, 1979 [10]
Los Angeles, CA [6]	156						118 (75.6)	35 (22.4)	3 (1.9)	0.869	Siglar, 1979 [10]
ORIENTAL											
Seattle, WA [7]	426	422 (99.1)	4 (0.9)	0							Motulsky and Morrow, 1968
Seattle, WA [8]	55	73 (100)	0	0							Whittaker, 1968d
San Francisco, CA [8]	111						17 (30.9)	30 (54.5)	8 (14.5)	0.582	Sensabaugh and Golden, 1976b
San Francisco, CA [9]	73						40 (36.0)	56 (50.5)	15 (13.5)	0.612	Sensabaugh and Golden, 1976b
ASIAN											
California and Hawaii	3,029						(41.6)	(44.2)	(14.2)	0.637	Grunbaum et al., 1978b

[1] Cited by Mourant et al., 1976

[2] Random sample of unrelated children

[3] About 15% were Mexican-American

[4] Mexican

[5] "Chicano/Amerindian"

[6] Primarily Mexican

[7] Primarily Japanese

[8] Japanese

[9] Chinese

[10] And see Shaler, 1978

★ Gene frequency

SECTION 32. CARBONIC ANHYDRASE

32.1 Recognition of Carbonic Anhydrase

Carbonic anhydrase (CA; carbonate dehydratase; carbonate hydro-lyase; E.C. 4.2.1.1) catalyzes the reversible conversion of carbonic acid to CO_2 and H_2O. Its physiological importance is based on the fact that much of the CO_2 produced in tissues as a product of cellular respiration is transported in blood as H_2CO_3 (or, more correctly, as $H^+ + HCO_3^-$). The enzyme plays an essential role in the conversion of dissolved carbonate to CO_2 in the lungs. The early physiologists thought that hemoglobin was primarily involved in CO_2 transport, and they were partly right. Some CO_2 is bound to Hb, but not the majority. Studies on the $H_2CO_3 \rightleftarrows H_2O + CO_2$ equilibrium and its rate of reaction indicated that the spontaneous rate was too slow to meet the needs of physiological function, and that hemolyzed blood significantly increased the conversion of bicarbonate to CO_2 (Henriques, 1928). He thought that Hb was catalyzing the reaction. In 1932, Meldrum and Roughton showed that the catalytic activity was distinct from hemoglobin and from its components, and proposed to call it "carbonic anhydrase" (Meldrum and Roughton, 1932a, 1932b and 1932c). The enzyme is found primarily in red cells, and Meldrum and Roughton (1933) purified it and described many of its properties. Stadie and O'Brien (1933a and 1933b) confirmed these findings, apparently independently. Keilen and Mann (1939 and 1940) established that CA was a Zn-enzyme, and this finding established a physiological role for zinc for the first time. The earlier studies on CA were reviewed by Roughton in 1935, and again in 1943.

32.2 Multiple Forms of Carbonic Anhydrase

32.2.1 Recognition of CA isoenzymes

In 1960, it began to be shown that human CA occurs in different molecular forms. At least four different laboratories, working independently and using somewhat different methods, were involved. Most of the purification work has been carried out on the human and the bovine red cell enzymes, and these have been extensively characterized. In 1960, Linskog found that two distinct forms of CA were isolated in the purification of the bovine red cell enzyme. This was soon found to be the case for the human enzyme by the Uppsala group (Nyman, 1961). Refinement of the purification procedures led to the finding of a third form of CA (Nyman and Lindskog, 1964). Around the same time, similar results were obtained in Derrien's laboratory in Marseilles (Laurent *et al.*, 1965 and 1966; Reynaud *et al.*, 1965), and in Edsall's laboratory at Harvard (Rickli and Edsall, 1962; Rickli *et al.*, 1964). Everyone used a somewhat different nomenclature for a time, but in 1964, all the laboratories had agreed to call the three components carbonic anhydrases A, B and C (Rickli *et al.*, 1964). CA-A was the minor, low specific activity, component, CA-B was the major low specific activity component, and CA-C was the high specific activity one. In 1962, Shaw *et al.* in Tashian's laboratory found two forms of CA in human red cells, which could be separated by starch gel electrophoresis at pH 8.7 in borate buffers. These forms were detected by their esterase activity, and were first called esterases "Da" and "Db", but it was strongly suspected, and soon proven (Tashian *et al.*, 1963) that these were carbonic anhydrases (see in section 31.3.2). On the basis of similarities and differences in the properties of the different forms and of their genetic control, Tashian *et al.* (1963) designated the two major CA's as CA I and CA II, and these correspond to the CA-B and CA-C, respectively, of Rickli *et al.* (1964).

In addition to the two major isozymes, different numbers of isozymes which occur in very much smaller amounts have been observed by different investigators. These forms may be seen as minor electrophoretic bands, or they can be obtained as separate fractions in purification sequences. Their nature is not completely clear, but there is evidence which strongly suggests that they are not the products of different genetic loci from those which control CA-I (CA-B) and CA II (CA-C). The minor component CA-A in the "A", "B", "C" nomenclature of Rickli *et al.* (1964) is designated CA I (+1) by Tashian (Tashian, 1969). This designation is based upon a systematic scheme for designating minor forms based upon electrophoretic mobilities (Tashian, 1969). The scheme is shown in Figure 32.1. CA-A is known to be identical in its properties to CA-B (Laurent *et al.*, 1966). Tashian (1969) suggested that these minor forms may be conformational isomers of the major CA isozymes, by analogy to the very similar behavior of chicken mitochondrial malate dehydrogenase observed by Kitto *et al.* (1966), who referred to the different forms as "conformers". Funakoshi and Deutsch (1969) have examined the multiple isozymic forms of CA in some detail. They found that all the forms are similar to one of the major ones, and that many of the "minor" forms can be generated *in vitro* by incubation of the major forms at relatively high pH. They use capital letter nomenclature (major isozymes are CA-B and CA-C), and they designated the minor forms with a series of letters as well. The correlation between these forms and those represented in Figure 32.1 is not clear. Funakoshi and Deutsch (1969) said that these forms may differ from one another in the number of amide groups, i.e., one or more of the Gln or Asn residues of the native molecule may be hydrolyzed to Glu or Asp.

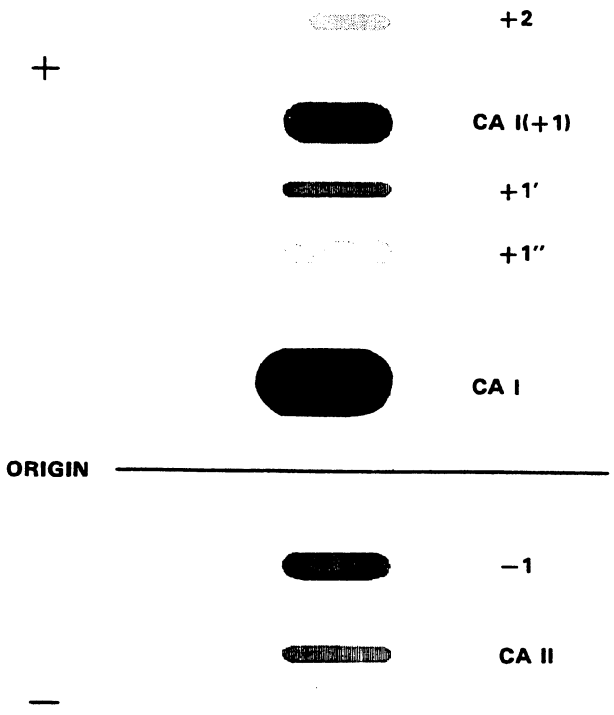


Figure 32.1 Designations of Minor Isozymes in Relation to the Major Isozymes of CA (after Tashian, 1969)

32.2.2 Genetics and nomenclature of CA isoenzymes

Based primarily upon the genetic variation that has been found in the carbonic anhydrases (section 32.3), the CA I (CA-B) and CA II (CA-C) isozymes are believed to be controlled by two different genetic loci. All the evidence supports this view. These loci will be designated CA_I and CA_{II}, after Hopkinson *et al.* (1974). The nomenclature issue is apparently still not settled (Tashian and Carter, 1976). The biochemists still tend to use the "A", "B", "C" nomenclature, while investigators with a genetic orientation have mostly adopted Tashian's nomenclature. The use of the symbols CA_I and CA_{II} to represent the genetic loci, and the designation of the different alleles, especially of the polymorphic CA_{II} locus, by superscript numbers is consistent with general nomenclature trends in human biochemical genetics. CA nomenclature is due for a revision, based upon some general agreement among all workers, and this may come when the sequences of the CA_I locus variants have been worked out.

32.3 Genetic Variation of CA

32.3.1 Genetic variation at the CA_I locus

The CA_I locus cannot be regarded as polymorphic in most populations. Most of the variants that have been found are exceedingly rare. Many of them have been thoroughly studied, and the results have given much information about the genetic control of CA's, but because none of them occur at polymorphic levels in populations in this country, they are not useful as genetic markers.

The first CA_I locus variant was detected by Shaw *et al.* in 1962 in a Mongoloid (trisomic 21) individual. The father and paternal grandmother of the propositus had the variant as well, and it was unrelated to the Mongolism. This variant was called "Da_2" at first, based on the original designation of CA I as "esterase Da". The name was soon changed, however, to CA Ib, where CA Ia is the designation of the usual kind of CA I (Tashian *et al.*, 1963). These same investigators also described the second variant, called CA Ic, in a group of people called Chamorros in Western Micronesia. There are now something more than 10 different variants known, all representing heterozygosity at CA_I of a variant allele with the common allele. Some of the variant enzymes have been characterized rather extensively, and in some cases the amino acid substitution has been determined. The variants are shown in Table 32.1, and have been well reviewed by Tashian and Carter (1976). An additional variant, CA $I_{Nagasaki\ I'}$ having the amino acid substitution 76 Arg → Gln, has been described by Goriki *et al.* (1979). There are occasional reports of quantitative variation, in which the synthesis of one of the isozymes appeared to be greatly reduced. Rieder and Weatherall (1964), for example, described a young Black girl in whom CA I activity was very low, but CA II was normal.

Table 32.1 CA_I Locus Variants

Variant	Ethnic Background	Where Found	Amino Acid Substitution	Reference(s)
Ib Michigan	Caucasian	USA	–	Show et al., (1962); Tashian (1969)
Ie Michigan	Caucasian	USA	–	Tashian et al. (1968)
^{P}Mut	Caucasian	USA	236 Asp—Val	Funakoshi and Deutsch (1970)
Ie Portsmouth	Caucasian	England	255 Thr—Arg	Carter et al., (1972); Tashian and Carter (1976)
Ie Hull	Caucasian	England	225 Gln—Lys or Arg	Carter et al. (1972); Tashian and Carter (1976)
If London	Caucasian	England	102 Glu—Lys	Carter et al. (1973)
Id Michigan; P	Negro	USA	100 Thr—Lys	Tashian (1965); Shows (1967); Moore et al. (1973); Tashian and Carter (1976)
Ih Jackson	Negro	USA		Tashian and Carter (1976)
Ih Jamaica	Negro	Jamaica		Tashian and Carter (1976)
Ic Guam	Asian	Mariana Islands	253 Gly—Arg	Tashian et al. (1963); Tashian et al. (1966)
Ic★	Asian	Phillipines	–	Lie-Injo (1967)
Ic★	Asian	Indonesia	–	Lie-Injo and Poey-Oey (1970)
Ic★	Asian	Malaysia	–	Lie-Injo et al. (1971)
I "Malaysia"	Asian	Malaysia	–	Lie-Injo et al. (1971)
Ih Hiroshima	Oriental	Japan	Maybe 16 Trp—Arg	Ueda (1974)

★ May be the same as CA Ic Guam

32.3.2 Genetic variation at the CA_{II} locus

In 1971, Moore *et al.* described a variant form of CA-C (CA II), which they called "H". Its electrophoretic mobility was close to that of CA-B (CA I); that is, the variant H isozyme ran anodically, whereas the usual kind of CA II isozyme ran cathodically. In some 222 Black people from various American cities, 18% appeared to be heterozygous for the H isozyme, and about 1% homozygous for it. The phenotypes were designated CC, CH and HH, and family studies which followed (Moore *et al.*, 1973) were consistent with a two allele, codominant, autosomal pattern of inheritance. Subsequent population and family studies have confirmed the postulated mode of inheritance, and the fact that the variant CA II occurs at polymorphic levels in a number of Black populations, including those in this country, in London and in several African nations (Carter, 1972; Moore *et al.*, 1973; Hopkinson *et al.*, 1974; Welch, 1975).

In 1974, Hopkinson *et al.* examined the products of the CA loci using fluorogenic substrates. They made the interesting (and, for phenotyping purposes, extremely useful) discovery that CA_I isozymes react preferentially with methylumbelliferyl acetate, while those of CA_{II} react preferentially with fluorescein diacetate. Thus, an electrophoretic plate stained with MUA reveals only the CA_I isozymes, while one stained with fluorescein diacetate reveals the CA_{II} isozymes. Fluorescein diacetate is, therefore, the preferred substrate for phenotyping CA_{II} isoenzymes (see in Figure 32.2). Hopkinson *et al.* (1974) referred to the "H" phenotype as a CA II 2, and to the "CH" heterozygote as a Ca II 2-1. The alleles determining these phenotypes are CA_{II}^{1} and CA_{II}^{2}, and this usage was adopted by Tashian and Carter (1976). The polymorphism is, thus far, restricted to Black populations. Hughes (1978) described a previously unrecognized phenotype in England, which was called CA_{II} 3-1.

32.4 Biochemical Studies on the CA Enzymes

Since the red cell is a convenient source of CA, many studies have been done on the enzyme from human blood. The major forms of both CA I and CA II have been extensively purified and characterized, as have a number of the variant forms (see in section 32.2.1). For reviews, see Edsall (1968), Linskog *et al.* (1971) and Tashian and Carter (1976). Most purifications have been carried out by subjecting hemoglobin-free hemolysates to gel filtration and/or ion exchange chromatography. CA I and CA II have also been purified by affinity chromatography (Johansen, 1976). The

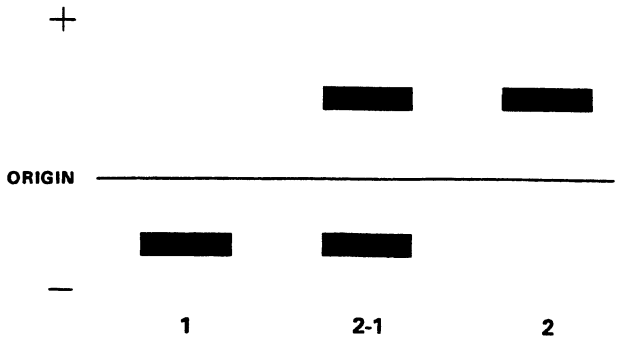


Figure 32.2 Electrophoretic Phenotypes of CA II Isoenzymes Electrophoresis in Gels at pH 8.6, and Staining with Fluorescein Diacetate.

MW's of both enzymes are close to 29,000. Both consist of single polypeptide chains of about 260 amino acid residues, and the sequences are known (see Tashian and Carter, 1976). There is one Zn associated with each molecule, and there are no intramolecular disulfide bridges. The high resolution crystal structures have been determined (see Lindskog *et al.*, 1971). CA will catalyze the hydration of aldehydes, and the hydrolysis of esters in addition to the reversible conversion of CO_2 and H_2O to carbonic acid. The turnover number for the reaction with CO_2 is among the highest known for any enzyme-catalyzed reaction. The Zn^{++} ion is required for catalytic activity, although the binding constant for several other divalent metal ions is higher (Lindskog and Nyman, 1964). CA is inhibited by a number of different things, including most monovalent anions, sulfonamides and some metal ions.

32.5 Red Cell CA Variation in Infrahuman Species—Phylogenetic Relationships

A large number of studies have been conducted on the CA isozymes of primate species other than humans in an effort to better understand the phylogeny of this genetic variation (Tashian, 1965 and 1969; Tashian *et al.*, 1968; Tashian and Carter, 1976). Many primate species have two CA loci, and a number of them show genetic variation at one or both of them. A number of mammals other than primates also have two CA loci, but there are some which appear to have only one. Based upon these studies, and on the sequences of a number of carbonic anhydrases from different species, Tashian has suggested that the two gene loci which occur in some mammals arose by gene duplication during evolution, the CA II probably being the "older" locus.

32.6 Medicolegal Applications

No published papers on the application of CA to disputed parentage were found in the literature that was examined. There is no reason why the CA II polymorphism could not be applied to disputed paternity cases involving Black people, and this application was, in fact, suggested by Moore *et al.* (1973). The gene frequencies of CA_{II}^{1} and CA_{II}^{2} appear to be about 0.9 and 0.1, respectively, in U.S. Black populations. The probability of excluding a falsely accused father with CA II alone would thus be about 8%. The enzymes are fully developed at birth (Moynihan, 1977).

CA_{II} typing in bloodstains has developed quite recently. Hughes (1978) described a procedure on 10% starch gels using a Tris-boric acid-EDTA buffer at pH 8.5 at different strengths for bridge and gel. A very similar procedure is given in MPFSL (1978). CA_{II} phenotypes can be determined on starch-agarose gels, using the multisystem procedure described by Wraxall and Stolorow (1978). This system is designed to allow simultaneous typing of PGM, ESD and GLO. The CA_{II} isozymes can be detected around the origin area of the plate, the CA_{II} staining being carried out along with ESD staining, and prior to PGM and GLO detection. The CA_{II} phenotypes can be observed in bloodstains in this system (Matthews and Stolorow, 1981). These authors have noted, however, that this electrophoretic system should be used to screen samples for CA_{II} phenotypes and not for positive diagnosis. Samples which appear to be types 2-1 or 2 can be confirmed in a more optimal typing system, such as that of Hughes (1978).

32.7 The Distributon of CA_{II} Phenotypes in U.S. Populations

There is very little data on this particular polymorphism. Moore *et al.* (1971) found 180 (81.1%) CA II 1, 39 (17.8%) CA II 2-1, and 3 (1.4%) CA II 2 among 222 Blacks from a number of cities. Tashian and Carter (1976) said they found 103 (80.0%) 1, 23 (18.0%) 2-1 and 2 (1.6%) 2 among 128 U.S. Blacks. Stolorow *et al.* (1979) found 423 (83.9%) 1, 75 (14.9%) 2-1 and 6 (1.2%) 2 types in 504 people in greater Detroit. Mortimer *et al.* (1978) tested 646 people in the greater Pittsburgh area and found the CA_{II}^{1} frequency to be 0.902. The CA_{II}^{1} frequencies in the other studies were 0.914 (Stolorow *et al.*, 1979), 0.899 (Moore *et al.*, 1971) and 0.895 (Tashian and Carter, 1976).

SECTION 33. GLUCOSE–6–PHOSPHATE DEHYDROGENASE AND 6–PHOSPHOGLUCONATE DEHYDROGENASE

33.1 Glucose–6–Phosphate Dehydrogenase (Glc6PD; Gd; Zwischenferment; D-Glc6P:NADP+ 1–oxidoreductase; E.C. 1.1.1.49)

33.1.1 Recognition of Glc6PD

The recognition of Glc6PD, the characterization of its properties, metabolic role, and those of other associated reactions, form a significant part of the history of biochemistry itself in the present century. The review by Noltmann and Kuby (1963) should be read for the details. The enzyme was first described in mammalian red cells by Warburg and Christian in 1931. At the time, the enzyme was regarded as having an "intermediary" function in the transfer of hydrogen from Glc–6P through NADP and "old yellow enzyme" to methylene blue (or oxygen) (Warburg and Christian, 1932), and it was given the name "zwischenferment" (Warburg and Christian, 1933). In 1935, Ogston and Green suggested that the name "zwischenferment" was uninformative as to the enzyme's function, and that it be called "hexose–6–phosphate dehydrogenase". Negelein and Haas (1935) objected to this suggestion, saying: "Das Zwischenferment ist aber so wenig eine Dehydrogenase, wie das Globin des Hämoglobins ein Sauerstoffüberträger ist." [Zwischenferment is as little a dehydrogenase as the globin of hemoglobin is an oxygen carrier]. They thought that Zwischenferment was the apoenzyme and that NADP (then called Coenzyme II) was the prosthetic group responsible for the oxidoreductase activity. There is some truth in this thinking, but the enzyme ultimately came to be known as Glc–6–phosphate dehydrogenase. The first example of Glc–6–PD that was highly purified and characterized came from brewer's yeast.

33.1.2 The Relationship of Glc6PD and Hemolytic Anemia—Recognition of Genetic Variation in Glc6PD

Genetic variation in Glc6PD came to be noticed and extensively studied as the result of clinical observations on the induction of acute episodes of hemolytic anemia in certain individuals by drugs. The first such observation came about when it was noted that the antimalarial drug pamaquine, an 8–aminoquinoline derivative introduced in 1926, caused acute, and even fatal, episodes of hemolytic anemia in certain patients. Dozens of studies failed to reveal the reasons for this reaction, and why only certain people were susceptible to it. A number of observations were made which were not understood at the time, but which seem significant in the light of hindsight. The development of Heinz bodies in the red cells of patients undergoing the drug reaction were noted. Heinz (or Heinz-Ehrlich) bodies are inclusion bodies seen in blood smears resulting from oxidative damage to hemoglobin. The pamaquine sensitivity was observed to be familial in some cases, and it was also noted that there were differences in the number of susceptible individuals in different racial and ethnic groups. These effects could not be sorted out, however, until after 1950, when it became possible to develop *in vitro* tests for the detection of the abnormalities.

In 1950, a therapeutically more effective antimalarial agent, called primaquine, was introduced (Edgcomb *et al.*, 1950). Primaquine, like its predecessor, is an 8–aminoquinoline derivative. In 1954, Dern *et al.* demonstrated that primaquine-induced hemolytic anemia was the result of some kind of defect within the red cells themselves. Biochemical studies of red cells from primaquine-sensitive individuals were carried out, and revealed that their reduced glutathione (GSH) content was uniformly lower than that of normal cells (Beutler *et al.*, 1955). In addition, administration of the drug to sensitive people caused rapid reductions in their red cell GSH levels (Beutler, 1957). Subsequent examination of the pathways of GSH metabolism in red cells disclosed that the primary defect in primaquine-sensitive red cells was a deficiency of Glc–6PD (Carson *et al.*, 1956; Gross *et al.*, 1958; Gross and Marks, 1958). Beutler (1957) found that the compound acetylphenylhydrazine would deplete primaquine-sensitive, but not primaquine-insensitive, red cells of their reduced glutathione *in vitro*, and used this as the basis for a hematological test for primaquine sensitivity. It was called the "glutathione stability test". This material was excellently reviewed in detail by Beutler in 1959.

Primaquine sensitivity tends to be more frequent in certain racial and ethnic groups than in others, and is nearly always associated with Glc6PD deficiency. As studies of this phenomenon progressed, it became clear that there is more than one kind of Glc6PD deficiency, and that the different Glc6PD variants were distinguishable on the basis of the biochemical properties of the enzymes, and in some cases on the resulting clinical manifestations.

Glc6PD deficiency, leading to primaquine sensitivity, was recognized early to occur in appreciable frequencies in Black people of African origin. Most people suffering from the deficiency are clinically asymptomatic unless challenged by certain drugs. Many substances, in addition to the 8-aminoquinoline antimalarials, are now known which will induce hemolytic anemia in susceptible subjects. Depending upon the individual, the clinical course of the hemolytic anemia may be relatively mild or quite severe. Glc6PD deficiency in non-Black populations is similar, in that symptoms

are not usually apparent unless there is exposure to drug stress. There are some hematological differences between the syndromes as manifested in Blacks and Whites, however, and Marks and Gross (1959) suggested that these were reflections of genetic differences.

In certain people, the ingestion of fava beans (*Vicia faba*) induces hemolytic anemia. This syndrome has been called favism, and some earlier workers drew attention to the similarity between it and antimalarial drug sensitivity (see in Beutler, 1966). Favism tends to be particularly characteristic of Mediterraneans, and it is known to be familial. It is also associated with GSH instability and with Glc6PD deficiency (Sansone and Segni 1956 and 1957; Szeinberg *et al.*, 1958a and 1958b). There is something more to it though, since many people with Glc6PD deficiency do not react to fava beans.

There are some cases of what is called congenital nonspherocytic hemolytic anemia (CNHA) associated with Glc6PD deficiency (Newton *et al.*, 1958; McGovern *et al.*, 1958; Zinkham and Lenhard, 1959; Beutler *et al.*, 1968). These cases represent less common forms of Glc6PD deficiency and many of these people suffer from anemia in the absence of drugs. The anemia is often mild, and seen primarily in the neonatal period.

Glucose is the major energy source of the red cell, and the hematological mechanisms underlying red cell enzyme disorders reflect disturbances in glucose metabolism. A simplified diagram of major glucose metabolizing pathways is shown in Figure 33.1. Glc-6PD represents a branchpoint. Glucose can be metabolized via the Embden-Meyerhof pathway (glycolysis) or via the hexose monophosphate pathway (pentose pathway). In normal, unstressed red cells, about 90% of the glucose is metabolized by glycolysis. The Glc-6PD reaction is the principal regulatory step for the hexose monophosphate pathway, the major physiological functions of which are the production of NADPH and the formation of five carbon sugars. Under normal circumstances, the Glc-6P level of the red cell is well below that required for maximal Glc-6PD activity, and so is the level of NADP. Further, NADPH competitively inhibits the enzyme. The rate of metabolism via the hexose monophosphate pathway can thus be readily altered. Perhaps the major function of the hexose monophosphate pathway is the production of sufficient NADPH to keep oxidized glutathione reduced by way of the glutathione reductase reaction (Figure 33.1). Glutathione reductase also reduces the mixed disulfides of glutathione and protein (including hemoglobin). Oxidized glutathione is produced by the glutathione peroxidase reaction which rids the cell of peroxides. These may arise through the action of superoxide dismutase (see section 37.4).

Red cell Glc-6PD deficiencies can thus have far reaching physiological consequences. The consequences in any given instance are complicated, however, by the fact that many of the genes producing deficiency syndromes code for Glc-6PD molecules with altered kinetic properties. This subject has been well reviewed by Beutler (1978).

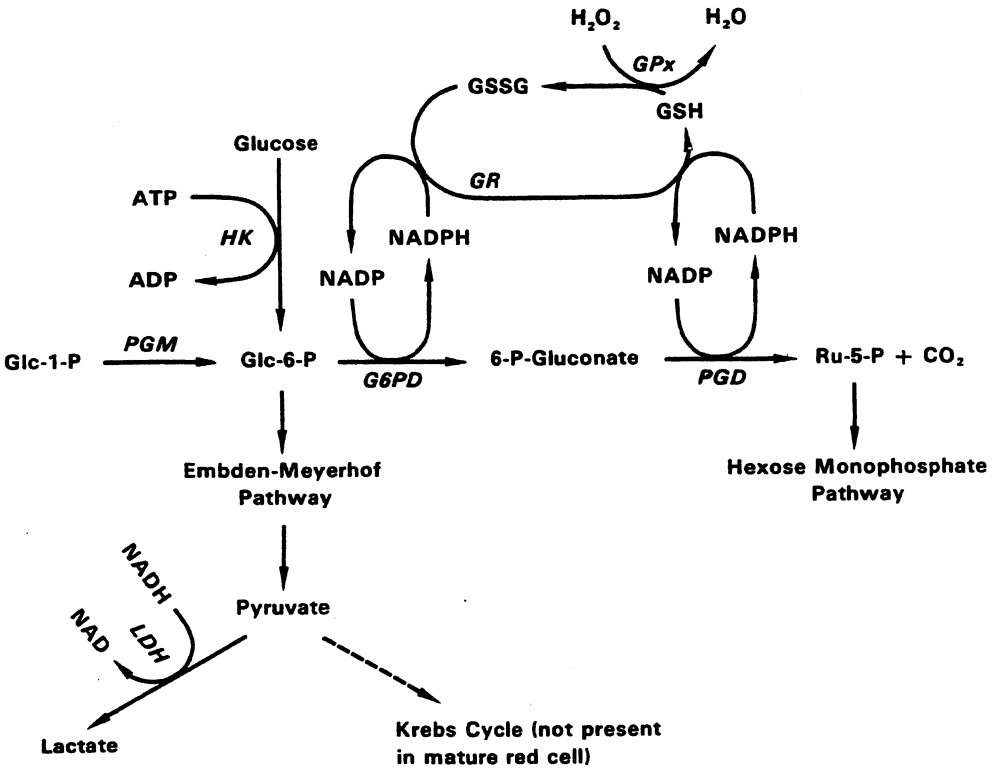
33.1.3 The genetic variants of Glc-6PD

A great deal of work has been carried out on the biochemical genetics of Glc-6PD, and many variant kinds of the enzyme have been described. Some differ from the normal enzyme in electrophoretic mobility, but many do not, and are characterized by differences in kinetic properties, activity or thermostability.

In 1962, Boyer and Porter reported that Glc-6PD in American Blacks showed electrophoretic heterogeneity. Three types could be distinguished, and were called A, B and AB. The sex distribution of the phenotypes was such that the only logical explanation for it was X-linkage of the Glc-6PD locus. Only women were ever heterozygous. Black males with Glc-6PD deficiency were electrophoretically type A (the Glc-6PD type could be determined from leucocytes, which are not deficient even in red cell-deficient subjects). This suggested that there were three alleles, an A-normal, an A-deficient and a B-normal. Further studies were conducted by Boyer *et al.* (1962). They noted that all Caucasian Americans were of type B, and that several Greek Glc-6PD-deficient subjects were of type B as well. Kirkman (1962a) reported almost identical findings, except that he called "type A" and "type B" as "fast" and "slow", respectively. Two Caucasians with CNHA were type B. The A-type enzymes from both normal and deficient people were partially purified, and compared for a number of different properties; they were found to be identical in all respects (Kirkman, 1959; Marks *et al.*, 1961).

A large number of variant forms of Glc-6PD have since been described. They have been named after the cities in which their possessors lived. Some differ in electrophoretic mobility from A and B while others do not. The latter must be distinguished on the basis of other properties, such as the K_m for Glc-6P and/or NADP, thermostability, affinity for 2-deoxyglucose-6P, pH optima, and so forth. Some of the variants are: Mediterranean (Kirkman *et al.*, 1964a), Tel-Hashomer (Ramot and Brok, 1964; Ramot *et al.*, 1964), Chicago I (Kirkman *et al.*, 1964b), Seattle (Shows *et al.*, 1964; Kirkman *et al.*, 1965), Ohio (Pinto *et al.*, 1966), Canton (McCurdy *et al.*, 1966), Kabyle (Kaplan *et al.*, 1967a and 1967b), Athens (Stamatoyannopoulos *et al.*, 1967), Kerala and West Bengal (Azevedo *et al.*, 1968), "New Guinea" variants (Kirkman *et al.*, 1968), Madrona (Hook *et al.*, 1968), Albuquerque and Duarte (Beutler *et al.*, 1968), Joliet I (Bowman *et al.*, 1969) and Hong Kong (Chan *et al.*, 1972). There are many additional variants. Reviews have been published periodically (Kirkman *et al.*, 1964c; Kirkman and Hanna, 1968; and Beutler, 1966).

By 1966, it was apparent that something ought to be done about standardizing the nomenclature of the many variants, and also about standardizing the methods used to characterize them, particularly because so many of them differ in kinetic and other properties. Most of them are not distinguishable by electrophoresis. An expert group was convened under the auspices of the World Health Organization to undertake this task (WHO Scientific Group on Standardization of Procedures for the Study of Glucose-6-Phosphate



Abbreviations: HK - Hexokinase GPx - Glutathione Peroxidase GR - Glutathione Reductase G6PD - Glucose-6-Phosphate Dehydrogenase PGM - Phosphoglucomutase PGD - 6-Phosphogluconate Dehydrogenase LDH - Lactate Dehydrogenase GSSG and GSH - Oxidized and Reduced Glutathione

Figure 33.1 Simplified Representation of Major Red Cell Pathways of Glucose Metabolism

Dehydrogenase, 1967; and World Health Organization, 1967). The nomenclature recommendations included retention of the designations "B", "A" and "A–" to indicate the usual forms of the enzyme, and the most common deficient A type seen in Black people. All other variants would be given trivial names after the city of origin. If there were more than one from a given city, arabic numbers would be used, e.g., Austin-1, Austin-2, etc. The genotype symbol for Glc-6-PD is *Gd*, and superscripts indicate specific genes, e.g. Gd^{A}, $Gd^{Mediterranean}$. Glc-6PD Mediterranean is the name assigned to the common deficient type with electrophoretic mobility identical to B. The activity level of the enzyme was to be designated as (−) 25% or less, (±) 25%–65%, (+) 65%–150% or (+ +) greater than 150% or 2 S.D. above the mean. These symbols could be incorporated into the phenotype designations if necessary, e.g., "Gd (+) B", "Gd (+) AB", "Gd (−) Canton", and so forth. Extensive standardized procedures were recommended for establishing the characteristics of variants. A complete discussion of the recommended testing procedures may be found in Motulsky and Yoshida (1969) as well.

A few more than 20 variants appeared in the 1967 WHO Technical Report. By 1971, the number had grown to almost 80, and a complete list was published by Yoshida *et al.* (1971). They placed the variants into one of five major classes: 1. Severe enzyme deficiency with chronic nonspherocytic hemolytic anemia; 2. Severe enzyme deficiency (<10% normal); 3. Moderate to mild enzyme deficiency (10–60% normal); 4. Very mild or no enzyme deficiency (60–100% normal); and 5. Increased enzyme activity (greater than normal). In 1973, Beutler and Yoshida published a supplement to the 1971 list, containing over 25 more variants. Glc-6PD Long Prairie, which was in the supplemental list as an unpublished variant, is fully described by Johnson *et al.* (1977). Two more recent variants are Glc-6PD Ube (Nakashima *et al.*, 1977) and Glc-6PD

Velletri (Mandelli *et al.*, 1977). A recent list of variants was given by Beutler (1978). Undoubtedly, there are a number of other new ones. Well over a hundred variants have thus been described. The vast majority of them are rare. The only relatively common variants appear to be A(+) and A(−) which are common in Black people, "Markham" in New Guinea, "Taiwan-Hakka" in Hakka Chinese, "Union" in Filipinos, "Camplellur" in Pakistanis (about 2%), "Debrousse" in Arabs, "Athens" in Greeks, and "Mediterranean" which has been observed in Greeks, Sardinians, Sephardic Jews and Asiatic Indians. The A(+) and A(−) are the only readily distinguishable variants since they have a different electrophoretic mobility from the normal B. "Mediterranean" might be detected as a "B" with exceedingly low activity. Since Glc-6PD A(+) is nearly always associated with Blacks, it is worth noting that Angelopoulos and Delitheos (1970) described an A(+) in a Greek woman. Grunbaum *et al.* (1978b) found a small number of Glc-6PD A(+) among 5,916 California Caucasians in their population survey as well.

33.1.4 X-Linkage of the *Gd* locus—X-chromosome inactivation

In 1957, studies were carried out at Johns Hopkins on the primaquine sensitivity characteristic in 296 Black people, a number of whom belonged to 17 families. The GSH stability test was used to assess sensitivity, and three classes of individuals were seen. One was insensitive, another was sensitive and there was a class having intermediate sensitivity (Browne, 1957; Childs *et al.*, 1958). All the data suggested a mother to son mode of inheritance, characteristic of X-linkage. The gene, however, showed variable penetrance, and there was some variability within a phenotypic class with respect to GSH stability. Kirkman (1962a) and Boyer and Porter (1962) soon established that the electrophoretic A variant, common in Blacks, segregated as if it were controlled at an X-linked locus. It has also been firmly established that *Gd* is linked to the gene for hemophilia A, which is a firmly established X marker (Boyer and Graham, 1965).

For years it was thought that both the X chromosomes in females were genetically active. If they were, one would expect to find more gene product in XX individuals than in XY ones, and one does not. In *Drosophila*, there are "dosage compensation" mechanisms that can account for the observations. In the late 1950's it began to appear that the situation in mammals might be quite different. The chromatin body in XX cells was demonstrated by Ohno *et al.* (1959) to consist of heterochromatic chromosomal material derived from one of the X chromosomes. Since heterochromatin was known to be genetically inactive in some insect species, this finding raised the prospect that XX cells contain an inactive X chromosome. Additional aspects of this subject were discussed in section 1.2.4.4., and a little more is said in section 49.2.

In 1962, Beutler *et al.* established that the expression of Glc-6PD in human beings was consistent with the Lyon hypothesis, i.e., with inactivation of one of the X chromosomes in XX cells. Inactivation is thought to occur fairly early in the embryonic development of females, and to be random with respect to which of the X chromosomes becomes inactivated. As a result, it is to be expected that the fully developed female body will contain two different populations of cells, one descended from cells in which the maternally-derived X chromosome became inactivated, and the other from the cells in which the paternally-derived one did. In 1962, Beutler *et al.* presented evidence that the red cells of females heterozygous for Glc-6PD deficiency behave exactly as expected on the basis of two separate cell populations, one deficient and the other normal. Similar results were obtained by Davidson *et al.* (1963). In 1965, Gall *et al.* were able to prove the existence of two red cell populations in heterozygous females using tests on individual erythrocytes. Beutler (1969a) has pointed out that minor modification of the inactivation hypothesis can account for some of the "variable penetrance" seen in Glc-6PD deficiency syndromes. During the embryological and post-natal developmental stages, some natural selection could be occurring in the two cell populations, which might lead to unequal numbers of the two kinds of cells in the adult. The original 1:1 ratio could be significantly altered by quite mild selective forces, and these need not be related to Glc-6PD activity *per se*. If a gene favoring red cell production were present, for example, on the X chromosome having the normal *Gd* gene, then cells having this chromosome would proliferate much more readily than those having the inactive X. The end result would be an adult with near normal Glc-6PD levels, even though she was heterozygous for a deficiency gene. This interesting subject was well reviewed by Beutler (1968 and 1969a).

33.1.5 Biochemical studies on Glc-6PD

The earlier biochemical studies on the enzyme have been reviewed by Noltmann and Kuby (1963). The normal enzyme and that of several variant types have been purified extensively in a number of laboratories and characterized (Kirkman, 1962b; Kirkman and Hendrickson, 1962; Yoshida, 1966, 1967 and 1968; Yoshida *et al.*, 1967; and others). Glc-6PD appears to be a polymer, consisting of subunits of MW 55,000. Estimates of the MW of the "native" protein have varied (Chung and Langdon, 1963; Ratazzi, 1968), apparently because the enzyme can exist in several different states of aggregation. Bonsignore *et al.* (1971) studied the interconversion of the different forms. The monomer is catalytically inactive, but the dimer and tetramer are both active. The catalytically active enzyme contains bound NADP, and removal of this cofactor leads to dissociation with concomitant loss of activity (Kirkman and Hendrickson, 1962; Chung and Langdon, 1963; Bonsignore *et al.*, 1971). The active dimer has 18 -SH groups, and no disulfide bridges (Yoshida, 1973). Oxidation of the sulfhydryls reduces activity, reduces the K_m for 2-deoxy-Glc-6P, deamino-NADP and NAD, and increases the anodal electrophoretic mobility. Some 70% of the -SH groups may be oxidized, however, without observable

effect. The A(+) enzyme from common variant Negro subjects differs from the B enzyme by a single amino acid, there being an Asp in the A(+) in place of an Asn (Yoshida, 1967). The deficient Negro variant A(−) enzyme apparently represents a structural variant different from the A(+) (Yoshida *et al.*, 1967; Yoshida, 1968).

It has been known for some time that red cell stroma has an inactivating effect upon Glc-6PD (Carson *et al.*, 1959), believed to be related to the stabilizing effects of NADP upon the enzyme. The stroma appear to be able to deplete the enzyme of NADP, thus inactivating it (Carson *et al.*, 1966). It is routine, therefore, to add exogenous NADP to hemolysates and/or to electrophoretic gels in order to prevent this phenomenon.

33.1.6 Medicolegal applications

Glc-6-PD variant typing is apparently not often employed for purposes of disputed parentage testing. No published reports of such application were found.

The only discussion of Glc-6PD phenotyping in bloodstains was found in Culliford (1971) and the system was not enthusiastically recommended. Only the major electrophoretic variant in Blacks, the A(+), would be of much routine value. Common variant patterns are shown in Figure 33.3. Glc-6PD may be detected in gels following electrophoresis by the addition of substrate, NADP, $MgCl_2$, MTT and PMS in pH 8 buffer. Zones of activity appear as blue formazan bands where MTT is reduced by NADPH (Figure 33.2). The other variants require much more involved protocols for characterization. The recommendations of Culliford (1971) closely followed those of the WHO Technical Report, No. 366 (1967). Electrophoresis in 15% thin starch gels, containing 2 mg NADP per plate, in Tris-EDTA-boric acid buffers was found to be generally satisfactory, although Tris-HCl, ph 8.8, buffers were used as well. Procedures for Glc-6PD typing on various forms of cellulose acetate membranes have been devised by Rattazi *et al.* (1967) and Ellis and Alperin (1972). Grunbaum and Zajac (1976) described a CAM procedure applicable to hemolysates and to bloodstain extracts. Culliford (1971) noted that loss of activity of the enzyme is a serious problem, but that bloodstains up to 4 weeks old could be attempted, and would sometimes give results. Although the A(−) and A(−)B variants could be detected by electrophoresis in fresh blood by their deficiency of activity, diagnosis in bloodstains is an uncertain exercise. Losses of activity resulting from drying, aging or other effects are indistinguishable from low intrinsic activity. BA heterozygous patterns are observed only in women. The A variant will almost always be observed in Black people, although it needs to be remembered that Angelopoulos and Delitheos (1970) found an A(+) pattern in a Greek woman, and Grunbaum *et al.* (1978b) found 0.1% and 0.4% of 2,071 White women and 3,845 White men, respectively, in California to be Glc-6PD A(+).

Sperm cells show Glc-6-PD activity, and there is some activity in seminal plasma as well, but the cells have more activity on a per volume basis assuming 8×10^7 cells/mℓ semen (Blake and Sensabaugh, 1976 and 1978). The activity in whole semen is only about 2% of that in whole blood, however. Blake and Sensabaugh (1978) estimated that the minimum amount of whole semen which would be required for Glc-6-PD determination would be 40 $\mu\ell$, and even more with seminal plasma alone. The comparable figure for whole blood was 1 $\mu\ell$.

33.1.7 The distribution of common *Gd* phenotypes in U.S. Black populations

There are not many U.S. population studies in which information about the frequency of the electrophoretic variants has been collected. The data are given in Table 33.1.

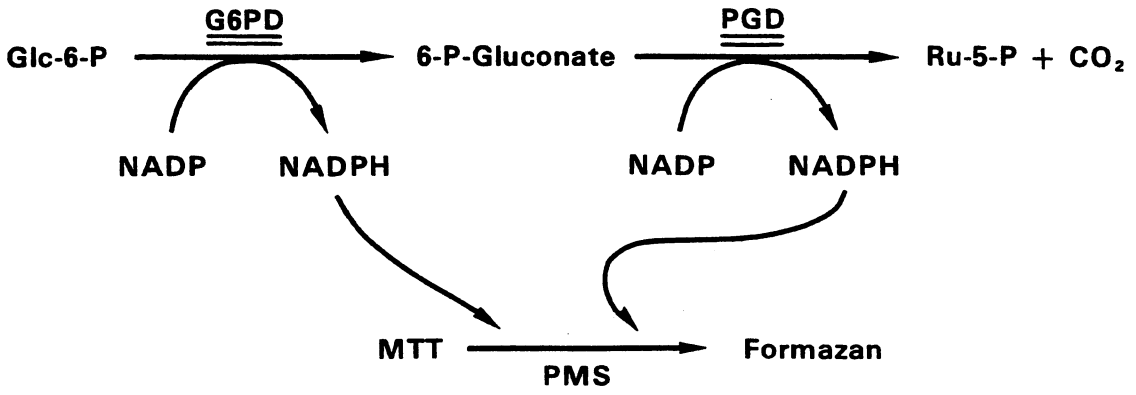


Figure 33.2 Basis of Detection Reaction Sequences for Glc6PD and PGD

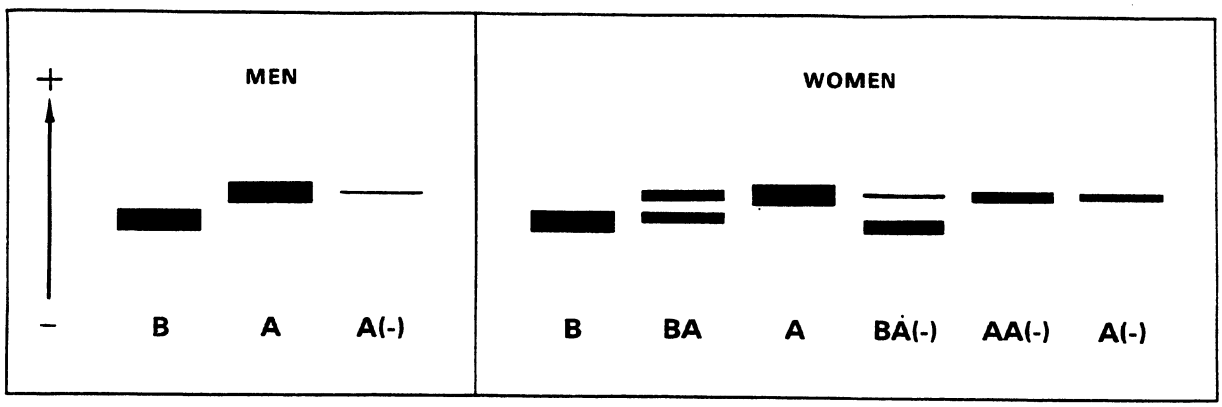


Figure 33.3 Electrophoretic Patterns of Glc-6PD Common Variant Isoenzymes

33.2 6–Phosphogluconate Dehydrogenase [PGD; 6–PGD; Phosphogluconate Dehydrogenase (decarboxylating); 6–Phospho–D–Gluconate: $NADP^+$ 2–Oxidoreductase (decarboxylating); E.C. 1.1.1.44]

33.2.1 Recognition of PGD

In connection with their pioneering studies on Glc–6–PD, discussed in section 33.1.1, Warburg *et al.* (1935) and Warburg and Christian (1936) realized that the oxidation of Glc–6P proceeded beyond 6–P–gluconate in yeast extracts. They, and Dickens (1936 and 1938), demonstrated that the further breakdown of 6–P–gluconate consisted of an oxidative decarboxylation reaction leading to a pentose-P, which could in turn be further metabolized to yield a complex mixture of products. NADP was shown to be a required co-factor for the reaction. The identity of the pentose phosphate product was not established for quite some time. Dickens (1938) thought that it was ribose–5–P, but this supposition was later shown to be wrong. Strong indications that the reaction product was ribulose–5P (Ru5P), and not ribose–5P (R5P), came from the work of Horecker and Smyrniotis (1950 and 1951) and of Horecker *et al.* (1951) with fairly pure PGD preparations from yeast. They were quite certain that Ru5P was the product of the PGD reaction, but that contamination of the preparation by pentose phosphate isomerase, which catalyzes the Ru5P $\rightleftarrows$ R5P interconversion, was leading to a rapid equilibrium in favor of the aldopentose-P. The equilibrium of the pentose phosphate isomerase reaction lies very far in the direction of R5P. Final proof of this idea came when Pontremoli *et al.* (1961) obtained a pure, crystalline preparation of PGD, free of PPI. PGD is the second enzyme in the hexose monophosphate pathway (section 33.1.2; Figure 33.1), and the earlier biochemical studies on the enzyme were reviewed by Noltmann and Kuby (1963).

33.2.2 Genetic variation of PGD

There are quite a number of PGD variants known, many of them quite rare. Some of the variant alleles give rise to isozymes which have normal levels of activity. These are discussed in section 32.2.2.1 in the order of their discovery. Other named phenotypic variants of PGD exhibit deficiency in activity. These are presented in section 32.2.2.2. A third class of PGD variant could be constructed, namely those in which the electrophoretic patterns are significantly different in the presence and absence of certain compounds. This class would have two members, PGD Friendship and PGD Natal. These have been included in section 32.2.2.1. The nomenclature history of PGD variants is quite confusing in certain respects (section 32.2.2.3), and it may be helpful to consult Table 33.2 while reading through section 32.2.2. Genetic variation of PGD is reviewed by Brewer (1969) and by Beckman (1978).

32.2.2.1 Variants exhibiting normal activity. In 1963, Fildes and Paar described the first electrophoretic variants of PGD. Electrophoresis at 10V/cm on starch gels in phosphate buffer, pH 7, revealed two electrophoretic types. Most people showed a single "A" band, but in 10 out of 150, a pattern with the "A" band and a slightly slower (more cathodal) "B" band was seen. Investigations of several families indicated that the variant pattern was the expression of heterozygosity at a simple autosomal locus. Individuals having the variant pattern showed no alteration in red cell PGD activity. As the electrophoretic procedures improved, Fildes and Paar realized that the heterozygote pattern also had a very weak "C" band, slower than the B band. This observation was first reported at the VI International Congress of Biochemistry in 1964, and was explained by Paar (1966). Bowman *et al.* (1966) examined hemolysates from 1,016 Americans, and confirmed the findings of Fildes and Paar (1963). They found a homozygous variant person in their survey as well. Bowman *et al.* (1966) suggested that the phenotypes be designated A, A–B and B, and the responsible alleles as Pd^A and Pd^B. Family studies fully sup-

Table 33.1 Distribution of Common *Gd* Phenotypes in U.S. Black Populations

Population	Total	Phenotypic Frequency — Number (Percent) MALES B	MALES A★	FEMALES B	FEMALES A★	FEMALES BA ☆	Gd^{A} ⊙	Reference
Baltimore, MD	311	206 (66.2)	105 (33.8)				0.34	Boyer et al., 1962
	100			52 (52)	13 (13)	35 (35)	0.31	
Oklahoma	135	88 (65.2)	47 (34.8)				0.34	Kirkman and Hendrickson, 1963
	39			23 (59.0)	3 (7.7)	13 (33.3)	0.24	
Chicago, IL	35	28 (80)	7 (20)				0.20	Shih and Hsia, 1969
	65			45 (69.2)	9 (13.8)	11 (16.9)	0.22	
California	896	(73.0)	(26.7)				0.270	Grunbaum et al., 1978b
	111			(63.1)	(9.9)	(23.4)	0.224	
Detroit, MI	252	173 (68.7)	78 (31.0)				0.31	Stolorow et al., 1979 and see Shaler, 1978

★ Includes A(-) ☆ Includes BA(-) ⊙ Gene frequency of Gd^{A} and Gd^{A^-} combined

ported the two allele mechanism of inheritance. Paar (1966) described several additional variants that had been found, some of which were more fully described in later papers. One of these showed a "fast" triplet pattern, while another showed a pattern quite similar but not identical to the common variant and had a weak A band and a weak C′ band, with a strong B′ band. The designations B′ and C′ were used to distinguish the bands in these variants from those in the common variant, the mobilities being just slightly different. Two homozygotes for the allele determining the usual variant had been seen. By 1966, the variants were acquiring names. The names were often used for the phenotypes, and, by analogy to the Glc-6PD variants, were those of the places where the possessors lived. The common variant, which had first indicated to Fildes and Paar (1963) that PGD was polymorphic, and which Brewer *et al.* (1966) called A–B, was still called the "common variant" by Paar. The homozygote for the common variant gene was named the "Canning variant". Bowman *et al.* (1966) called it "B". The fast triplet pattern was named "Richmond", and was more fully described by Carter *et al.* (1968). The other rare pattern, a triplet with weak A and C′ bands, but a strong B′, was called "Hackney", and it, too, was more fully described by Carter *et al.* (1968). Paar and collaborators had also proposed a series of Greek letter superscripts for the alleles, using "PGD" as the locus symbol. All the variants mentioned thus far, except Canning, were heterozygotes of the usual allele. Thus, the common type was genotypically $PGD^{\alpha}PGD^{\alpha}$, the common variant was $PGD^{\alpha}PGD^{\beta}$. The Canning $PGD^{\beta}PGD^{\beta}$, the Richmond $PGD^{\alpha}PGD^{\gamma}$, and the Hackney $PGD^{\alpha}PGD^{\delta}$. In 1967, Davidson described an additional mutant type, called "Friendship". Family studies indicated that it represented heterozygosity of the Friendship allele with the normal allele. "Friendship" is peculiar, in that it was detected in gels from which NADP had been excluded. Some workers routinely add NADP to their gels (primarily to stabilize the Glc-6PD which may be simultaneously determined), while others do not. This matter is discussed below. If "Friendship" is run in an NADP-containing gel, however, it exhibits the pattern of a usual PGD. Further, leucocytes from the original "Friendship" propositus show only the fastest band of the red cell pattern. Davidson (1967) said, therefore, that "Friendship" should be diagnosed by electrophoretic runs in both NADP-containing and NADP-less gels, and by comparing red cell and white cell patterns. In 1968, Tuchinda *et al.* described a variation in several unrelated people in Thailand. The variant pattern resembled that of "Richmond" and was called "PGD-Thai". It was not the same as the Friendship variant. It is possible that it is the same as Richmond. Blake and Kirk (1969 found a new variant in Australian aborgines, which occurred in polymorphic frequencies in these people,

especially in the Elcho Islands. This phenotype was called PGD Elcho. An Elcho phenotype has recently been observed in Finland (Virtaranta-Knowles and Nevanlinna, 1979). Tariverdian *et al.* (1970b) found a variant phenotype in a German population sample which looked very much like "Friendship" electrophoretically, but its pattern was not altered by the addition of NADP to the gel. The gene for this variant was segregating in two families, and the phenotype was called PGD–Freiburg. In 1973, Blake *et al.* found a new phenotype in a Chinese resident of Singapore, which was named PGD–Singapore. The allele codes for isoenzymes which run cathodic to (slower than) the usual A on electrophoretic gels (Blake *et al.*, 1973b). Blake *et al.* (1974) carried out an extensive population survey, and discovered six new PGD alleles. All represented heterozygous expression of unusual alleles in combination with the usual one, and the variants were called Wantoat, Canberra, Kadar, Caspian, Bombay and Natal. Wantoat and Canberra were slow electrophoretic variants, while the others were fast, relative to PGD A. Natal is a peculiar variant. Treatment of Natal samples with mercaptoethanol causes them to change to a form which looks "usual" electrophoretically. Blake *et al.* (1974) found a homozygote for the Kadar variant, as well as a Kadar/C heterozygote and a Natal/C heterozygote. In 1974, Jenkins and Nurse surveyed 34 distinct populations in southern Africa, and found a further PGD variant with a slow electrophoretic pattern. This was called PGD Oshakati.

32.2.2.2 Variants exhibiting deficiencies in activity—PGD^{o} and PGD^{w}. In 1964, inherited PGD deficiencies were observed independently in this country and in England. Brewer and Dern (1964a and 1964b) observed a Black woman whose red cells showed about 50% the normal PGD activity. Extensive studies of her family revealed a large kindred in which the 50% PGD activity condition was segregating in four generations. The most logical genetic explanation was heterozygosity in the affected members for the usual allele and an inactive one. This pedigree is quite an interesting one, because Glc-6PD deficiency was also segregating in this family. Paar and Fitch (1964) found a man in London with about 50% normal PGD activity. This condition was found in members of four generations of his family. The propositus was extraordinary, because his electrophoretic pattern was that of a homozygous Canning variant, and his genotype was imagined to be PGD^{g}PGD^{o} in the old terminology (PGD^{C}PGD^{o} in the newer terminology—see below). His phenotype was named the "Half-Canning" for the time being. Dern *et al.* (1966) extended the earlier population studies of Brewer and Dern (1964a and 1964b), and found quantitative variants in a number of families. In several, the affected people showed 50% of normal activity, while in others, the deficiency did not appear to be fully expressed. The latter had greater than 50% of the usual activity. One person had the common variant type (according to the nomenclature of Paar; the A/B phenotype, according to the nomenclature of the authors), and both bands of activity were 50% reduced. The result suggested a mutation in a regulatory gene, but this could not be proven in the particular family. In 1967, Paar and Fitch reported several additional phenotypes in the growing English population sample and summarized the studies that had been carried out thus far. In this paper, the newer nomenclature also appears for the first time (see in section 32.2.2.3). The common type was given the genotypic designation *PGD^{A}PGD^{A}*. The Canning variant allele was designated *PGD^{C}*. The common variant would thus be *PGD^{A}PGD^{C}* while the homozygous Canning variant would be *PGD^{C}PGD^{C}*. The phenotype previously called "Half-Canning", *PGD^{C}PGD^{o}*, was re-named the "Newham" type. An electrophoretically usual type, with 50% normal activity, was reported. The probable genotype was *PGD^{A}PGD^{o}*, and this was called "Ilford". The 50% activity subjects seen by Dern *et al.* (1966) are probably "Ilford" types. The London family in which the *PGD^{o}*-like allele was segregating was fully described by Paar and Fitch (1967). Another English family was described in this paper in which two fully deficient individuals, a brother and sister, occurred. These individuals were mentioned by Paar (1966). The activity assays and genetic data indicated that these people were not homozygotes for *PGD^{o}*, but rather that the deficiency had a different genetic basis. The fully deficient phenotype was called "Whitechapel" and the genotype denoted *PGD^{w}PGD^{w}*. Heterozygotes for this allele and *PGD^{A}*, i.e. the *PGD^{A}PGD^{W}* types, were called "Dalston" and they have about 75% of normal PGD activity, while the Ilford types have about half. The people in the sample of Dern *et al.* (1966) who had more than 50% enzyme activity, but less than or about 75% activity, may well have been Dalston phenotypes (Brewer, 1969).

Paar and Fitch (1967) raised an important point in connection with the assessment of these deficiency phenotypes. Enzyme activity in the red cell must be expressed in terms of some basis that reflects the amount of blood, or the number of red cells, for which the value is quoted. Units of activity alone are quite meaningless. A common way of expressing activity is in terms of "per gram Hb". Under some circumstances, PGD activity per g Hb will be quite satisfactory, but Paar and Fitch (1967) pointed out that if one of the individuals being compared is anemic, and has a low Hb concentration, the PGD activity expressed as Units/g Hb might turn out to be quite misleading. It is good practice, therefore, to assay another enzyme (in the case of PGD, the other one is usually Glc-6PD), and express the activity of the enzyme under study and the reference enzyme as ratios. In the case of PGD, the PGD/Glc-6PD ratio would correctly reflect a deficiency in an anemic person which a PGD activity in Units/g Hb might fail to disclose.

33.2.2.3 The nomenclature of PGD variants. The PGD variant nomenclature is somewhat confusing, but most of the time the designations are clear. Table 33.2 gives a summary of the variants. The electrophoretic patterns in the table are not really comparable to one another, since many of them were found in different laboratories, and everyone does not employ the same electrophoretic conditions. The

Table 33.2 Nomenclature and Properties of PGD Variants

Phenotype Designation	Previous Phenotype Designation(s)	Genotype Designations: Previous[1]	Genotype Designations: Current	Electrophoretic Mobility Relative to PGD-A[2]	PGD Activity % of Usual	Reference(s)
Usual; Type A	Normal; Type I	$PGD^{m}PGD^{m}$	$PGD^{A}\,PGD^{A}$[3]; $Pd^{A}\,Pd^{A}$[4]		100	–
Common Variant; Type AB[4]	Type II	$PGD^{m}PGD^{\beta}$	$PGD^{A}\,PGD^{C}$[3]; $Pd^{A}\,Pd^{B}$[4]		80-100	Fildes and Paar (1963); Bowman et al. (1966)
Canning; Type B[4]	Type III	$PGD^{\beta}\,PGD^{\beta}$	$PGD^{C}\,PGD^{C}$[3]; $Pd^{B}\,Pd^{B}$		70-80	Bowman et al. (1966); Paar (1966)
Newham	Half Canning; Type VII	$PGD^{\beta}\,PGD^{0}$	$PGD^{C}\,PGD^{0}$		40-50	Paar and Fitch (1964 and 1967)
Richmond	Richmond; Type IV	$PGD^{m}\,PGD^{\gamma}$	$PGD^{A}\,PGD^{B}$		100	Paar (1966); Davidson (1967)
Hackney	Hackney; Type V	$PGD^{m}\,PGD^{\delta}$	$PGD^{A}\,PGD^{Hackney}$; $PGD^{A}\,PGD^{N}$		100	Paar (1966)
Ilford	Half-Activity; Type VI	$PGD^{m}\,PGD^{0}$	$PGD^{A}\,PGD^{0}$		50-60	Paar (1966); Brewer and Dern (1964a and 1964b); Dern et al. (1966)
Fully Deficient	Fully Deficient; Type VIII	$PGD^{0}\,PGD^{0}$	$PGD^{0}\,PGD^{0}$		–	Not observed
Whitechapel	Fully Deficient		$PGD^{\gamma}\,PGD^{\gamma}$		3%	Paar (1966); Paar and Fitch (1967)
Dalston			$PGD^{A}\,PGD^{\gamma}$		75%	Dern et al. (1966); Paar and Fitch (1967)
Friendship	Friendship		$PGD^{A}\,PGD^{F\text{-}Friendship}$; $PGD^{A}\,PGD^{F}$	+NADP −NADP	100	Davidson (1967); Brinkman (1971)
Thai[5]			$PGD^{A}\,PGD^{Thai}$	like Richmond	100	Tuchinda et al. (1968)
Elcho			$PGD^{A}\,PGD^{Elcho}$		100	Blake and Kirk (1969)
Freiburg			$PGD^{A}\,PGD^{F\text{-}Freiburg}$; $PGD^{A}\,PGD^{F'}$	±NADP	100	Terriverdian et al. (1970b); Brinkman (1971)
Singapore			$PGD^{A}\,PGD^{S}$		100	Blake et al. (1973b)
Wantoat			$PGD^{A}\,PGD^{Wantoat}$		100	Blake et al. (1974)
Canberra			$PGD^{A}\,PGD^{Canberra}$		100	Blake et al. (1974)
Kadar			$PGD^{A}\,PGD^{Kadar}$		100	Blake et al. (1974)
Caspian			$PGD^{A}\,PGD^{Caspian}$		100	Blake et al. (1974)
Bombay			$PGD^{A}\,PGD^{Bombay}$		100	Blake et al. (1974)
Natal			$PGD^{A}\,PGD^{Natal}$	$-CH_2CH_2SH$ $+CH_2CH_2SH$	100	Blake et al. (1974)
Oshakati			$PGD^{A}\,PGD^{Oshakati}$; $PGD^{A}\,PGD^{S}$		100	Jenkins and Nurse (1974)

[1] According to the earlier papers of Paar and collaborators

[2] Patterns are given only to indicate the relative mobilities of variant isozymes and PGD-A; the types have not all been run under the same conditions, and, as such, are not directly comparable to one another

[3] Revised nomenclature according to Paar and Fitch (1967) and Davidson (1967)

[4] Nomenclature according to Brewer, Dern and collaborators

[5] May be equivalent to Richmond; Jenkins and Nurse (1974) said it may be equivalent to Elcho

representations have been constructed from published photographs and drawings.

The only variant that is at all common is the "common variant". The genotype of such persons is now generally denoted $PGD^{A}PGD^{C}$, and the phenotype is usually denoted as "AC". Some workers employ the designations of Bowman *et al.* (1966), in which the common variant allele is denoted Pd^{B}. The genotype of the common variant heterozygote is thus $Pd^{A}Pd^{B}$, and the phenotype is called "AB". Both the "Freiburg" and the "Friendship" alleles have been denoted PGD^{F}, and Brinkmann (1971) suggested that they might be distinguished by using PGD^{F} and $PGD^{F'}$ for Friendship and Freiburg, respectively. It is probably best to use the complete name if there is any chance of confusion. The majority of variants are very rare, although some occur in appreciable frequencies in certain isolated populations.

32.2.2.4 Linkage relations and chromosomal localization of PGD. The data of Weikamp *et al.* (1970) indicated a linkage between *PGD* and *Rh* loci. Renwick (1971a) reported that *PGD* was syntenic to *Rh* and PGM_1 loci, thus localizing it on chromosome 1. This finding has been confirmed (Westerveld and Meera Khan, 1972; Jongsma *et al.*, 1973; Douglas *et al.*, 1973).

33.2.3 Biochemical studies on PGD

The earlier studies on PGD have been reviewed by Noltmann and Kuby (1963). Not too much detailed biochemical work appears to have been carried out on purified red cell preparations. Paar and his collaborators, in the earlier papers, thought that the active enzyme was probably a dimer, and that at least PGD^{A} and PGD^{C} were coding for different monomers. Bowman *et al.* (1966) did not completely accept this idea. Some of the variant enzymes show variable thermal denaturability as well as variable denaturability in the presence of urea or iodoacetamide, the PGD-A enzymes being more stable than the PGD-C (Paar and Paar, 1965; Carter *et al.*, 1966 and 1968). The PGD-A enzyme did not differ in these respects from Hackney and Richmond, however (Carter *et al.*, 1968). Shih *et al.* (1968b) partially purified PGD from A, AC and C red cells. The A and C forms differed in their K_m for 6-phosphogluconate but not for NADP. Pearse and Rosemeyer (1974) may have obtained the purest preparation of PGD from red cells. Their preparation was homogeneous in the ultracentrifuge, but showed some heterogeneity upon starch gel electrophoresis. Some of the properties of the purified enzyme were described by these workers, but they were not concerned with genetic variants of PGD. The MW of PGD is about 80,000 (Kazazian, 1966).

Carson *et al.* (1966) discovered that incubation of PGD with NADP in the presence of red cell stroma resulted in a significant loss of enzyme activity. Neither stroma, nor NADP alone, caused the effect. Subsequent studies by this group (Ajmar *et al.*, 1968) revealed that the stroma-NADP inactivation effect is an indirect one. Stroma contains an NAD(P)-ase. The designation "NAD(P)-ase" means that the enzyme will act on NAD or on NADP. The enzyme acts on NADP to produce, as one of the products, 2′-phosphoadenosine diphosphate ribose (P-ADPR). This molecule modifies PGD in some way leading to inactivation. If hemolysate, containing stroma, is incubated with NADP for increasing periods of time, and the electrophoretic mobility of PGD examined as a function of time of incubation, the PGD-A band is seen to become weaker with the simultaneous appearance of a slower band. The final pattern greatly resembles that seen with a homozygous Canning variant. It should be noted that this effect of NADP is quite opposite to the stabilizing influence this cofactor exerts on Glc-6PD (section 33.1.5).

33.2.4 Medicolegal applications

The PGD polymorphism is utilized in a number of laboratories in cases of disputed parentage. Since the frequency of the PGD^{C} allele does not reach very high frequencies in most populations, PGD is not one of the more powerful systems for the exclusion of true nonfathers. In German populations, the system may be expected to exclude 2%-3% of falsely accused fathers (Brinkmann, 1971). In England, the figure is 2.5% (Boorman *et al.*, 1977). In this country, the values are about 2.3% for Caucasians and 3.3% for Blacks (Polesky *et al.*, 1976; Dykes and Polesky, 1978). In the more recent work, the figure for Caucasians is given as 1.8%, and is probably based upon gene frequencies calculated for a larger population sample. PGD isozymes are fully developed in fetal blood (Chen *et al.*, 1977).

PGD isozymes have been determined in bloodstains. Procedures for typing the enzyme have been described by Culliford (1971) and by Brinkmann (1971). The methods differ only slightly. Brinkmann (1971) utilized phosphate buffers at pH 6.8 and thicker starch gels, where Culliford (1971) recommended the use of thin starch gels, and a slightly different buffer concentration. Except for slight differences in the pH of buffers, these procedures closely resemble the original method used by Fildes and Paar (1963). PGD isozymes are detected in gels after electrophoresis by the addition of an overlay of substrate, NADP, $MgCl_2$, PMS and MTT in pH buffer, and looking for formazan bands (Figure 33.2). NADP in pre-treating solutions or incorporated into gel buffers is to be scrupulously avoided with bloodstains, since the stroma are still present. Carter *et al.* (1968) noted that additional weak bands, anodal to PGD-A, may appear if NADP is present, and Culliford (1971) confirmed this finding. These same bands may be seen in old lysates (Culliford, 1971). PGD is quite labile in bloodstains. Brinkmann (1971) said that he could differentiate A and AC types in stains kept at room temperature for up to four weeks. Welch (1972b) could detect the phenotypes for up to 15 days in dried bloodstains. Henke (1979) recently recommended caution in the interpretation of PGD patterns, especially where unusual variants are involved. These variants can exhibit variability with respect to the retention of the pattern characteristics, even upon freezing and thawing.

Other electrophoretic procedures have been described for PGD phenotyping (see in Brewer, 1969 and 1970). Sonneborn (1972) described a cellulose acetate membrane technique. A number of procedures have been devised for the simultaneous determination of PGD along with other isozyme systems: with AK (Brinkmann and Thoma, 1970); with AK and ADA in lysates and bloodstains (Brinkmann and Dirks, 1971); with GPT, PGM and AK (Goedde and Benkmann, 1972); and with ACP on thin layer agarose gels (Martin and Voss, 1978).

Suyama and Imai (1975) reported that PGD types could be determined from tooth parts, and Oya *et al.* (1978) reported successful PGD typing in human hair bulbs up to two weeks after the hair was extracted.

PGD isozymes occur in sperm cells, and the type is the same as that seen in red cells (Brinkmann and Koops, 1971; Blake and Sensabaugh, 1976). The concentration of PGD is much lower in seminal plasma, however (Culliford, 1971; Blake and Sansabaugh, 1976 and 1978). Whole semen contains only about 4% of the PGD activity seen in whole blood on a per volume basis, and it was estimated that a minimum quantity of 25$\mu\ell$ semen would be needed for PGD determination, as against about 1$\mu\ell$ for blood (Blake and Sensabaugh, 1978).

Gibbs (1968) reported that PGD is present in "vaginal fluid", but the activity levels observed were not compared with those in blood, nor were they referenced to a defined amount of "vaginal fluid". The studies were undertaken to explore a possible relationship between PGD levels in vaginal fluid and cervical carcinoma.

33.2.5 The distribution of PGD phenotypes in U.S. populations

The distribution of PGD variants for world populations was reviewed by Tills *et al.* (1970b). A ten-fold decimal error, causing the frequencies to be 10 times too large, was subsequently corrected (Tills *et al.* 1971b). The frequency of PGD^C is low in most Caucasian populations. It can reach much higher levels (10–20%) in some Asian Indian, African and Middle Eastern populations. The data for U.S. populations appears in Table 33.3.

Table 33.3 Distribution of PGD Phenotype in U.S. Populations

Population	Total	Frequency of Occurrence — Number (Percent) A	AC	C	PGD^{A} ★	Reference
CAUCASIAN						
Unspecified locale	58	57 (98.3)	1 (1.7)		0.99	Dern et al., 1966
Unspecified locale	600	554 (92.3)	45 (7.5)		0.961	Bowman et al., 1966
Buffalo, NY	1,377	1,313 (95.3)	62 (4.5)	2 (0.2)	0.976	Davidson, 1967
Chicago, IL	101	97 (96)	4 (4)		0.98	Shih and Hsia, 1969
Seattle, WA	647	624 (96.5)	22 (3.4)	1 (0.1)	0.98	Giblett, 1969
California	4,472	(96.2)	(3.7)		0.98	Grunbaum et al., 1978b
Detroit, MI	503	482 (95.8)	20 (4.0)	1 (0.2)	0.978	Stolorow et al., 1979 and see Shaler, 1978
NEGRO						
Unspecified locale	296	278 (93.9)	18 (6.1)		0.97	Dern et al., 1966
Unspecified locale	416	385 (92.5)	31 (7.5)		0.963	Bowman et al., 1966
Buffalo, NY	1,226	1,141 (93.0)	83 (6.8)	2 (0.2)	0.964	Davidson, 1967
Chicago, IL	101	93 (92.1)	8 (7.9)		0.96	Shih and Hsia, 1969
Seattle, WA	506	452 (89.3)	52 (10.3)	2 (0.4)	0.945	Giblett, 1969
California	787	(92.6)	(7.2)	(0.4)	0.964	Grunbaum et al., 1978b
Detroit, MI	504	462 (91.7)	39 (7.7)	2 (0.4)	0.955	Stolorow et al., 1979 and see Shaler, 1978

★ Gene frequency

SECTION 34. GLYOXALASE I

34.1 Recognition of Glyoxalase

Glyoxalase I [GLO;. GLO I; Lactoyl-glutathione lyase; S-lactoyl-glutathione methylglyoxal-lyase (isomerizing); E.C. 4.4.1.5] catalyzes the reversible conversion of reduced glutathione and methyl glyoxal to S-lactoylglutathione. In conjunction with its companion enzyme, glyoxalase II (GLO II; hydroxyacylglutathione hydrolase; S-2-hydroxyacylglutathione hydrolase; E.C. 3.1.2.6) which catalyzes the hydrolytic conversion of S-2-hydroxyacyl glutathione compounds to glutathione and the corresponding 2-hydroxy acid, the overall conversion of methyl glyoxal to lactate can be effected.

The presence of an enzyme activity in animal tissues which catalyzed the conversion of methyl glyoxal to lactate, and of phenyl glyoxal to mandelic acid, was simultaneously recognized by Neuberg (1913) and by Dakin and Dudley (1913a). The latter authors are responsible for the name "glyoxalase" (Dakin and Dudley, 1913b), and also noted that the enzyme was present in the red cells but not in the serum of dog blood. In 1932, Lohmann established that reduced glutathione was required for glyoxalase activity. Jowett and Quastel (1933) found that human red cells contain substantial GLO activity, and proposed that S-lactoylglutathione was an intermediate in the reaction leading to lactate. Yamazoye (1936) demonstrated the formation of the glutathione-methyl glyoxal adduct in crude liver extracts. In 1948, Hopkins and Morgan obtained evidence from partially purified beef heart preparations that a protein "factor" accelerated the formation of lactate from the methyl glyoxal and glutathione in the presence of glyoxalase. In 1951, Racker demonstrated that yeast contains two glyoxalases, which he called I and II. Glyoxalase I catalyzed formation of the S-lactoyl glutathione, and glyoxalase II catalyzed its hydrolytic breakdown into lactate and reduced glutathione. A further description of the methods used to study yeast glyoxalases was given by Racker in 1955. Crook and Law (1952) showed that beef heart contains both glyoxalases as well. Human erythrocytes contain substantial amounts of glyoxalase I, but no glyoxalase II (Cohen and Sober, 1945; Valentine and Tanaka, 1961; Paar *et al.*, 1977). Glyoxalase enzymes are widely distributed in nature.

34.2 Genetic Variation of Glyoxalase I

GLO polymorphism is a relatively recent finding. In 1975, Kömpf *et al.* in Germany, and Bagster and Paar in England, independently discovered that GLO exhibited genetically determined electrophoretic variation in red cells. Three phenotypes were observed, and were called "1", "2-1" and "2" by the German workers, and "slow", "intermediate" and "fast", respectively, by the English investigators. These could be explained by a pair of codominant autosomal alleles, and family and population studies were consistent with this idea (Kömpf *et al.*, 1975; Kömpf and Bissbort, 1975; Bagster *et al.*, 1975; Paar *et al.*, 1977). The alleles determining the common phenotypes are now denoted GLO^1 and GLO^2. The GLO^1 frequencies in Britain and Germany were very similar, 0.42 to 0.43. In Gambians, the GLO^1 was about 0.25 (Bagster *et al.*, 1975).

Additional population and family studies have been consistent with the codominant autosomal allelic pair hypothesis of inheritance (Olaisen *et al.*, 1976b; Martin and Ott, 1976; Meera Khan and Doppert, 1976; Brinkmann and Püschel, 1978). Ranzani *et al.* (1979) reported a new phenotype of GLO, which could be attributed to heterozygosity of GLO^2 with a new allele, GLO^3. There is, additionally, a silent allele, GLO^0. Olaisen *et al.* (1976b) mentioned that they had found a woman with no detectable GLO activity, but further studies could not be pursued. Rittner and Weber (1978) found a family, however, in which a GLO^0 allele was segregating in three generations. An unusual HLA haplotype was also segregating in this family, making it even more interesting, particularly in view of the established *GLO:HLA* linkage. Rubinstein and Suciu-Foca (1979) reported a GLO^0 allele segregating in three generations of a family. The *GLO* locus has been mapped on chromosome 6, and is linked to *HLA* and PGM_3. *GLO* is thought to lie between the PGM_3 and *HLA-B* loci (Olaisen *et al.*, 1976a; Weitkamp, 1976; Pretorius *et al.*, 1976). The electrophoretic patterns of GLO isozymes are shown diagrammatically in Figure 34.1.

34.3 Assay and Detection Methods for GLO

Assay procedures for glyoxalases were given by Racker (1955). These are applicable to the assay of red cell GLO. Spectrophotometric assay of GLO is based upon the fact that S-lactoyl glutathione, the product of the GLO I reaction, absorbs light of 240 nm. GLO I can thus be assayed by following the increase in absorbance at 240 nm, while GLO II can be assayed by following the A_{240} decrease (Racker, 1951 and 1952). GLO I can be assayed in crude hemolysates utilizing this principle (Paar *et al.*, 1977).

A number of different detection procedures for GLO isozymes in gels have been employed. Apart from its characteristic UV absorption, the product of the GLO I reaction has no other properties that make it readily detectable. The impracticality of spectrophotometric detection procedures in gels has prompted the employment of other procedures. The methods used have taken advantage of the fact that reduced glutathione occurs everywhere in the gel except at the sites of GLO activity. Two procedures, based upon the color reactions of unreacted GSH have been devised. In one

of these, MTT tetrazolium salt is dissolved in agar gel in the presence of either PMS (Bagster and Paar, 1975), or of DCPIP (Kömpf *et al.*, 1975), and overlaid on the electrophoretic gels. This overlay may contain the GLO substrates, or may be added after incubation of the electrophoretic gel with a filter paper overlay containing the substrates. Sites of activity appear as clear zones on an otherwise violet (formazan) background. In the second, introduced by Paar *et al.* (1977), the gel is overlaid with the GLO substrates on filter paper, and incubated to allow the reaction to occur. It is then overlaid with an iodine solution in agar gel. The unreacted glutathione in the electrophoretic gel reduces the iodine to iodide except at the sites of GLO activity, where the iodine reacts with the starch in the electrophoretic gel giving the familiar deep blue color. This procedure is applicable only to electrophoretic gels made from starch or containing it. Another procedure, used by Bagster and Paar (1975), is to add the substrates along with exogenous GLO II, LDH, NAD, PMS and MTT to the gel overlay. The GLO II catalyzes the formation of lactate from S-lactoyl glutathione at the sites of GLO I activity, and the lactate is oxidized to pyruvate by LDH with the concomitant reduction of NAD to NADH. The NADH then reduces MTT tetrazolium to formazan in the presence of PMS. The detection schemes are summarized in Figure 34.2.

34.4 Biochemical Studies on Glyoxalase I

No reviews of glyoxalase biochemistry were found. The earlier studies were mentioned in section 34.1. The first glyoxalase purified was a yeast enzyme (Racker, 1951). Glyoxalases from animal sources were not purified and characterized until relatively recently. Davis and Williams (1966) obtained relatively pure preparations from calf liver and from yeast. Their yeast enzyme was purified to a greater extent than was Racker's in 1951. More recently, affinity chromatography steps have been introduced into purification procedures, and very pure preparations have been obtained from pig erythrocytes (Mannervik *et al.*, 1972; Aronnson and Mannervik, 1977; Aronnson *et al.*, 1978), rat erythrocytes (Han *et al.*, 1977), and the livers of mice (Kester and Norton, 1975), rabbits (Elango *et al.*, 1978) and sheep (Uotila and Koivusalo, 1975). The yeast enzyme, and that from human red cells, have also been purified (Aronnson *et al.*, 1978). Most mammalian GLO I appear to be dimers, with MW about 43,000 to 48,000. The pig red cell enzyme exhibits two components when pure, and treatment with reduced glutathione converts them to a single form, suggesting that one of the "forms" is a mixed disulfide with GSH (Aronnson and Mannervik, 1977). This enzyme contains two -SH per mole. Davis and Williams (1966) found that the enzyme was inactivated by EDTA, but that activity could be more or less restored by incubation with Mg^{++} or Mn^{++}. The yeast enzyme was similarly inactivated, but its activity could not be restored by Mg^{++}. Atomic absorption analysis of the yeast enzyme detected Mg^{++}, and they concluded that this was a Mg^{++}-enzyme. All the mammalian

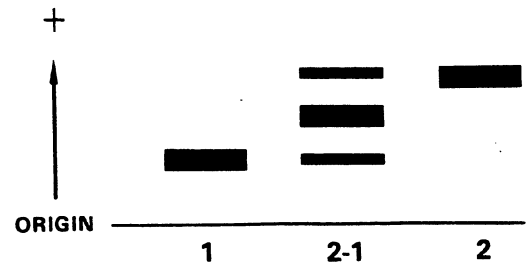


Figure 34.1 Electrophoretic Patterns of GLO Phenotypes

enzymes are inactivated by chelating agents as well, but activity can be restored at least to some extent by the addition of divalent metal ions, Mg^{++} generally being the most effective. Aronnson *et al.* (1978), after extensive purification of the human and pig red cell, rat liver and yeast enzymes, and atomic absorption analysis, said that all of them contained Zn^{++}. The mammalian enzymes have one Zn^{++} per subunit, or two per molecule. The fact that Mg^{++} restores activity to the apoenzyme more effectively than does Zn^{++} in most cases, if GLO is really a zinc metalloenzyme as indicated by the studies of Aronnson *et al.* (1978), seems to be a bit puzzling.

Schimandle and Vander Jagt (1979) purified red cell GLO from all three red cell phenotypes, obtaining 12,000 to 20,000 fold purifications. All the purified samples had MW 44,000, as estimated by gel filtration. The GLO from phenotypes 1 and 2 were indistinguishable kinetically, although the preparation from 2-1 red cells appeared to exhibit greater stability than that from either homozygous type.

Martin and Ott (1976) noted that substantial loss of GLO activity in hemolysates kept for several weeks can be slowed or prevented by the addition of bovine serum albumin at a concentration of 150 $\mu g/m\ell$ lysate. Racker (1951) had found that bovine albumin had a stabilizing influence on the yeast enzyme as well.

34.5 Medicolegal Applications

34.5.1 Disputed parentage

There are few papers as yet on the application of GLO to disputed parentage, because it is relatively new polymorphism. Because of the frequency of distribution of its phenotypes, however, GLO will undoubtedly turn out to be one of the better two allele systems available. Brinkmann and Püschel (1978) recommended the use of GLO typing in these cases, as did Eriksen (1979) who said that the exclusion probability was of the order of 18% in Denmark. The figure is very similar in Vienna (Pausch *et al.*, 1979), and in Poland (Jakliński and Koziol, 1979). There is little data on gene frequencies in U.S. populations as yet, but it does not seem unreasonable to expect that the probability of excluding a

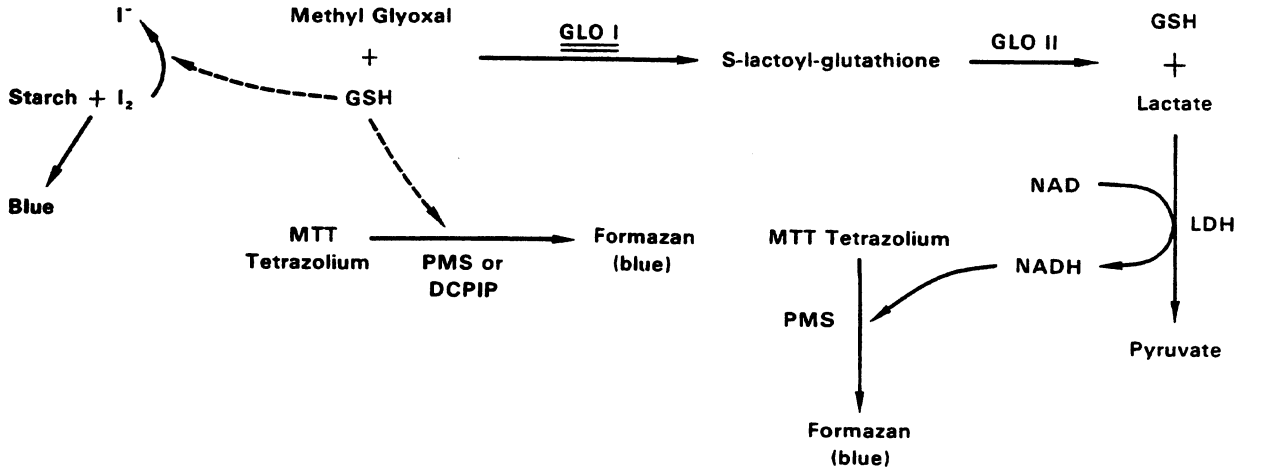


Figure 34.2 Detection Reaction Sequences for GLO

falsely accused father with the GLO system alone will be about 0.18 for Whites and about 0.16 for Blacks.

34.5.2 Electrophoretic methods for bloodstain typing

GLO has been phenotyped on starch gels and on several other electrophoretic media. Paar *et al.* (1977) used phosphate buffers at pH 6.7 for this system, while Kömpf *et al.* (1975) used a Tris-histidine buffer system at pH 7.8. The isoenzymes are quite acidic and both systems are perfectly suitable. Martin and Ott (1976) ran GLO isoenzymes on agarose gels in Tris buffers at pH 7.8. Migration is faster in agarose than in starch, and the plates could be run in less than 1½ hours. Meera Khan and Doppert (1976) gave a procedure for GLO typing on gelled cellulose acetate in pH 8 buffers.

Bloodstains can be phenotyped for GLO isoenzymes (Wraxall and Stolorow, 1978; MPFSL, 1978). The MPFSL utilized 0.2M phosphate buffers, pH 6.8, for the purpose. Stains are treated with dilute mercaptoethanol, and they said that mercaptoethanol and dithiothreitol were not interchangeable in this system. This procedure is given by Emes and Parkin (1980) as well.

Wraxall and Stolorow (1978) described a technique in which GLO phenotyping is combined with that of PGM and ESD in the same electrophoretic gel. The running gel is made from 2% starch and 1% agarose, and the original PGM buffer system of Spencer *et al.* (1964b) is employed. GLO isoenzymes run ahead of (anodal to) PGM and ESD isozymes in this system, and can be separately detected. Experience thus far indicates that GLO is quite labile in bloodstains, although detailed stability studies on bloodstains were not found in the literature. GLO is one of the better systems for bloodstain individualization. The DI for the White population is about 0.6, and that for the Black population will probably be a bit lower.

Burgess (1979) and Burgess and Twibell (1979) described a procedure for GLO phenotyping in hair root sheath cells using Cellogel membranes.

Burgess and Twibell (1979) said that Parkin had reported on GLO phenotyping in semen at the 7th International Congress of Forensic Haematogenetics in Hamburg in 1977 (I had no access to the proceedings of this conference). Blake and Sensabaugh (1978) reported that there is about 4 times more GLO in sperm cells than in red cells on a per cell basis, and that phenotypic expression in the two is identical. They said, however, that the GLO in seminal plasma showed different patterns which were difficult to interpret, and which tended to mask the clear patterns from the sperm cells. They suggested that GLO typing in whole semen would present very difficult problems. Stöhlmacher and Haferland (1980) recently showed that a variety of human tissues contained GLO activity, and that the same phenotype is seen in all the tissues (including red cells) from the same person. There was some variation in the concentration of GLO in the various tissues, which included skin, skeletal muscle, brain, tongue, tonsil, lung, heart, stomach, intestine, liver, spleen, kidney, various glands, testicle, uterus and bone marrow.

34.6 Distribution of GLO Phenotypes in U.S. Populations

Only two population studies were found before Table 34.1 was prepared. The table shows the data. Grunbaum *et al.* (1980) have added some further data for California Whites and Blacks, Mexicans from California and Mexico City and Asians from California and Hawaii. These frequencies were: For Whites (n=313): 54(17.3%) 1, 165 (52.7%) 2-1 and 94(30%) 2, GLO^1 = 0.436; For Blacks (n=308): 39(12.7%) 1, 125(40.6%) 2-1 and 146(47.4%) 2, GLO^1 = 0.327; For Mexicans (n=1080): 111(10.3%) 1, 444(41.1%) 2-1 and 525(48.6%) 2, GLO^1 = 0.308; For

Table 34.1 Distribution of GLO Phenotypes in U.S. Populations

Population	Total	Phenotype Distribution — Number (Percent) 1	2-1	2	*GLO*¹★	Reference
CAUCASIAN						
Buffalo, NY	101	21 (20.7)	42 (41.6)	38 (37.6)	0.42	Weitkamp, 1976
Detroit, MI	503	100 (19.9)	260 (51.7)	143 (28.4)	0.457	Stolorow and Wraxall, 1978; Stolorow et al., 1979
NEGRO						
Buffalo, NY	107	10 (9.3)	40 (37.4)	58 (54.2)	0.28	Weitkamp, 1976
Detroit, MI	504	75 (14.9)	212 (42.1)	217 (43.1)	0.359	Stolorow and Wraxall, 1978; Stolorow et al., 1979

★ Gene frequency

Asians (n=884): 4(0.5%) 1, 125(14.1%) 2-1 and 755 (85.4%) 2, $GLO^1 = 0.075$.

The gene frequency of GLO^1 in western European Caucasians is of the order of 0.4–0.5. It is lower in Negroes. GLO^1 is quite high in Japanese, about 0.9 (Toyomasu *et al.*, 1977), and apparently quite low in some Asians (Grunbaum *et al.*, 1980).

SECTION 35. GLUTAMIC-PYRUVIC TRANSAMINASE

35.1 Recognition of GPT

Glutamic-pyruvic transaminase (GPT; glutamate-pyruvate transaminase; glutamic-alanine transaminase; alanine aminotransferase; L-aspartate:2-oxoglutarate aminotransferase; E.C. 2.6.1.2) catalyzes the reversible conversion of L-alanine and 2-oxoglutaric acid to pyruvic acid and L-glutamic acid. 2-oxoglutaric acid was formerly called α-ketoglutaric acid. GPT is a representative of one category of amino group transferring enzymes, of which a number of categories are now known. There is a vast literature on transaminases. The review by Braunstein (1973) should be consulted for further details. "Glutamic-pyruvic transaminase" is an older name for this enzyme, but it is still commonly used by workers interested in genetic polymorphism because the designation "GPT" is the basis for the genetic variation nomenclature. Biochemists tend to call the enzyme "alanine aminotransferase", which is preferred by the Enzyme Commission as the trivial name.

Enzyme catalyzed aminotransferase activity involving amino acids and the corresponding 2-oxo acids was first described in 1937, and it was GPT activity that was detected (Braunstein and Kritzmann, 1937a, 1937b and 1937c). It was clear soon afterward that transaminase activities played a major role in amino acid metabolism. In 1944, Snell suggested that the vitamin B_6 related compounds pyridoxal phosphate and pyridoxamine might play a principal role as cofactors in these reactions, and his suggestion has turned out to be correct. Snell (1953) reviewed the development of knowledge about the role of pyridoxal phosphate in aminotransferase reactions. Green *et al.* (1945) presented evidence that pyridoxal phosphate was a required cofactor for pig heart GPT. GPT is widely distributed in nature. It has been known for quite some time that red cells possess GPT activity (Karmen *et al.*, 1955; Rapoport, 1961; Löhr and Waller, 1961; Radhakrishnamurty and Sabry, 1968). It is also known that GPT occurs in two forms in many animal tissues, one form being associated with mitochondria, and the other form being soluble (Hopper and Segal, 1964; Saier and Jenkins, 1967). GPT shares this property with GOT (E.C. 2.6.1.1). Biochemical studies on the aminotransferases have been periodically reviewed (Braunstein, 1947, 1957 and 1973; Meister, 1955). Many other reviews are cited by Braunstein (1973).

35.2 Genetic Variation of GPT

Genetic variation in GPT was discovered by Chen and Giblett in 1971. They detected three electrophoretic patterns in the bloods of different people after vertical starch gel electrophoresis of hemolysates in a Tris-citrate, pH 7.5, buffer system. The three phenotypes were designated "1", "2-1" and "2". Family studies indicated that a pair of autosomal codominant alleles was responsible for the phenotypes. These alleles were originally designated *Gpt*^{1} and *Gpt*^{2}, but are now more generally designated *GPT*^{1} and *GPT*^{2}. In a Seattle population, *GPT*^{1} showed a frequency of about 0.5 in Caucasians, 0.8 in Negroes and 0.6 in Orientals, making the system particularly powerful in discriminating members of a population. Family and population studies in a number of laboratories were consistent with the genetic explanation for the GPT polymorphism (Kömpf, 1971; Benkmann and Goedde, 1972; Chen *et al.*, 1972). An extensive study of the GPT phenotypes in a number of populations was carried out by Chen *et al.* (1972). Six additional rare phenotypes were observed in the course of the survey, which could be explained on the basis of heterozygosity of one of four new rare alleles with *GPT*^{1} or *GPT*^{2}. The new alleles were designated *GPT*^{3}, *GPT*^{4}, *GPT*^{5} and *GPT*^{6}. The *GPT*^{3} allele has been observed in a number of German populations (Brinkmann *et al.*, 1972c; Gussmann and Schwarzfischer, 1972; Martin and Niebuhr, 1973b; Jungwirth and Woll, 1974), and we found four examples of its expression in New York and London populations (Welch *et al.*, 1975). An additional allele has been described by Olaisen (1973a) segregating in four generations of a Norwegian family. This was called *GPT*^{7}, and examples of both GPT 7-1 and 7-2 were observed. The electrophoretic patterns shown by the different GPT phenotypes are indicated in Figure 35.1. A further allele, *GPT*^{8}, was observed by Santachiara Benerecetti *et al.* (1975) in an Italian population.

There are reports of silent alleles of GPT. Olaisen (1973b) found an apparent *GPT*^{0} segregating in a large family, and the presumed heterozygotes exhibited about half the GPT activity characteristic of the homozygous normal types. Spielmann *et al.* (1973) found another example in a survey of a German population. In this case, an apparent GPT 1 mother and GPT 2-1 father had a GPT 2 child, and the explanation appeared to be that this mother was *GPT*^{1}*GPT*^{0}, and her child, *GPT*^{2}*GPT*^{0}. The question of a silent allele at the *GPT* locus was called into question by the work of Kömpf and Bissbort (1974) and Kömpf *et al.* (1974). These investigators have presented evidence for inherited quantitative variability of the GPT isozyme activities. This matter is discussed more fully in section 35.4.2. Under some circumstances, typing results with certain quantitative 2-1 variants were almost indistinguishable from those that would be predicted for heterozygotes carrying a silent allele, unless densitometric traces were carefully examined. It does not appear that the issues surrounding *GPT*^{0} are fully settled as yet, and the subject is discussed further in section 35.5.1.

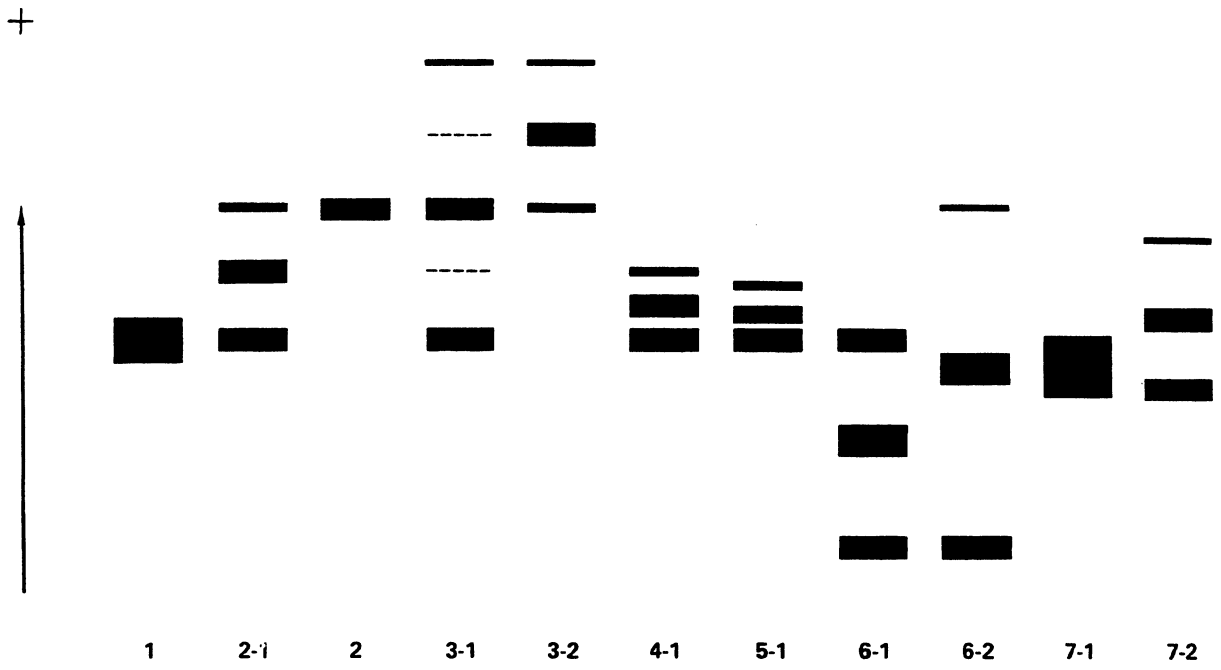


Figure 35.1 Electrophoretic Patterns of GPT Phenotypes

35.3 Procedures for GPT Phenotyping

The original studies which disclosed the GPT polymorphism (Chen and Giblett, 1971) employed vertical starch gel electrophoresis, but horizontal starch gel electrophoresis procedures have been successfully used as well (Radam and Strauch, 1972;. Welch, 1972a and 1972b; and others). Most workers use Tris-citrate buffers at pH 7.5 for electrophoresis, although systems have been described in which GPT is combined with other isoenzyme systems for typing in the same gel. GPT can be typed simultaneously with PGM, using the PGM buffer system (Gussmann and Rames, 1972), or with PGD, PGM and AK in Tris-citrate, pH 7.5, buffers (Goedde and Benkmann, 1972). Thick starch gels are normally employed for GPT typing, and they require slicing prior to staining. We have generally preferred to use thin starch gels, as described by Wraxall and Culliford (1968), for isoenzyme phenotyping, but this technique was not found to be very successful for GPT phenotyping. Part of the difficulty may have been that the red cell content of GPT is lower than that for many other polymorphic erythrocyte enzymes, and the thicker gel allows the application of more material for typing.

The detection of GPT in gels following electrophoresis has also been carried out in a number of different ways. Perhaps the most common is the original (Chen and Giblett, 1971) procedure (Figure 35.2, Sequence A). This method takes advantage of the fact that NADH fluoresces under the UV, whereas NAD does not, and zones of GPT activity show up under UV illumination as non-fluorescent bands on an otherwise fluorescent background. Methods in which MTT tetrazolium is added have been used as well. Since the zones of GPT activity are occupied by NAD, and since NADH is the reductant, these procedures result in clear bands on an otherwise purple-blue formazan background. Kömpf (1972) used this procedure. He incubated the gel with all the components except PMS, which was added in a second step. Anger *et al.* (1974) utilized a similar procedure except that they added all the components except MTT and PMS in the first overlay, incubated for about 2 1/2 hours, and then put on a second overlay containing MTT and PMS, after having removed the first one (Figure 35.2, Sequence A). Martin and Niebuhr (1973a) utilized a procedure based on a different reaction sequence. Alanine and 2-oxoglutarate were added as substrates, along with L-glutamic acid dehydrogenase, acetylpyridine adenine dinucleotide (APAD), MTT and PMS. The L-glutamate formed as the product of the GPT reaction is converted back to 2-oxoglutarate by L-glutamate dehydrogenase, with the concomitant reduction of APAD, which can reduce MTT to formazan. APAD is an NAD analog (Figure 35.2, Sequence B).

GPT typing can sometimes present problems (see, for example, Berg *et al.*, 1974a). The GPT^1 product is somewhat more active than that of GPT^2, and may stain more rapidly on the gels. The GPT 1 type may thus tend to become overstained if sufficient time is allowed for the full development of the GPT 2-1 and GPT 2 types. Overstained GPT 1 can

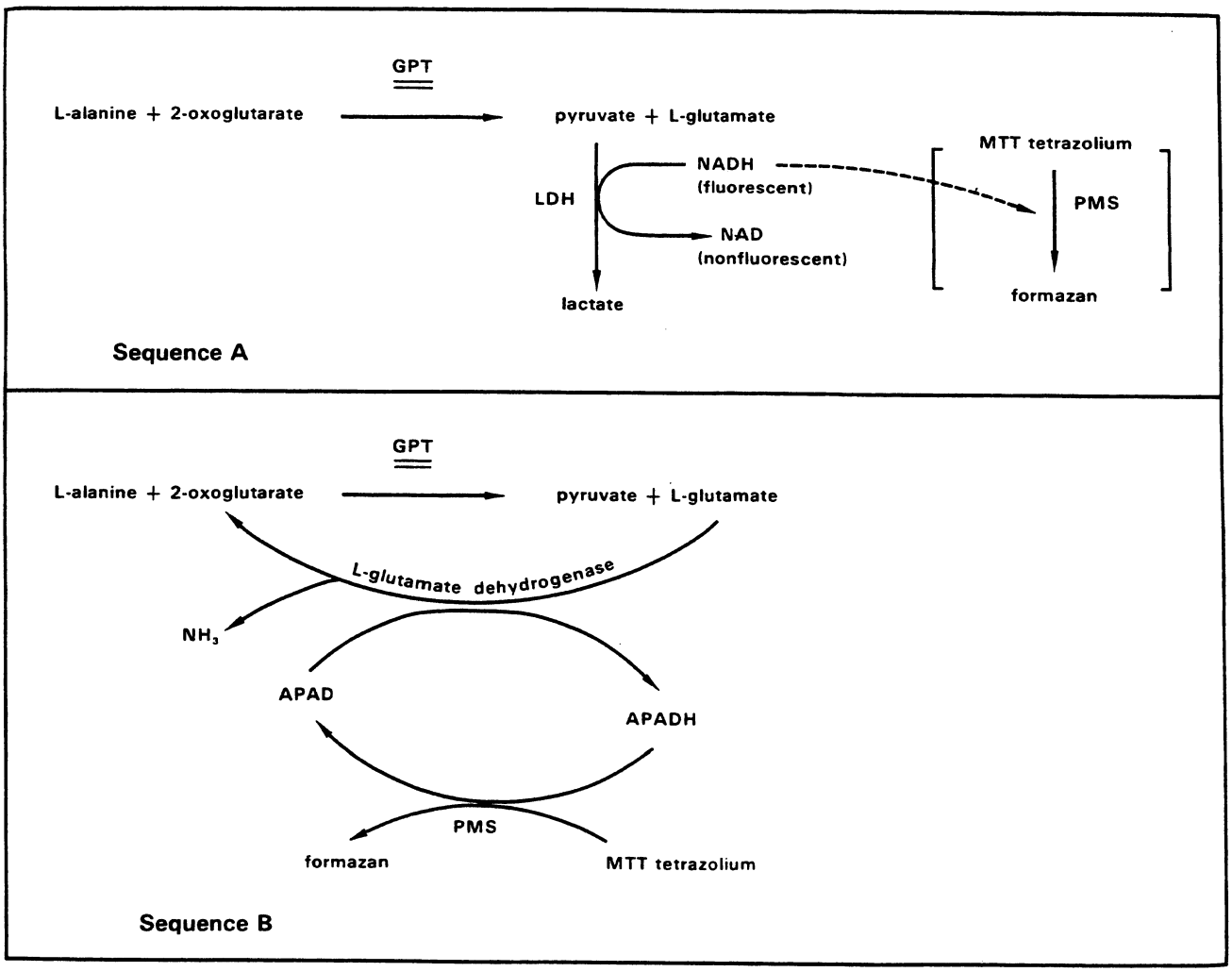


Figure 35.2 Detection Reaction Sequences for GPT

resemble GPT 2-1 at times. This difficulty can usually be avoided if the gels are examined several times during the course of development.

35.4 Biochemical Studies on GPT

35.4.1 Purification and properties of GPT

The biochemical studies on transaminase enzymes, including GPT, were reviewed by Braunstein in 1973. Transaminases, including GPT, require pyridoxal phosphate as a cofactor, and it is usually tightly bound to the enzyme. The cofactor is intimately involved in the catalytic mechanism, and Braunstain's (1973) review may be consulted for details.

The rat liver enzyme has been extensively purified, and crystallized (Gatehouse *et al.*, 1967; Matsuzawa and Segal, 1968). The molecule has a MW of 114,000 and contained 25 to 30 titrable -SH groups. There were two pyridoxal phosphate per molecule, and the enzyme exhibited a series of differently aggregated forms in the absence of mercaptoethanol. No reports of purification of the red cell enzyme were found.

35.4.2 Activity studies of the red cell GPT isozymes—quantitative variation

In 1972, Chen *et al.* carried out activity studies on the major GPT phenotypes. It was very clear that the *GPT*^{1} product had something more than twice the activity of the *GPT*^{2} product. The mean activity of heterozygote red cells fell in between the homozygous red cell values. Welch (1972a) conducted similar experiments, and his results also clearly showed that the *GPT*^{1} product was considerably more active than that of *GPT*^{2}. There was a fairly broad range of activity values within each phenotypic class, the 2-1 range overlapping both homozygous ranges. The 1-1 range did not overlap the 2-2 range, however. These activity differences were not based on any stability differences in the gene products that could be demonstrated by differential thermal

denaturation. The mean level of GPT 2-1 activity was in quite good agreement with the computed value from the mean activity levels of the homozygous phenotypes. Similar relationships between the activities of the isozymes were described by Ueda *et al.* (1979) using pyruvate and L-glutamate as substrates, i.e., measuring the "reverse" reaction.

In 1974, Kömpf *et al.* reported quantitative densitometric studies on the GPT phenotypes. In many cases, the three bands of the GPT 2-1 phenotype showed 4:3:2 activity ratios. But in a few cases, a different but consistent ratio was seen. These "variant" 2-1 types were seen not just in individuals, but in members of certain families. They were regarded, therefore, as genetic quantitative variants. One variant 2-1 exhibited an activity ratio of 1:1:1 in the three bands, and another variant showed 1:2:3. The latter was called a "2-1M" or "2-1 Marburg". The 2-1M, they said, would not be recognized as a 2-1 on an electrophoretic gel by visual reading, and they themselves had thought for some time that the pattern represented the product of a GPT^2GPT^0 combination. Quantitative assay data were collected by Kömpf and Bissbort (1974), following upon the densitometric work. Six discreet groups of activity level could be distinguished within the two homozygous phenotypic classes, they said, and four groups of activity level were present within GPT 2-1. They regarded these different levels of activity as being a manifestation of quantitative genetic variation of GPT (but see below in section 35.5.1).

35.5 Medicolegal Applications

35.5.1 Disputed parentage

GPT phenotypes are quite well distributed in many populations. The system is one of the better ones for population discrimination, and the GPT system is employed in a number of laboratories in disputed parentage investigations. Radam and Strauch (1972) noted that, in Berlin, 18.7% of falsely accused putative fathers would be excluded by GPT alone. The figure for England is very comparable at 19%, as noted by Welch and Dodd (1974), who reported their results in over 250 cases. The New York population data (Welch *et al.*, 1975) suggest that the value for American Caucasians would be 18 to 19%, while that for Blacks would be about 13%. The isozymes are fully developed in fetal blood (Chen *et al.*, 1977).

Although silent alleles are exceedingly rare, they can cause difficulty in the interpretation of disputed paternity results if encountered. Some of the material on GPT^0 was discussed in the foregoing sections, and there is some disagreement in the literature about interpreting the presence of a silent allele at the *GPT* locus. Heide *et al.* (1974) noted that the variation found in GPT activity within individual phenotypic classes was sufficiently great that enzymatic assays in individual samples could not be regarded as accurate in diagnosing the presence of a silent allele, especially in a medicolegal case. Their paper prompted Ritter to comment on the entire subject, based primarily on the work of Kömpf and others discussed in section 35.4.2 (Ritter, 1975a and 1975b). The comments were very technical, based upon the way the enzyme assays were carried out and their results interpreted. Heide *et al.* (1975) replied to Ritter's comments. They did not appear to accept the notion of genetically controlled quantitative variation in GPT phenotypic classes. The question of activity variation in GPT phenotypes, and the role of a silent allele in this variation, do not seem to have been completely settled as yet.

35.5.2 GPT phenotyping in bloodstains

GPT can be phenotyped in very fresh bloodstains. The system requires quite a bit more material for typing than many other systems, and more difficulty is encountered in trying to type GPT in bloodstains than in fresh blood. In 1972, Welch reported that GPT could be typed in bloodstains up to about 2 weeks old (Welch, 1972b). The electrophoretic system was very similar to that used for the typing of hemolysates. Pieces of bloodstained material about 15 mm^2 were briefly soaked, and inserted directly into the gel. Jungwirth and Woll (1974) also reported on GPT typing in bloodstains up to about a week old. Most laboratories do not carry out GPT typing in bloodstains, in part because only the freshest stains can be typed, and probably also in part because GPT is usually typed on thick starch gels. Most of the laboratories in this country use thin starch gels for enzyme typing, and it is difficult to put a sufficient amount of material into a thin gel for GPT typing. If suitable procedures could be devised for this enzyme, and if typing became possible in somewhat older stains, however, GPT would be one of the best enzymes for this kind of work because of its phenotypic distribution. The Seattle (Chen and Giblett, 1971; Chen *et al.*, 1972) and New York (Welch *et al.*, 1975) data suggest that the DI would be about 0.6 in Caucasians, and about 0.48 for Black Americans.

35.5.3 GPT in other tissues

GPT activity has been reported to occur in semen (Povda, 1962; Ishibe, 1975). It is not clear whether or not the "red cell" GPT types can be determined from seminal plasma or not. It has also been reported that GPT can be phenotyped in tooth parts (Suyama and Imai, 1975).

35.6 Distribution of GPT Phenotypes in U.S. Populations

Only two surveys were found for U.S. populations, and these data appear in Chen and Giblett (1971) and Chen *et al.* (1972) for Seattle, and in Welch *et al.* (1975) for New York.

SECTION 36. PEPTIDASES

36.1 Introduction to Peptidases

The peptidases found in human red cells and tissues are members of a diverse class of enzymes which catalyze the hydrolysis of peptide bonds. In the older literature, a distinction was made between endo- and exo-peptidases, the former being able to catalyze the hydrolysis of internal peptide bonds in peptide chains, while the latter catalyze the hydrolysis of terminal peptide linkages. Some of the endopeptidases show appreciable esterase activity, while many exopeptidases do not. The peptidases to be discussed in this section are exopeptidases, which can act on a variety of di- and tripeptides as substrates. They are also aminopeptidases, i.e., hydrolyzing the N-terminal peptide bond, rather than carboxypeptidases which hydrolyze C-terminal peptide linkages. The earlier literature on peptide bond cleavage was reviewed by Smith (1960), and the more recent review by Delange and Smith (1971) contains some information on the types of peptidases which are discussed in this section.

Peptidases A, B, C, E, F and S are also called dipeptidases, tripeptidases, and aminopeptidases, and are classified by the Enzyme Commission under E.C. 3.4.11.X and 3.4.13.X, where the "X" can be one of several different numbers. Peptidase D is also called proline dipeptidase, prolidase and imidodipeptidase, and is classified under E.C. 3.4.13.9.

36.2 Peptidases of Human Red Cells and Tissues—Multiple Genetic Loci Determining Peptidases

In 1952, Adams *et al.* showed that four distinct di- and tripeptidases were present in human red cells. The substrates used for the different enzymes included gly-pro (for prolidase), L-leucinamide (for aminopeptidase), and di- and triglycine. Haschen (1961) characterized the gly-leu peptidase activity of red cells in terms of its kinetics and activation mechanism, and showed that the enzyme requires Zn^{++} as an essential cofactor. In 1963, Haschen studied the kinetic properties of human red cell prolinase and prolidase.

In 1967, Lewis and Harris subjected red cell hemolysates to starch gel electrophoresis, and stained the gels for peptidase activity with a series of di- and tripeptides as substrates. Based upon electrophoretic mobility and substrate specificity, five distinct types of peptidase could be identified in human red cells, and these were designated A, B, C, D and E. Peptidases A and C showed a broad range of substrate specificity, but did not hydrolyze any of the tripeptides tested (leu-gly-gly, leu-gly-phe, tyr-tyr-tyr and leu-leu-leu). Peptidase B was the only one that hydrolyzed the tripeptides (it was most active with leu-gly-gly), and it also hydrolyzed phe-leu, phe-tyr, pro-phe and lys-leu. Peptidase B may correspond to the "tripeptidase" described by Adams *et al.* (1952), which was detected with triglycine. Peptidase D reacted only with leu-pro, of the substrates tested, and corresponds in all probability to the "prolidase" of Adams *et al.* (1952) and Haschen (1963). Prolidases are peptidases that catalyze the hydrolysis of various peptides having proline as C-terminus. Peptidase E reacted with dileucine, lys-leu, phe-leu and phe-tyr, but its activity was relatively weak. It was the only one of the peptidases that hydrolyzed leucyl-β-naphthylamide. The list of substrates tested with the various peptidases has since been expanded (see Table 36.1). The peptidases are designated "PEP", followed by the appropriate letter, i.e., PEPA, PEPB, etc.

The designations "A" through "E" were based partly on electrophoretic mobility, A being the slowest and E being the fastest (most anodal) in the system used in these experiments (Figure 36.1). Electrophoresis was carried out on starch gels, with bridge buffer consisting of 0.1M Tris-maleic acid, pH 7.4, and gel buffer consisting of 1:20 dilute bridge buffer at the same pH. Detection of peptidase activity following electrophoresis was carried out by allowing the L-amino acids produced in the reaction to react with oxygen via the L-amino acid oxidase reaction, and detecting the peroxide thus formed with o-dianisidine in the presence of peroxidase (Figure 36.2 A). In 1969, Harris reported a zone of peptidase activity which had an electrophoretic mobility more anodal than E, and it was called PEP F. It showed very weak activity compared to the other peptidases, and utilized only trileucine and trityrosine as substrates.

In 1971, Rapley *et al.* carried out an extensive study of tissue distributions of various peptidases. The peptidases were found to be distributed in most tissues examined, although different tissues showed differing amounts of the various peptidase isozymes in most cases. A further peptidase activity, designated PEP S, was found in all tissues examined, except red cells, skin and saliva. Its electrophoretic mobility is slower than (cathodal to) PEP A in the pH 7.4 buffer system of Lewis and Harris (1967). PEP S showed a broad range of substrate specificity, and was especially active with dileucine, phe-leu and trileucine.

The data collected in these studies, coupled with the biochemical studies (section 36.5) and the observation of genetic variation in a number of the different peptidases (section 36.4), have led to the conclusion that the different peptidases are under the control of different genetic loci, and all subsequent experimental data is consistent with this data. The genetic loci are designated *PEPA, PEPB, PEPC,* and so forth.

The relative electrophoretic mobilities of peptidases A through F and S are shown diagramatically in Figure 36.1.

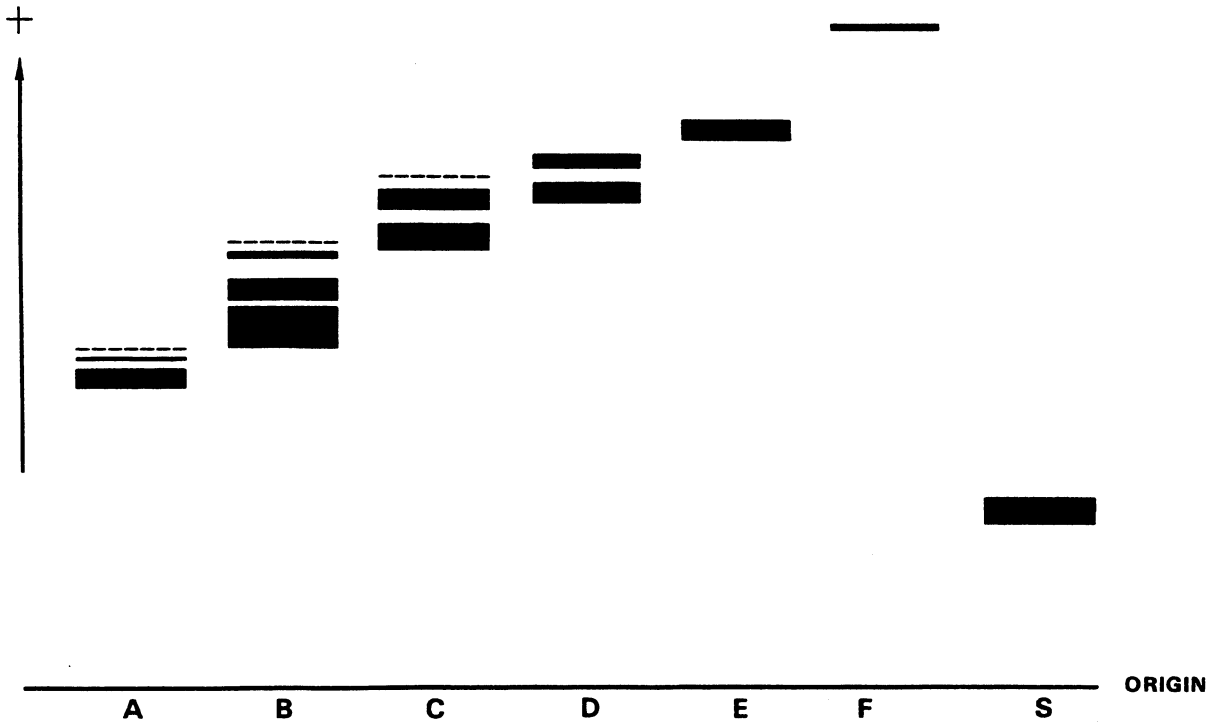


Figure 36.1 Relative Electrophoretic Mobilities of Peptidases A through F and S (after Harris and Hopkinson, 1976)

The activities of the various peptidase isoenzymes toward a variety of substrates is shown in Table 36.1. This information may also be found in Harris and Hopkinson (1976).

36.3 Detection of Peptidase Activity

Lewis and Harris (1967) detected peptidase activity through the use of a coupled reaction involving the L-amino acid oxidase reaction. The scheme is shown in Figure 36.2 A. In this sequence, it is the peroxide resulting from the L-amino acid oxidase reaction that is actually detected by the addition of oxidizable dye (o-dianisidine) and peroxidase. The L-amino acid oxidase normally available comes from snake venom, and this enzyme is only reactive with leucine, isoleucine, methionine, phenylalanine, tyrosine or tryptophan to any significant extent. This feature of the detection scheme, therefore, restricts the di- and tripeptides which can be used as substrates for the peptidases. Substrates must be selected which will produce one of the amino acids as products which can, in turn, react with the snake venom L-amino acid oxidase. Dyes other than o-dianisidine have also been employed. For spectrophotometric assay of the enzyme, Sinha *et al.* (1970) used the same scheme that is represented in Figure 36.2 A, but the dye was o-tolidine. The oxidized o-tolidine product absorbs visible light at 415 nm. Baker (1974) introduced another dye for the staining procedure, called 3-amino-9-ethyl carbazole. This substance is oxidizable to a brown-colored product. Harris and Hopkinson (1976) recommended its use, rather than o-tolidine or o-dianisidine.

In 1971, Rapley *et al.*, in connection with their extensive study of the tissue peptidases, introduced a second detection system based upon the GPT reaction (compare Figure 35.2, Sequence A). GPT reacts with 2-oxoglutarate and alanine, and this detection scheme, which is shown in Figure 36.2 B, allows another whole range of di- and tripeptides which release alanine to be used as substrates for the peptidase isoenzymes.

For routine work, Hopkinson and Harris (1976) recommended the use of the following substrates for the peptidase isozymes: leu-ala for PEP A and PEP C; leu-gly-gly for PEP B; leu-pro or phe-pro for PEP D; and trileucine for PEP S, B, E and F. It should be noted that the peptidases are specific for peptides made from L-amino acids. Glycine, of course, is not optically active.

Kühnl *et al.* (1979) noted that PEP activity with glycine-containing peptides can be detected by allowing the glycine produced to react with o-phthalaldehyde. This method was used to detect PEP A activity following electrohoresis.

Table 36.1 Activities of Various Peptidases with a Number of Substrates (after Rapley et al., 1971)

	Activity of Peptidase						
Substrate	**A**	**B**	**C**	**D**	**E**	**F**	**S**
Val-Leu	+++	−	−	−	−	−	++
Val-Ala	+++	−	−	−	−	−	+
Ala-Gly	+++	−	−	−	−	−	++
Gly-Leu	+++	−	+	−	−	−	−
Leu-Gly	+++	−	+	−	−	−	+
Gly-Phe	++	−	+	−	−	−	−
Ala-Lys	++	−	+	−	−	−	+
Ala-Glu	++	−	+	−	−	−	−
Leu-Ala	++	−	+++	−	−	−	++
Gly-Ala	++	−	++	−	−	−	−
Leu-Leu	++	−	++	−	+	−	+++
Gly-Trp	++	−	++	−	−	−	+
Ala-Ala	+	−	±	−	−	−	−
Lys-Leu	+	+	+++	−	+	−	++
Lys-Tyr	+	+	+++	−	+	−	++
Pro-Phe	++	+	+++	−	−	−	±
Pro-Leu	++	+	+++	−	−	−	−
Phe-Leu	++	++	+++	−	+	−	+++
Phe-Tyr	++	++	+++	−	+	−	+++
Leu-Tyr	+++	++	+++	−	+	−	+++
Ala-Tyr	++	+++	+	−	±	−	+
Ala-His	++	−	++	−	−	−	+
Leu-Gly-Gly	−	+++	−	−	±	−	+
Ala-Gly-Gly	−	+++	−	−	−	−	++
Ala-Ala-Ala	−	+++	−	−	+	−	++
Leu-Leu-Leu	−	++	−	−	+	+	+++
Leu-Gly-Phe	−	+	−	−	±	−	+
Tyr-Tyr-Tyr	−	+	−	−	±	+	+
Phe-Gly-Phe-Gly	−	−	−	−	++	−	+
Leu-Pro	−	−	−	++	−	−	−
Phe-Pro	−	−	−	++	−	−	−
Ala-Pro	−	−	−	++	−	−	−
Leu-β-Naphthylamide	−	−	−	−	+	−	+
Leucinamide	−	−	−	−	±	−	+
Leu-Nitroanilide	−	−	−	−	+	−	±

36.4 Genetic Variation of the Peptidases

36.4.1 PEP A

Lewis and Harris (1967) in the original paper on red cell peptidases found five PEP A patterns in the course of examining a larger number of hemolysates. These were designated PEP A 1, 2-1, 2, 3-1 and 4-1, and family studies indicated that they could be explained by four alleles, *PEPA*^{1}, *PEPA*^{2}, *PEPA*^{3} and *PEPA*^{4}, at an autosomal locus. *PEPA*^{3} and *PEPA*^{4} are very rare. *PEPA*^{2} is relatively frequent in Black populations, but is exceedingly rare in Europeans. Some 15%–20% of Blacks in many African populations are PEP A 2-1. In 1968, two additional rare alleles of PEP A, *PEPA*^{5} and *PEPA*^{6}, were described by Lewis *et al.*. Both were seen as heterozygotes, PEP A 5-1 and 6-1. In 1969, Harris described another rare allele, *PEPA*^{7}, which had been found as a 7-1. In 1973, Lewis added *PEPA*^{8} to the list. The *PEPA*^{8} product exhibits very low activity in red cells, but can be detected in leucocytes, placentae and fibroblasts as well as in other tissues. *PEPA*^{8} occurs at polymorphic frequency (about 0.25) in Europeans, but its frequency is much lower in Nigerians (about 0.08). The *PEPA*^{8} phenotypes are discussed further in what follows in connection with the quantitative variation of PEP A in red cells.

In 1970, Sinha *et al.* undertook studies of the activity of PEP A in the red cells of a number of people. The common PEP A 1 types showed a considerable variation in activity levels, which was much less pronounced in white cells. Parent-child studies of this variation suggested that the activity levels were under genetic control, and Sinha *et al.* (1970) proposed that there might be two *PEPA*^{1} alleles, which they called *PEPA*^{1S} and *PEPA*^{1W} (where the "S" and "W" indicated "strong" and "weak"). In 1971, Sinha *et al.* carried out follow-up studies on the activity variation. PEP A isozymes from the red cells of persons with "strong" and "weak" activity were partially purified, and their properties compared in the hope of finding evidence of qualitative differences in the enzymes. Both types of enzyme, however, were completely similar with respect to pH-activity profile, K_m, elution profiles from ion exchange media and thermostability. In 1973, Lewis noted in his description of *PEPA*^{8} that the products of *PEPA*^{1} and *PEPA*^{8} cannot be separated adequately by the usual electrophoretic system. He used 0.3M potassium phosphate, pH 5.9, bridge buffer with an 0.02M imidazole-citrate, pH 5.9, gel buffer system on 11% starch gels to distinguish the PEP A 8-1 and 8 phenotypes from PEP A 1. The finding of very low activity of the *PEPA*^{8} product in red cells, the fact that *PEPA*^{1} and *PEPA*^{8} products were not separated by the usual electrophoretic

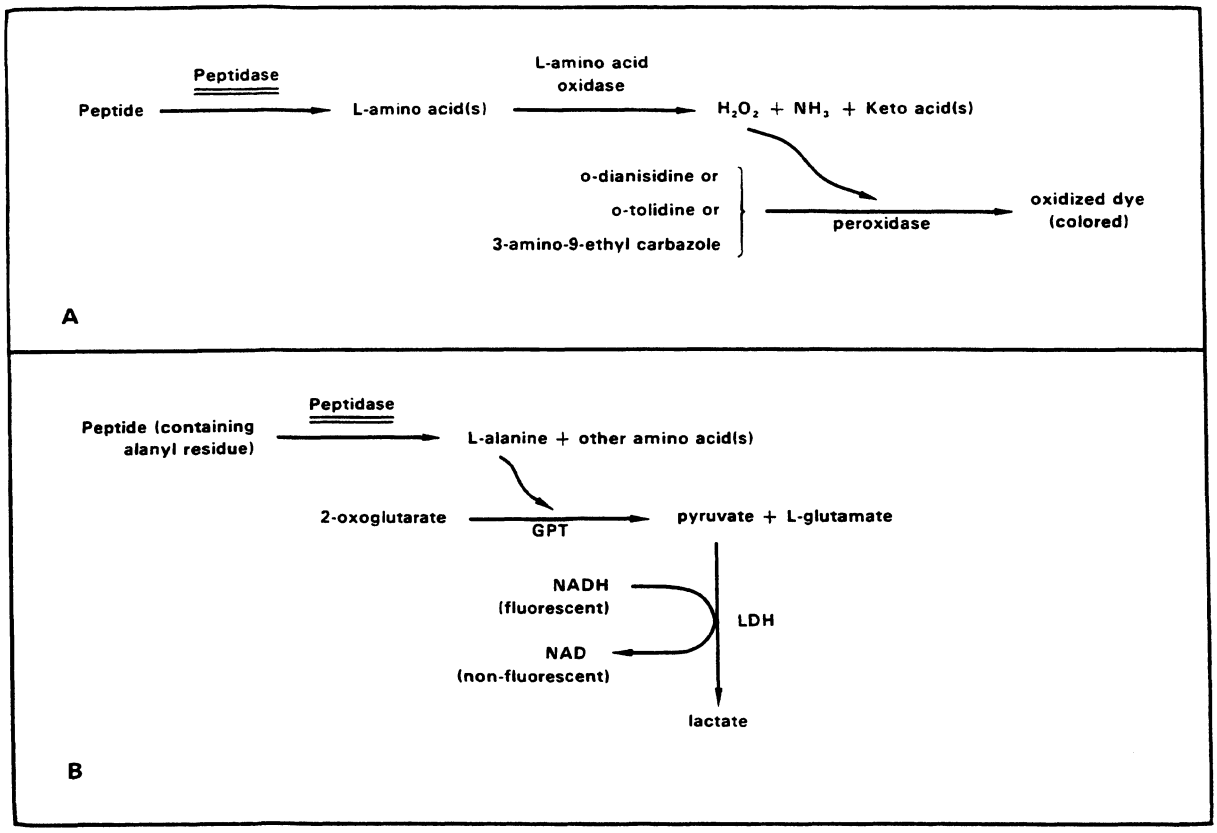


Figure 36.2 Schemes for the Detection of Peptidase Activity

system, and the finding that PEP A 8–1 occurred in appreciable frequencies in Europeans, led him to suggest that the *PEPA*^{1S} of Sinha *et al.* (1970 and 1971) was in fact *PEPA*^{1}, while the "*PEPA*^{1W}" was, in reality, *PEPA*^{1}. This notion appears to be the currently accepted one (Harris and Hopkinson, 1976). *PEPA*^{1} phenotypes are difficult to detect in red cells, because of the low activity of the *PEPA*^{1} product, and it is necessary to use a different electrophoretic system, as described by Lewis (1973), in order to get clear separations. An additional allele of PEP A, called *PEPA*^{5}, which gives rise to an unstable isoenzyme product *in vivo*, has been described by Kühnl *et al.* (1979) in a German family.

36.4.2 PEP B

All the variants of PEP B are very rare. Lewis and Harris (1967) detected four phenotypes of PEP B, and called them 1, 2–1, 3–1 and 4–1. Family studies indicated that they were the expression of four alleles at an autosomal locus, *PEPB*^{1}, *PEPB*^{2}, *PEPB*^{3} and *PEPB*^{4}. Only 9 Europeans in a survey of 2,197 people showed a phenotype other than PEP B 1. In 1970, Blake *et al.* reported four additional types, explicable on the basis of two additional rare alleles, *PEPB*^{5} and *PEPB*^{6}. Examples of PEP B 6–1, 7–1 and an apparent homozygous PEP B 6, were seen in Australian aborigines, and a PEP B 5–1 was detected in a survey of Nigerians.

36.4.3 PEP C

In 1970, Santachiara Benerecetti found genetic variation in PEP C in the Babinga pygmies of Africa. Two additional phenotypes, besides the common one, which were called 2–1 and 2, could be explained by a second allele at the *PEPC* locus, *PEPC*^{2}. The frequency of this allele was fairly low (0.014). In addition, 11 of 261 people had no detectable PEP C activity in hemolysates, and this was attributable to homozygosity for a silent allele, *PEPC*^{0}, which had a frequency in these people of about 0.21.

In 1972, Povey *et al.* described five additional phenotypes of PEP C, called 3–1, 4–1, 3, 4 and 5–1. These were accounted for by the additional alleles *PEPC*^{3}, *PEPC*^{4} and *PEPC*^{5}. *PEPC*^{5} is very rare, and *PEPC*^{3} and *PEPC*^{4} occur in Europeans at frequencies of less than 0.01. The *PEPC*^{4} product has the same electrophoretic mobility as the *PEPC*^{3} one. The *PEPC*^{4} isozyme is unstable in red cells, presumably because it is labile in the anucleate phase of red cell life, and it is almost inactive in hemolysates. In red cells, therefore, PEP C 4–1 and 1–1 are not distinguishable, and white cells or tissues must be examined for this purpose. *PEPC*^{4} had a frequency of about 0.08 in the Black people examined by Povey *et al.* (1972). *PEPC*^{4} may be identical to the "*PEPC*^{0}" of Santachiara Benercetti (1970), since the latter investigator examined only red cells.

36.4.4 PEP D

Lewis and Harris (1969a) reported three phenotypes of PEP D in addition to the usual PEP D 1. These were called PEP D 2–1, 2 and 3–1, and represented the expression of two additional alleles, *PEPD*^{2} and *PEPD*^{3}. Both of these alleles are quite rare, but have frequencies of about 0.024 and 0.021 in African Black populations (Harris and Hopkinson, 1976).

A rare disorder, apparently inherited as a recessive trait, and characterized by the absence of red cell PEP D activity, was reported by Powell *et al.* (1974). There were a considerable number of peptides (15 were found in a 24 hour sample) excreted in the urine of the subject, and all were either di- or tripeptides with C-terminal proline.

36.4.5 Linkage relations of the *PEP* loci

Family investigations by Lewis and Harris (1967) suggested that the *PEPA* and *PEPB* loci were not closely linked. It has since been shown by means of human-rodent somatic cell hybrid studies that *PEPA* is on chromosome 18 (Creagan *et al.*, 1973a), *PEPB* is on chromosome 12 (Chen *et al.*, 1973), and *PEPC* is syntenic to *PGM*$_1$ and *Rh* on chromosome 1 (Ruddle *et al.*, 1972).

36.5 Biochemical Studies on the Peptidases

The electrophoretic patterns observed with PEP A 1, 2–1 and 2 phenotypes suggested to Lewis and Harris (1967) that PEP A was a dimeric molecule, and Lewis and Harris (1969c) carried out molecular hybridization experiments with PEP A from different phenotypes to establish that this was indeed the case. The product of the rare *PEPA*^{5} allele is affected by sulfhydryl reagents in a manner suggestive of a free -SH group in the protein (Lewis *et al.*, 1968; Sinha and Hopkinson, 1969). The enzyme undergoes changes upon storage which can be mimicked by treatment with oxidized glutathione (GSSG), and which are reversed by treatment with mercaptoethanol. The electrophoretic patterns of PEP B and PEP C variants suggest that these are monomeric isozymes, while those of PEP D indicate that it, like PEP A, is dimeric.

The MW of all the peptidases have been estimated by gel filtration chromatography (Lewis and Harris, 1969b; Rapley *et al.*, 1971; Harris, 1969): PEP A, 92,000; PEP B, 55,000; PEP C, 64,400; PEP D, 100,000; and PEP S, 245,000. PEP E from all tissues except liver has a MW of 85,000, but the liver PEP E is about 177,000.

36.6 Medicolegal Applications

No reports on the application of the peptidase polymorphisms to disputed parentage investigations were found. Chakraborty *et al.* (1974) said that their calculations indicated an exclusion probability of about 7% for falsely accused Black men with the PEP A system. The PEP isozymes are fully developed in fetal blood (Chen *et al.*, 1977).

The PEP A polymorphism has been applied to bloodstain grouping, but it is not widely used. PEP A is not significantly polymorphic in Caucasians, but the *PEPA*^{2} allele occurs at polymorphic frequency in many Black populations. Culliford (1971) noted that PEP A phenotypes could be determined in bloodstains, but that the system taken alone was not very economical, because of the expense of the reagents and the low frequency of variants. The system is

apparently in routine use, at least in selected cases, in London at the present time, however (MPFSL, 1978). PEP A phenotyping is carried out on 10% thin starch gels in 0.1M Tris-phosphate bridge buffer, pH 7.4, and 0.01M Tris-maleate gel buffer, pH 7.5. This procedure is also described by Parkin (1978). The enzyme is detected using the scheme shown in Figure 36.2 A, with aminoethylcarbazole.

In 1976, Neilson *et al.* described an electrophoretic procedure in which PEP A phenotyping was combined with that of PGM and AK on the same electrophoretic gel. The original PGM buffers, described by Spencer *et al.* (1964b) were employed, with 10% thin starch gels, except that the EDTA was omitted in the bridge buffer, and combined instead with the PGM reaction buffer, and the bridge buffer was diluted 1:10 to make gel buffer. The procedure was applicable to bloodstain typing.

The data of Blake (1976) and Blake and Sensabaugh (1976) clearly show that PEP A is expressed in seminal plasma and in spermatozoa. The majority of the activity occurs in the seminal plasma rather than in the cells, and PEP A is unusual in that semen contains considerably more of it than a comparable volume of blood (some 25 times more) (Blake and Sensabaugh, 1978). Accordingly, quantity is not a limiting factor in seminal PEP A typing. The only variant type that is comparatively well distributed and can be detected relatively easily by electrophoresis, however, is largely limited to Black populations. Development of a reliable procedure for the detection of $PEPA^{a}$ (or $PEPA^{1W}$) would make the system a useful marker in White populations as well. Parkin (1979) said that PEP A can be typed in semen, but that vaginal swabs containing semen are typable only if they were taken within a few hours after intercourse.

Neilson *et al.* (1976) reported that 157 Black Americans, from the greater Pittsburgh area, had been phenotyped for PEP A with the following results: 139(88.5%) 1, 13(8.3%) 2-1, 4(2.5%) 2 and 1(0.6%) 3-1. The gene frequencies for this population would thus be $PEPA^{1} = 0.93$ and $PEPA^{2} = 0.07$. Grunbaum *et al.* (1980) tested 301 Whites and 492 Blacks from California, 766 Mexicans from California and Mexico, and 108 Asians from California and Hawaii. 300 of the Whites were PEPA 1 (1 was rare) and all but 5 of the Mexicans were PEPA 1 (3 were 2-1; 2 were rare). The Asians were all PEPA 1. Among the Blacks were 442(89.8%) 1, 41 (8.3%) 2-1, 5(1%) 2 and 4(0.8%) rare, and $PEPA^{1} = 0.948$, $PEPA^{2} = 0.052$. The patterns of the commoner PEP A types seen in Black populations are shown in Figure 36.3.

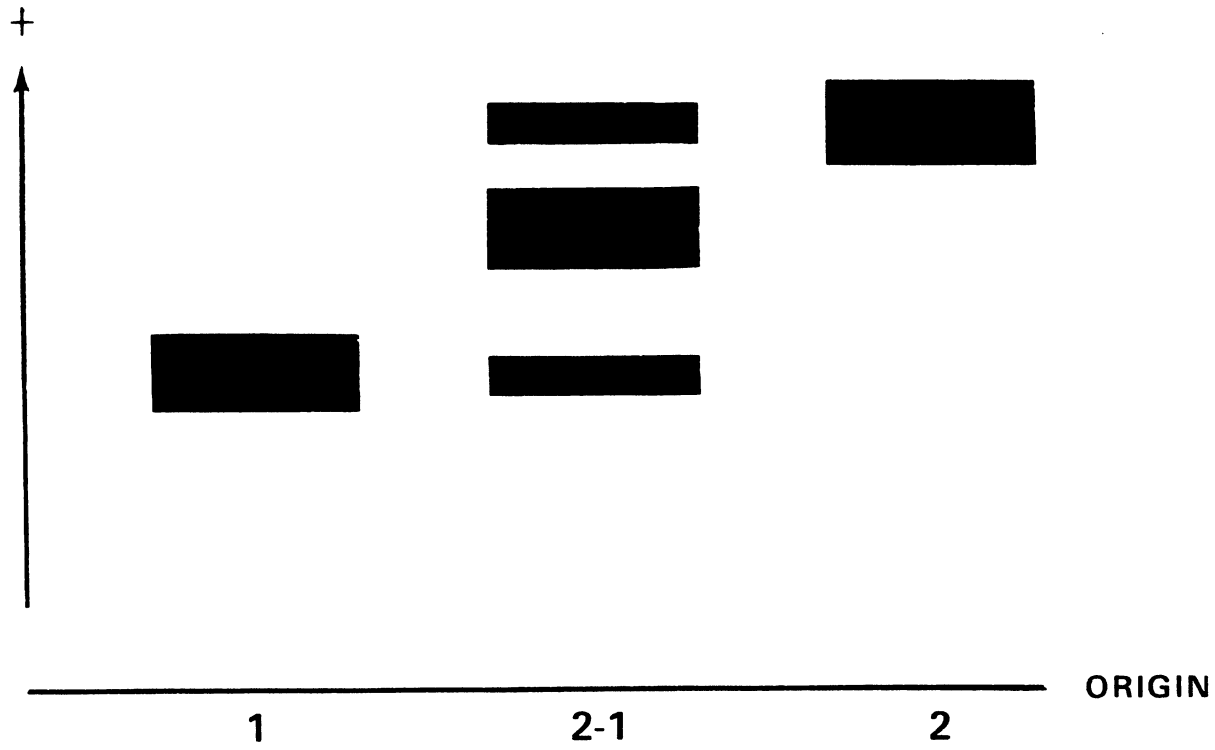


Figure 36.3 Electrophoretic Patterns of *PEPA* Phenotypes in Black Populations

SECTION 37. OTHER ISOENZYME SYSTEMS

37.1 Introduction

There are a considerable number of human enzymes, besides those discussed in sections 27 through 36, which exhibit genetically determined multiple molecular forms. A number of these are not expressed either in blood or in body fluids of the kinds encountered in criminal investigations, nor in tissues or fluids which would be accessible in disputed parentage cases. Accordingly, they are not useful genetic markers from a medicolegal point of view. Some of these isozymes are expressed in blood, however. In certain cases, procedures for their routine use in medicolegal cases have not been worked out. In other cases, the genetic variation does not occur with a high enough frequency in populations to make them worth the investment of time and material required for their phenotyping. A number of these additional systems are discussed briefly in this section.

37.2 Alkaline Phosphatase [ALP; orthophosphoric-monoester phosphohydrolase (alkaline optimum); E.C. 3.1.3.1]

37.2.1 Alkaline phosphatases of serum and tissues

A number of different alkaline phosphatases have been described in human serum and tissues. These enzymes have been of interest to clinical chemists and biochemists for years. There is a very large literature on the subject, which will not be developed in detail here. There are a number of excellent reviews which may be consulted for details. The material up to 1959 was reviewed by Gutman (1959), one of the pioneers in the field. More recently, Posen (1967), Fishman and Ghosh (1967) and Fishman (1974) have reviewed the subject.

Several different organs are now known to contribute to the alkaline phosphatase activity of serum, notably bone, liver, intestine and placenta. These different isoenzymes are distinguishable from one another on the basis of a combination of properties, including electrophoretic mobility, inhibition by specific inhibitors such as L-phenylalanine and L-homoarginine, immunological cross reactivity, and the effect of treatment with neuraminidase. All the evidence is consistent with these forms being under the control of different genetic loci. Following Harris and Hopkinson (1976), the liver, bone and intestinal enzymes are designated ALP_L, ALP_B and ALP_I, respectively. These isozymes have not been reported to exhibit genetic variation. From a medicolegal standpoint, therefore, they do not serve as genetic markers. There has been reported a rare familial type of low serum ALP activity (hypophosphatasia), which may be accompanied by widely varying degrees of skeletal abnormalities in affected individuals. The condition is inherited as an autosomal recessive, and is also characterized by the excretion of phosphoethanolamine in urine. It is apparently the skeletal, or bone, isoenzyme (ALP_B) that is deficient in this syndrome, because levels of intestinal enzyme (ALP_I) are normal (Rathbun *et al.*, 1961; Danovitch *et al.*, 1968; Warshaw *et al.*, 1971).

Efforts have been made to utilize the concentration of alkaline phosphatase in body fluids as a tissue marker, especially in the case of saliva. There is no doubt that saliva exhibits ALP activity (Giri, 1936; Chauncey *et al.*, 1954; Levitskii *et al.*, 1973; Lindqvist *et al.*, 1974; Pini Prato, 1970). This matter has been discussed in section 11.2. The placental alkaline phosphatase has been shown to exhibit genetic polymorphism (section 37.2.2). This enzyme is designated "PL" (Harris and Hopkinson, 1976). The placental enzyme is responsible for an elevation of serum ALP activity during pregnancy (Fishman *et al.*, 1972), and this fact has been used as the basis of medicolegal identification tests for the diagnosis of blood from pregnant women (Oya *et al.*, 1973; Stafunsky and Oepen, 1977; and see in section 8.2.4).

37.2.2 Placental alkaline phosphatase (PL)

In 1961, Boyer observed several different electrophoretically distinguishable phenotypes of the alkaline phosphatase from placentae. In 1965, Robson and Harris established that there were six relatively common phenotypes, distinguishable by electrophoresis provided the sample were run at both pH 8.6 and pH 6. At either of these pH values alone, certain of the phenotypes are indistinguishable from one another. The phenotypes were originaly called S, FS, SI, I, FI and F, and the designations referred to "slow", "intermediate" and "fast" electrophoretic mobilities. In 1967, further variants were discovered by Robson and Harris, and the nomenclature was revised to include numerical subscripts to distinguish between certain types, e.g. F_1S_1 and F_1S_2. An extensive study by Donald and Robson (1974) revealed a large number of rare variants. They described 44 phenotypes in all, and these could be accounted for by a series of 18 separate alleles at the *PL* locus, all except three of which are rare in all populations studied. The nomenclature was revised by Donald and Robson (1974) to a numerical system, the three common alleles being designated PL^1, PL^2 and PL^3. The common phenotypes, PL 1, 2-1, 2, 3-1, 3-2 and 3 correspond to the older designations S_1, F_1S_1, F_1, S_1I_1, F_1I_1 and I_1, and to the still older designations S, FS, F, SI, FI and I, respectively. The remainder of the *PL* alleles have been designated PL^4 through PL^{18}. The expression of placental alkaline phosphatase is controlled by the genotype of the fetus, and not of the mother. It has been

suggested (Gladkikh, 1976) that this interesting polymorphism might find medicolegal applications in certain types of cases.

37.2.3 Other notes on alkaline phosphatases

The placental enzyme has been studied by many workers, including Beckman and Beckman (1968), Beckman (1970), Posen *et al.* (1969) and Beratis and Hirschhorn (1972). The enzyme has also been purified and characterized in comparison with the ALP from other tissues (Ghosh, 1969). There is an established relationship between the level of intestinal ALP and the ABO type and secretor status of individuals (Beckman and Beckman, 1970). This enzyme is rarely found in the serum of nonsecretors, and among secretors, the highest levels are seen in persons of group A and O. There is no such relationship between ABO group, secretor status and the placental enzyme. The function of the alkaline phosphatases *in vivo* is not really known. The ALP isozymes from the different tissues may become elevated in serum in certain disease states involving the tissue of origin, and this has been a subject of great clinical interest because of the obvious diagnostic implications. There has been great interest, too, in the finding that a placental-like ALP isozyme occurred in the serum of a male cancer patient. This isoenzyme has been called the "Regan isoenzyme" after its first known possessor (Fishman *et al.*, 1968a and 1968b; Fishman, 1969). Other ALP isozymes resembling, or having properties in common with, placental alkaline phosphatase have been found in the sera of other cancer patients as well. Fishman (1974) regarded the issue of the identity of the placental isozyme and that which occurs in the serum of cancer patients as settled, and, in fact, uses the term "carcinoplacental isoenzyme antigen" to describe them because of their immunological identity. Beckman (1978), however, did not seem to regard the identity of the Regan isoenzyme and placental alkaline phosphatase as proven.

37.3 α-Amylase [AMY; diastase; ptyalin; 1,4-α-D-glucan glucanohydrolase; E.C. 3.2.1.1]

37.3.1 Activity and occurrence of α-Amylase

α-Amylase catalyzes the endohydrolysis of 1→4 α-glycosidic linkages in starch, glycogen and related polysaccharides containing three or more sugar residues, in a random manner. Amylase is one of the oldest known enzymes, its activity in pancreatic extracts having been noted and measured by Roberts in 1881. Amylases are primarily of two types, pancreatic and salivary, and the amylase activity of serum and urine is believed to be primarily pancreatic in origin. Skude (1977) gave a thorough discussion of the amylases in human serum. The isoenzymes which occur in serum can give clinical indications (Warshaw, 1977). Salivary amylase is the basis of several identification tests for the presence of saliva (see section 11.3).

37.3.2 Genetics of salivary and pancreatic amylases (AMY_1 and AMY_2)

It has been known for some time that both salivary and pancreatic amylases exhibit multiple molecular forms (McGeachin and Reynolds, 1961; Ogita, 1966; McGeachin, 1968). In 1965, Kamarýt and Laxova reported observations on an unusual band pattern in the serum amylases, and studies on the family indicated that the pattern was inherited. Further variants, which appeared to be segregating in families, were reported the following year (Kamarýt and Laxova, 1966). These workers used agar gel electrophoresis for the separaton of the isoenzymes, and the serum patterns were believed to be the result of both salivary and pancreatic contributions to amylase activity. Starch gels cannot be used for amylase electrophoresis for obvious reasons, and most workers have employed either agar or polyacrylamide gels for the purpose. The polyacrylamide gels appear to give better resolution. Vacikova and Blochova (1969) confirmed the finding of a number of variant amylase patterns by agar gel electrophoresis.

In 1969, a horizontal polyacrylamide gel electrophoretic procedure for amylases was described by Boettcher and De La Lande (1969b). Using the technique, they observed patterns similar to those which had been seen by Ogita (1966). Studies on salivary (Boettcher and De La Lande, 1969a) and serum (Boettcher and De La Lande, 1971) amylase isoenzymes revealed several variant types, which appeared to be inherited. They also concluded from their studies that salivary amylases do not occur in serum, and that the isozymes of serum originate in the pancreas. This view was reinforced by observations on a person with very unusual salivary and serum isoamylase characteristics (Boettcher and De La Lande, 1971).

In 1971, Ward *et al.* described a polyacrylamide gel electrophoretic system which, they thought, gave very good resolution of salivary isoamylases. Six to eight bands were observed in specimens from 700 people, and three variant types could be seen. These variant types were shown by family studies to be inherited. At the time, the variants were designated according to the surnames of the people in whom they were observed. In 1973, this group of workers extended their studies of amylase isozymes (Merritt *et al.*, 1973a). Electrophoretic studies were carried out on salivary and pancreatic enzymes in saliva, serum and urine in a number of persons from ethnically distinct populations. Agar gel techniques were compared with the polyacrylamide gel technique. The isozymes of salivary origin were designated "Sa 1" through "Sa 6", in order from origin to anode. Similarly, pancreatic isozymes were denoted "Pa 1" through "Pa 4". A total of 7 salivary phenotypes could be distinguished, each on the basis of an additional isozyme band closer to the origin than (cathodal to) Sa 1, and these were given capital letter designations. In a similar way, three pancreatic amylase phenotypes could be distinguished. The patterns are shown in Figure 37.1. The pedigree data indicated that the amylase isozymes are under the control of two sepa-

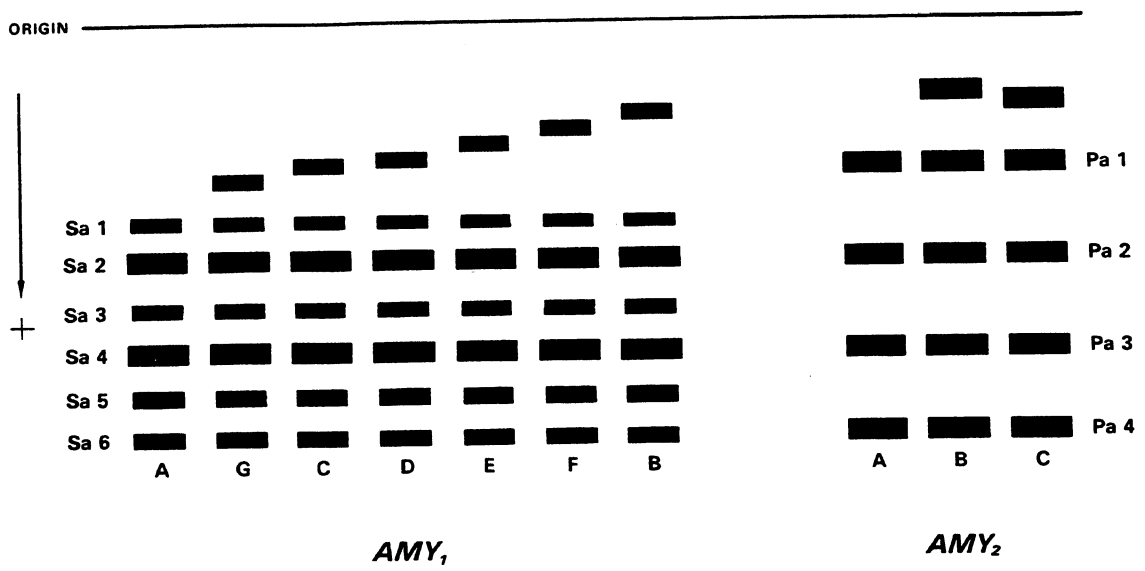


Figure 37.1 Electrophoretic Patterns of *AMY*$_1$ and *AMY*$_2$ Phenotypes following Vertical Polyacrylamide Gel Electrophoresis (after Merritt et al., 1973a)

rate genetic loci, called *AMY*$_1$ and *AMY*$_2$. *AMY*$_1$ codes for the salivary amylases, while *AMY*$_2$ codes for the pancreatic ones. *AMY*$_1$ isozymes are not very strongly expressed, nor very easily detectable in serum or in urine. The readily detectable amylase isozymes in these fluids are products of *AMY*$_2$, and correspond to pancreatic enzyme. In very concentrated specimens, minor components with the mobility of Sa 2 and Sa 4 isozymes of *AMY*$_1$ could be observed. The variant phenotypes could be explained most readily as the expression of heterozygosity of a variant allele with the common allele at the locus. The ordinary (common) alleles are designated *AMY*$_1^A$ and *AMY*$_2^A$. The *AMY*$_1$ variant alleles are designated *AMY*$_1^B$ through *AMY*$_1^G$. *AMY*$_2$ shows a variant type relatively common in Caucasians, and this was called *AMY*$_2^B$, while the other variant, seen only in Blacks, was called *AMY*$_2^C$.

Population studies were conducted on a number of White and Black people in the United States, and on a limited number of Orientals and Nigerians. *AMY*$_1$ variants are uncommon in Caucasians. Only 7 variant phenotypes were seen in 961 people. In Black Americans, however, about 5.3% of 208 people were AMY$_1$ E, and there were several other variants as well. 2 out of 10 Nigerians were AMY$_1$ E. The *AMY*$_1^E$ allele was not observed in Whites. *AMY*$_1^F$ was detected in a Chinese individual. As to *AMY*$_2$ variants, Caucasians show a frequency of about 10% for AMY$_2$ B. Black Americans showed AMY$_2$ B less frequently, but nearly 5% were AMY$_2$ C, a type not seen in Caucasians. These workers recommended that saliva be utilized for AMY$_1$ typing, and that serum or urine be used only for AMY$_2$ typing. It may be noted that *AMY*$_2$ is expressed in seminal plasma (Blake and Sensabaugh, 1976 and 1978), a finding which might find medicolegal applications in time.

The data of Merritt *et al.* (1973a) were consistent with their previous findings (Merritt *et al.*, 1972) that *AMY*$_1$ and *AMY*$_2$ are closely linked. This linkage was not apparent to the Czechoslovakian workers (Kamarýt *et al.*, 1971), but a probable linkage of *AMY*$_1$ with a chromosome 1 marker was noted. Merritt *et al.* (1973b) established that the closely linked *AMY* loci are indeed linked to *Fy* (Duffy blood group locus), thereby assigning *AMY*$_1$ and *AMY*$_2$ to the first chromosome.

37.3.3 Biochemical studies on the amylase isoenzymes

The amylase isozymes of saliva were resolved by polyacrylamide disc gel electrophoresis into 5 to 7 bands by Kauffman *et al.* (1970). Further characterization of the isoenzymes on gel filtration media indicated that they could be arranged into two "families" on the basis of MW. The "A" family consisted of the "odd" bands, 1, 3 and 5, while the "B" family contained 2 and 4, and two forms designated "z". These relationships were found in purified, crystalline preparations of human parotid gland amylase as well (Keller *et al.*, 1971). The "A" family isozymes had MW values of about 62,000, and were glycoproteins (the carbohydrate moiety consisting of 6 moles $GlcNH_2$, 3 moles Fuc, 2 moles Man, and 2 moles Gal per mole glycoenzyme). The "B" family isozymes contained no carbohydrate, and had MW about 56,000. Incubation of the isozymes at pH 9 gave rise to more anionic forms, and the transformation appeared to involve a deamidation reaction. The "A" and "B" family designations of Keller and her colleagues should not be confused with the electrophoretic phenotype designations used by Merritt and his collaborators. The latter group designate the "families" of isozymes as "odds" and "evens". Stiefel

and Keller (1973) purified the pancreatic amylase, and showed that the purified preparation exhibited up to 6 isozymes on polyacrylamide gels. The properties of the enzyme were very similar to but not completely identical with those of the salivary enzymes.

There is good biochemical and immunochemical evidence from the studies of Karn *et al.* (1973, 1974 and 1975) that the complex isozyme patterns exhibited by both AMY_1 and AMY_2 loci can be explained on the basis of post-translational modification of primary gene products. Karn *et al.* (1973) isolated an enzyme from the oral bacterial flora which could convert "odds" to "evens", and it appeared that this "salivary enzyme modifier" protein was responsible for the removal of carbohydrate from the "odd" isozymes, thus yielding the "even" group. The evidence for this and the other studies indicate that some of the primary salivary amylase (AMY_1 locus) product undergoes glycosidation. This modification is followed by deamidation of both glycosylated and nonglycosylated enzyme, and eventually, in whole saliva, glycosylated forms may be deglycosylated. The pancreatic enzyme appears to undergo only deamidation in the course of its post-translational modification. The pancreatic and salivary enzymes are immunologically identical, and all the genetic evidence is consistent with their production by separate, but closely linked, loci, although no crossovers were observed. The most tempting explanation for this situation is that a gene duplication occurred at some point in the evolutionary history of the original *AMY* locus.

37.4 Superoxide Dismutase

Superoxide dismutase [SOD; indophenol oxidase; tetrazolium oxidase; "white patch enzyme"; superoxide:superoxide oxidoreductase; E.C. 1.15.1.1] catalyzes the dismutation of O_2 radicals to yield H_2O_2 and O_2. The enzyme has had a variety of names over the years. Brewer, in 1967, discovered genetic variation in SOD. In the course of staining hundreds of starch gels for various isozymes with PMS-MTT containing mixtures, clear bands in a bluish background had been noted consistently. These "achromatic regions" had always given a similar pattern, until a variable pattern was observed in a single sample. This observation prompted further study, and the protein responsible for the "achromatic regions" turned out to have indophenol oxidase activity. In addition, the "variant" pattern observed turned out to have been inherited.

SOD can be detected in a variety of ways, as Brewer (1967) demonstrated. Assay techniques were elaborated upon by Beauchamp and Fridovich (1971). Most workers put in MTT and PMS and allow the gel to be exposed to light. Sites of SOD activity are clear zones on a blue-purple formazan background. NBT can be used in place of MTT (Beauchamp and Fridovich, 1971). The enzyme also shows a DCPIP oxidase activity in the presence of NADH (Brewer, 1967).

In 1973, Beckman found that certain northern Swedish and some Finnish people showed the variant described by Brewer (1967) in polymorphic frequency. He called the enzyme "superoxide dismutase", and designated the common phenotype as "SOD 1". The variant, seen by Brewer (1967) and relatively frequent (3–4%) in the northern Swedish and Finnish populations, was SOD 2-1. These phenotypes are the result of a pair of alleles, SOD^1 and SOD^2, at an autosomal locus. An example of the rare SOD 2 phenotype has been reported (Beckman *et al.*, 1973a). In 1973, Beckman and his collaborators investigated the tissue distribution and genetic control of human SOD isozymes (Beckman *et al.*, 1973b). All tissues studied had two isozymes, A and B, except red cells and polymorphonuclear leucocytes. The SOD A is a soluble isozyme, and the SOD B originates in the mitochondria. The two forms are controlled by separate genetic loci, SOD_A and SOD_B. No genetic variation has been reported at the SOD_B locus. Red cells lack SOD B, while polymorphonuclear leucocytes lack SOD A. Briggs and Fee (1978) have described the purification and properties of red cell SOD.

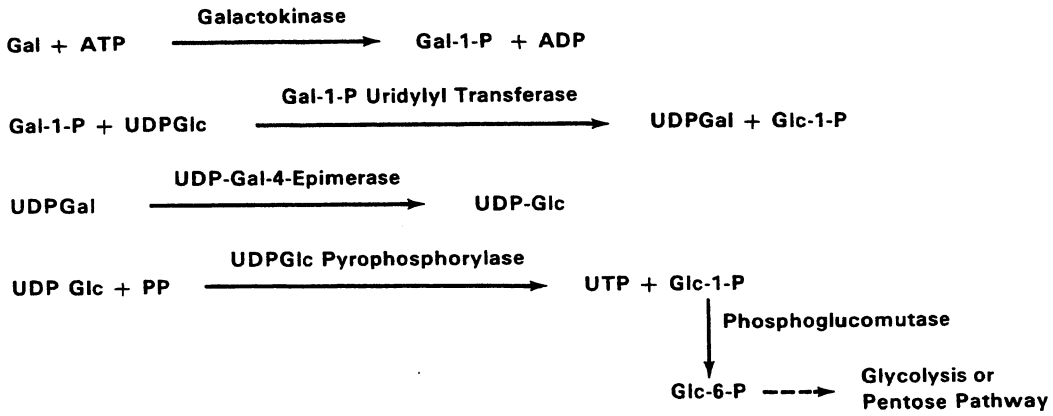
The polymorphism of SOD A does not appear to be very widespread. The only populations with significant frequencies of $SOD_A{}^2$ are the Finnish and northern Swedish (Beckman and Pakarinen, 1973) and the people of Westray (Orkney) (Welch, 1973). No evidence of $SOD_A{}^2$ was seen in Nigerians, nor in Chinese, Japanese, Polynesians and Filipinos in Hawaii (Beckman and Pakarinen, 1973). South African Bantu were all SOD 1 as well (Kirk *et al.*, 1971). There is one report of SOD^2 in three generations of a Japanese family (Shinoda, 1970), and Teng and Lie-Injo (1977) reported a variant in one Filipino in an Asian population survey.

Beckman and Beckman (1975) have reviewed the genetics and function of superoxide dismutase. The SOD_A locus has been assigned to chromosome 21 (Tan *et al.*, 1973) and the SOD_B locus to chromosome 6 (Creagan *et al.*, 1973b; Van Someren *et al.*, 1974).

37.5 Galactose-1-Phosphate Uridylyl Transferase

37.5.1 Metabolic role of the enzyme

Galactose-1-phosphate uridylyl transferase [GALT; uridyl transferase; Gal-1-PUT; hexose-1-phosphate uridylyl transferase; UDP galactose:α-D-galactose-1-phosphate uridylyl transferase; E.C. 2.7.7.12] is one of the key enzymes in the major pathway of galactose metabolism, the net result of which is the conversion of galactose to glucose-1-phosphate. The essential features of this pathway are indicated in Figure 37.2. These reactions were first recognized in microorganisms by Leloir and his collaborators and by Kalckar and his collaborators (Leloir, 1951; Munch Petersen *et al.*, 1953; Kalckar *et al.*, 1953), and the pathway was subsequently found to be operative in mammals as well. Existence of GALT was predicted by Leloir in 1951, and experimentally established by Kalckar *et al.* (1953). Lactose, or milk sugar, is a principal source of galactose in the diet. Lactose is a disaccharide consisting of galactose and glucose residues. Conversion of galactose to



Abbreviations: Gal = Galactose Gal-1-P = Galctose-1-Phosphate
UDPGlc = Uridine Diphosphoglucose UDPGal = Uridine Diphosphogalactose
Glc-1-P = Glucose-1-Phosphate PP = Pyrophosphate Glc-6-P = Glucose-6-Phosphate
ATP = Adenosine Triphosphate ADP = Adenosine Diphosphate UTP = Uridine Triphosphate

Figure 37.2 Principal Reactions of Galactose Metabolism

glucose-1-phosphate allows galactose to be metabolized by way of the glycolytic or pentose phosphate pathways (Figures 37.2 and 33.1).

37.5.2 Genetic variation of GALT

37.5.2.1 Galactosemia. The first kind of genetic variation described for GALT was a deficiency syndrome, known as galactosemia. The condition is a classical "inborn error of metabolism" (see section 1.2.2.1). The existence of galactosemia has been recognized for many years. In 1935, Mason and Turner described extensive studies on a patient affected with it, and they recognized that the normal metabolic pathway of galactose metabolism was affected in some way, and that withholding galactose from the diet was effective in reducing the severity of the clinical consequences. It was not until 1956, however, that Isselbacher *et al.* demonstrated conclusively that the condition represented a deficiency of GALT. Galactosemia is inherited as an autosomal recessive characteristic, and it is characterized by cirrhosis, cataract, failure to grow and develop normally, and mental retardation. The clinical consequences are the result of toxicity of galactose or of one of its metabolites. Although the exact nature of the pathogenesis is not fully clear, accumulation of galactitol and galactose-1-phosphate have been implicated as being responsible. The clinical and genetic aspects of galactosemia have been well reviewed by Segal (1978). The gene for galactosemia is now designated $GALT^{G}$ and the common allele as $GALT^{A}$. In the past, it has been designated *gt* or Gt^{G}, while the common allele was designated Gt^{+}.

37.5.2.2 Further genetic variation of GALT. In 1965, Beutler *et al.* found another genetic variant of GALT. This variant was detected as the result of studies on screening tests for galactosemia heterozygotes. In classical galactosemia, since it is recessive, homozygotes are afflicted with the condition. The heterozygotes are asymptomatic, but their red cell GALT activity is on the order of one-half that normally seen. Beutler *et al.* (1965a) encountered some people in their family studies who had enzyme activity levels significantly higher than expected for heterozygous carriers of galactosemia, and yet significantly lower than that seen in the general population. These activity levels were inherited, and the phenotype was described as the "Duarte variant". The gene responsible was designated Gt^{D}, and it is now usually called $GALT^{D}$. It has sometimes been called Gt^{2}, where Gt^{1} then denotes the normal allele (Kühnl *et al.*, 1974b). It remained to be established that the Duarte variant gene was an allele of the galactosemia gene. An informative family, in which both genes were segregating, served to establish the allelic relationship between $GALT^{C}$ and $GALT^{D}$ (Beutler *et al.*, 1966a). The $GALT^{D}$ allele gives rise to a lower than usual activity form of Gal-1-P uridylyl transferase, and Duarte heterozygotes ($GALT^{A}GALT^{D}$) exhibit about 75% of the usual enzyme activity. Duarte homozygotes ($GALT^{D}GALT^{D}$) exhibit about 50% activity levels. Heterozygotes for galactosemia ($GALT^{A}GALT^{G}$) also exhibit about 50% normal activity levels, whereas galatosemics ($GALT^{G}GALT^{G}$) have no activity. People heterozygous for both galactosemia and Duarte genes ($GALT^{D}GALT^{G}$) have about 25% normal levels of enzyme activity. Duarte variants are healthy, and the enzyme they make is similar to many of its properties to the normal one (Beutler *et al.*, 1966b).

In 1965, Beutler and his collaborators found that the

Duarte variant GALT was electrophoretically distinguishable from the normal enzyme on starch gels, exhibiting a slightly greater anodic mobility (Beutler *et al.*, 1965c; Mathai and Beutler, 1966). This finding enabled a number of workers to confirm the genetic hypothesis by means of population and family studies (Bissbort and Kömpf, 1973b; Kühnl *et al.*, 1974; Martin and Kienzler, 1975). Improvements in the electrophoretic and staining procedures were also forthcoming (Bissbort and Kömpf, 1973a; Ng *et al.*, 1969).

In 1973, Ng *et al.* reported another variant of GALT, which was called the "Los Angeles" variant. This phenotype was characterized by normal to higher than normal enzyme activity, and an electrophoretic pattern very similar to that of the Duarte variant. The differences in intensities of the triplet band patterns were sufficient to distinguish the two variants (see in Figure 37.3), but Ng *et al.* (1973) said that "Los Angeles" homozygotes were difficult to distinguish from "Los Angeles/Duarte" heterozygotes by electrophoresis. Sparkes *et al.* (1977) reported an improved electrophoretic procedure which, they said, made it easier to distinguish 'Los Angeles' and 'Duarte' variants.

A rare variant of GALT, designated the 'Berne' variant, was described in a Swiss population by Scherz *et al.* (1976). Only three people with this phenotype were seen in a survey of 1,668 persons.

Two other variants of GALT have been reported, both exceedingly rare. In 1969, Schapira and Kaplan described two sibs in a French family who suffered from clinical galactosemia, but who exhibited an incomplete enzyme deficiency. Electrophoresis revealed an enzyme with a slower anodal mobility than normal GALT, and thus, apparently, a structurally different form. This variant was called "Rennes". The structurally different enzyme could also be demonstrated in cultured fibroblasts (Hammersen *et al.*, 1975). In 1971, Chacko *et al.* found an unstable form of GALT in a patient with clinical galactosemia. In fresh material, the enzyme was about 40% as active as normal enzyme, but it lost activity rapidly upon storage. It had an electrophoretic mobility slower than normal GALT, but was different from 'Rennes', and was called the "Indiana" variant. Family studies indicated that the proband in this study was heterozygous for the 'Indiana' variant allele and the classical galactosemia allele ($GALT^G$).

37.5.3 Population studies on the GALT variants—A further type of galactosemia and a GALK variant

There is, and has been, considerable clinical interest in galactosemia syndromes. It is important to detect the condition in newborns as soon as possible, so that treatment can be initiated; further, from a genetic counseling point of view, it would be desirable to be able to detect heterozygotes. Screening and population studies on GALT have often been carried out with these objectives in mind.

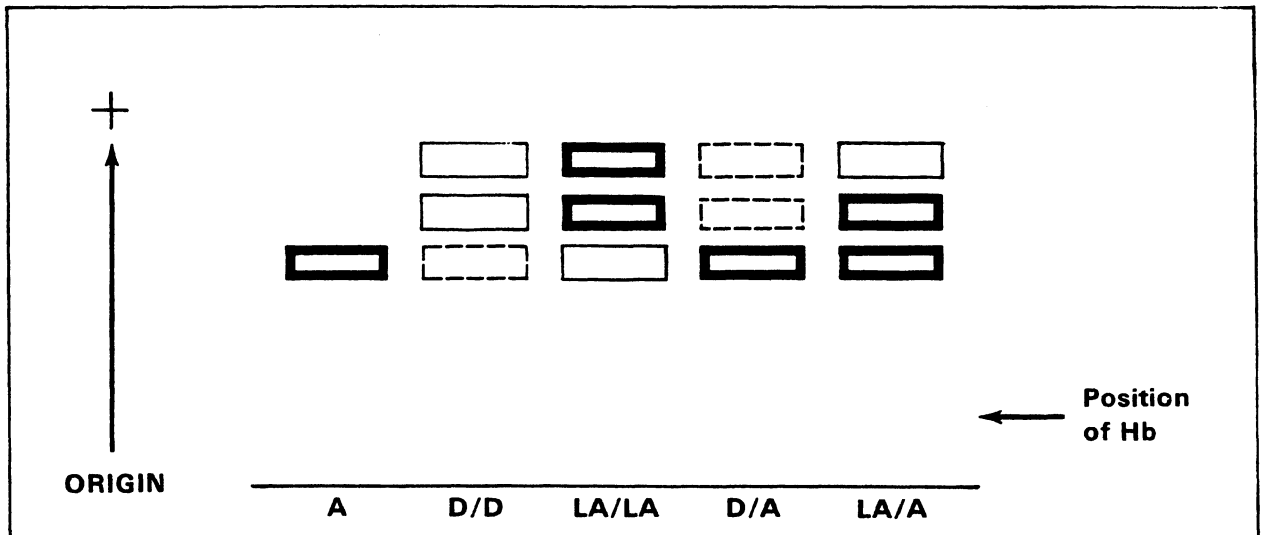


Figure 37.3 Electrophoretic Patterns of GALT Variants (after Ng et al., 1973)

Beutler (1969b) has reviewed the assay techniques for GALT used for screening hemolysates for clinically significant GALT deficiency syndromes. Until the recognition of the Duarte variant, screening tests based on activity determinations gave misleading results. Duarte homozygotes show about 50% normal activity levels, while heterozygotes show about 75%. Likewise, heterozygotes for galactosemia show about 50% activity. Duarte homozygotes are indistinguishable from galactosemia heterozygotes, therefore, on the basis of enzyme assay tests alone. The Duarte allele occurs with a much higher frequency in many populations than the galactosemia gene, and estimates of the frequency of $GALT^{G}$ must be corrected for the $GALT^{D}$ in the gene pool. The Duarte allele is relatively common, some 8% to 15% of the population being heterozygous for it (Beutler *et al.*, 1965b; Mellman *et al.*, 1968; Martin and Kienzler, 1975). The 'Los Angeles' variant is less frequent, occurring in about 4% to 5% of the population (Ng *et al.*, 1973). The galactosemia gene ($GALT^{G}$) occurs with a frequency of about 0.002 to 0.005. It is thus expected that about 0.4% to 1.0% of the population would be heterozygous for galactosemia, and that galactosemia should occur in about 1/40,000 to 1/250,000 people. In actual fact, the incidence of galactosemia has been recorded as 1/70,000 in England, 1/35,000 in New York State and 1/190,000 in Massachusetts (Segal, 1978). This material has been reviewed by Beutler and Mathai (1968).

Gitzelmann (1965 and 1967) described several patients with galactokinase (GALK) deficiency, which had been inherited (see in Figure 37.2). These patients were older, and the principal clinical manifestation of the syndrome seemed to be the development of cataracts, even in the earliest years. The association of this syndrome with cataracts seems established, but relationships to other abnormalities are not very clear. In 1975, Tedesco *et al.* conducted a survey of 1,700 pregnant women in the Philadelphia area for GALT and GALK activities. A variant of GALK, which leads to lower activity of the enzyme, was found and named the 'Philadelphia' variant. It was relatively common in Black, but rare in White people. The responsible allele was designated $GALK^{P}$. Segal (1978) suggested that classical galactosemia be called "transferase deficiency galactosemia", and that the syndrome described by Gitzelmann (1965 and 1967) be called "galactokinase deficiency galactosemia".

37.5.4 Other notes on GALT

Tedesco (1972) purified the GALT from liver and red cells, and compared the properties of normal, Duarte and galactosemia gene products. They were all similar, and had MW about 90,000. Sodium dodecyl sulfate polyacrylamide gel electrophoresis suggested that the enzyme might be a trimer. All three showed immunological identity with a liver anti-GALT antibody. The antibody could also be used to demonstrate that galactosemic individuals make a protein which is immunologically indistinguishable from the normal one, but catalytically inactive. Marcus *et al.* (1977) carried out biochemical studies on the reaction mechanism with purified human red cell enzyme. Williams (1978) purified red cell GALT to a higher degree. The enzyme had a MW of 67,000 as estimated by Sephadex chromatography, but of 88,000 by ultracentrifuge studies. It consists of two similar subunits with MW 44,000.

No reports on the typing of GALT in bloodstains were found in the literature. Hammersen and Levy (1977) showed, however, that blood dried on filter paper could be used for activity and electrophoretic screening measurements. The enzyme is thus stable, at least in freshly dried blood. The Duarte variant occurs with a high enough frequency to make GALT a useful genetic marker, if suitable procedures were available for typing the enzyme in bloodstains.

Eriksen and Dissing (1980) have recently described the application of the GALT system to disputed parentage cases. They denoted the GALT genes $GALT^{1}$ (usual; normal) and $GALT^{2}$ (representing both $GALT^{D}$ and $GALT^{\text{Los Angeles}}$). Treating the system in this way resulted in a probability of exclusion for a true non-father of about 6.6%, based on gene frequencies determined in over 2,000 Danish people. On apparent mother-child incompatibility in the studies was most easily explained by the presence of an inactive allele at *GALT*.

37.6 Glucose Phosphate Isomerase

Glucose phosphate isomerase (GPI; glucose phosphate isomerase; phosphohexose isomerase; D-glucose-6-phosphate ketol-isomerase; E.C. 5.3.1.9) catalyzes the reversible interconversion of D-glucose-6-phosphate and D-fructose-6-phosphate. The enzyme has been shown to exhibit genetic variation, but the variants are all extremely rare. The enzyme survives well in dried blood, and can be typed in bloodstains (Culliford, 1971). It is a very poor genetic marker, however, because variants are observed so seldom.

In 1968, genetic variation in GPI was reported almost simultaneously by Fitch *et al.* and by Detter *et al.* Fitch *et al.* (1968) found five different phenotypes in a survey of 1,650 people. Detter *et al.* (1968) surveyed 3,397 people, and identified 11 phenotypes, which could be accounted for by 10 alleles at an autosomal locus. These are now usually called GPI^{1} through GPI^{10}, and GPI 1-1 is by far the most common type. Only 20 people of the 3,397 surveyed were other than GPI 1-1. Welch (1971) said that eight variants had been seen in 3,300 bloods, and this total probably included the data of Fitch *et al.* (1968). Blake and Omoto (1972) identified nine GPI variants in a survey of 4,000 people from various populations in Asia and Oceania.

Quantitative variation has also been observed with GPI. In 1968, Baughan *et al.* found a patient suffering from congenital nonspherocytic hemolytic anemia, and he was found to have a GPI deficiency. The enzyme deficiency was inherited as an autosomal recessive, and the heterozygotes in the family showed intermediate levels of enzyme activity. A number of cases of GPI deficiency associated with hemolytic anemia have since been reported. Some of these have

been given trivial names based on the cities of origin of the possessors, while others have not. Some variant types have altered electrophoretic mobility, while others do not. Sixteen variants were listed in the review of GPI deficiency by Paglia and Valentine (1974). Welch (1971) reported two half activity variants, called 'Cowen' and 'Hay', the former of which showed altered electrophoretic mobility. Rotteveel *et al.* (1974) reported a variant child in a Dutch family who had hemolytic anemia, and they called the phenotype 'Nijmegen'. Family studies indicated that this person was heterozygous for two different rare PGI alleles. Family members who were heterozygous for one or the other of these exhibited intermediate levels of enzyme activity but not hemolytic anemia.

It may be noted that GPI is present in seminal plasma (Blake and Sensabaugh, 1976 and 1978), and in significant quantitites, approaching its concentration in blood on a per volume basis. The enzyme would be expected to be readily detectable in semen, therefore, but its value as a genetic marker is very limited because none of the variant alleles occur in significant frequencies in major populations studied thus far.

37.7 Glutathione Reductase

Glutathione reductase (GSR; NAD(P)H:oxidized-glutathione oxidoreductase; E.C. 1.6.4.2) catalyzes the reduction of oxidized glutathione by NADPH to yield reduced glutathione and NADP (see Figure 33.1). In 1967, Long reported a GSR variant characterized by a greater anodal mobility than the common type. The variant was found to be relatively common in Blacks, 6 homozygotes being found among 196 subjects. The variant is inherited in a straightforward autosomal, codominant way. Kaplan (1968) confirmed these findings. The gene frequency for the variant allele in American Blacks is about 0.13. The electrophoretic system consisted of 0.1M Tris-HCl, pH 9.6, containing 4.5 mM EDTA for bridge buffer, and a 1:10 dilution of this solution for the gel. The GSR is detected by adding an overlay containing GSSG, NADPH, DCPIP and MTT in 0.25M Tris-HCl buffer, pH 8.4. The reaction mixture also detects NADPH diaphorase (section 37.9). The diaphorase runs well ahead of the GSR, however, and can be located by omitting the GSSG from the staining reaction mixture.

There are a number of reports in the literature on inherited GSR deficiency, associated with hemolytic anemia, and sometimes with other disorders. This subject was reviewed by Waller (1968) and by Brewer (1969). The deficiency syndromes are inherited autosomally (Blume *et al.*, 1968), and heterozygotes have intermediate levels of GSR activity in red cells, white cells and platelets.

GSR has been purified quite extensively. It is a flavoprotein with a MW of 100,000, and the active enzyme is a dimer. A low resolution x-ray structure has been determined (Zappe *et al.*, 1977).

37.8 Glutathione Peroxidase

Glutathione peroxidase (GPX; glutathione:hydrogen-peroxide oxidoreductase; E.C. 1.11.1.9) catalyzes the oxidation of reduced glutathione by H_2O_2 and certain organic peroxides (see Figure 33.1). It is perhaps the only known enzyme having selenium as an essential cofactor (Godwin, 1975).

Studies by Beutler and West (1974) and Beutler *et al.* (1974) disclosed an electrophoretic variant of GPX having faster (more anodal) mobility than the usual form. Electrophoresis was carried out on starch gels in 0.1M citric acid-phosphate, pH 7.5, buffer diluted 1:10 for the gel. The enzyme zones were detected in an overlay of t-butyl hydroperoxide, GSH, NADPH, EDTA and glutathione reductase in phosphate buffer, pH 7, and were represented as defluorescent bands on an otherwise fluorescent background under UV light. Hydrogen peroxide cannot be used in the detection reaction on gels because the spontaneous (nonenzymatic) oxidation reaction rate is too greater. The electrophoretic variant, called the 'Thomas' variant, is relatively common in Blacks, but rare in Whites. About 6.4% of 392 Black blood donors were 'Thomas' heterozygotes, while less than 1% of 388 Caucasian donors showed the variant phenotype. No variants were seen in a small number of Orientals. "Storage" bands can develop in hemolysates, but the ones that could interfere with the diagnosis of the Thomas phenotype are removed by mercaptoethanol treatment. The Thomas variant is inherited as an autosomal codominant allele of the usual GPX gene. Kelly and Schedlbauer (1978) reported that GPX activity could be assayed in dried blood, indicating that the enzyme is stable at least in freshly dried stains.

In 1975, Beutler and Matsumoto reported that GPX levels of activity in red cells varied in systematic ways in certain populations. The lower activity enzyme, characteristic of Jewish and Mediterranean populations, had different thermostability characteristics, and may represent the expression of another allele of GPX, characterized by decreased RBC activity.

37.9 Hexokinase

Hexokinase (HK; ATP:D-Hexose-6-phosphotransferase; E.C. 2.7.1.1) may be regarded as the first enzyme in the Embden-Meyerhof pathway (glycolysis), and catalyzes the phosphorylation of glucose at the expense of ATP to yield ADP and Glc-6-P (Figure 33.1). Mannose and glucosamine can also act as substrates for some of the hexokinases.

Red cell HK exhibits a series of isoenzymes, their number and characteristics being dependent on the methods employed for electrohoresis and staining (Eaton *et al.*, 1966; Holmes *et al.*, 1967; Brewer and Knudsen, 1968; Holmes *et al.*, 1968; Schröter and Tillman, 1968).

Four distinct kinds of HK have been found in rat tissues, and these have been distinguished on the basis of various properties. Different tissues vary in the distributions of the different kinds (Katzen and Schimke, 1965). Four kinds of HK may be distinguished in human tissues as well, and they are called HK I through HK IV. HK IV has the greatest anodal electrophoretic mobility. HK IV has a high K_m for glucose, while the other three have relatively low K_m for it (Rogers *et al.*, 1975; Povey *et al.*, 1975). A variant kind of

HK III was found in fresh leucocytes (Povey *et al.*, 1975) and the data indicated that HK III was determined by an independent locus HK_3. The variant allele was designated HK_3^2, and 10 of 330 English people were HK_3 2-1. No genetic variants of HK I or HK II were found in a survey of 800 Europeans (Rogers *et al.*, 1975), but it is thought likely that they are under the control of separate genetic loci.

Patients with congenital hemolytic anemia who lack red cell HK have been reported (Valentine *et al.*, 1967 and 1968). The deficiency appeared to be inherited as an autosomal recessive characteristic.

37.10 Lactate Dehydrogenase

Lactate dehydrogenase (LDH; lactic dehydrogenase; L-lactate:NAD^+ oxidoreductase; E.C. 1.1.1.27) was one of the first enzymes found to exhibit multiple molecular forms, and the early studies on LDH isoenzymes represented the beginning of an explosive stage of growth and development in biochemical genetics. This aspect of LDH was discussed in section 26. Genetic variation of LDH is fairly rare in most populations, and it is not a very useful population marker. The variation in tissue distribution of LDH has led to its application as a specific tissue marker in medicolegal identification problems. These applications were discussed in sections 8.1.5 and 10.8.

Native LDH isozymes are tetramers, consisting of two pairs of nonidentical subunits. The separate subunits are under the control of different autosomal genetic loci, called LDH_A, LDH_B and LDH_C. LDH_C codes for LDH-X of spermatozoa (section 10.8) and the structure of LDH-X may be designated C_4. LDH isozymes in other tissues are the result of random associations of A and B subunits: B_4, AB_3, A_2B_2, A_3B and A_4. These isoenzymes are designated LDH-1 through LDH-5, respectively. The relative amounts of LDH_A and LDH_B products vary from tissue to tissue, so that various LDH isozymes tend to predominate in certain tissues. In liver and skeletal muscle, for example, LDH-5 predominates, whereas LDH-1 and LDH-2 are the major isozymes of cardiac muscle.

Allelic variants at both loci are known. They are detected as multiplicity of bands after electrophoretic separation. The large number of bands can be explained as follows: An individual heterozygous for a variant allele at LDH_B makes B subunits as well as "variant B" subunits (let us call these "β"). Such a person can make 15 separate isozymes of LDH, i.e. B_4, $B_3\beta$, $B_2\beta_2$, $B\beta_3$, β_4, B_3A, $B_2\beta A$, $B\beta_2A$, β_3A, B_2A_2, $B\beta A_2$, β_2A_2, BA_3, βA_3 and A_4. A heterozygote for an LDH_A locus variant can likewise make 15 different isoenzymes. The isoenzymes seen with a particular variant depends on the electrophoretic system and on the tissue examined.

In 1963, Nance *et al.* reported the first A variant in a Brazilian family. Boyer *et al.* (1963) found the first B variant in a young Nigerian man. Kraus and Neely (1964) described four variants, one of which was similar to that of Boyer *et al.* (1963). These were found in people from Memphis, and were designated $LDH\text{-}A_{Mem\text{-}1}$, $LDH\text{-}A_{Mem\text{-}2}$, $LDH\text{-}B_{Mem\text{-}3}$ and $LDH\text{-}B_{Mem\text{-}4}$. Vessell (1965) described a homozygous B variant. An A variant was described in an English population by Davidson *et al.* (1965). Blake *et al.* (1969) examined a New Guinea population and found a further A variant called 'New Guinea'. Several additional variants have been described in Asian Indian populations where, in certain caste groups, they can reach frequencies of 3% to 4% (Ananthakrishnan *et al.*, 1970; Das *et al.*, 1972). A B-variant similar to 'Madras-1' and 'Memphis-3' has been described in a person from Sofia, Bulgaria (Ananthakrishnan *et al.*, 1972). Lie-Injo *et al.* (1973) described some additional variants in Kuala Lumpur, Malaysia.

Kitamura (1971) found a 64 year old Japanese man with a complete deficiency of LDH-B. The condition was inherited, and this interesting patient made only LDH-5 (A_4) isoenzyme.

37.11 Pepsinogen (Pg)

Pepsinogens are precursors of the gastric digestive enzyme pepsin. This particular protein is not uniquely clasified by the Enzyme Commission, but would be in the 3.4.23 sub-sub-class. This interesting polymorphism in Pg is mentioned briefly because some of the isozymes are excreted in urine, and urine may be used to determine the phenotypes. The urine has to be concentrated about 20-fold, in order to detect the Pg isozymes, and it may or may not be possible to devise procedures applicable to the kinds of samples encountered in medicolegal cases. Nevertheless, urine is sometimes encountered, and this polymorphism is well distributed in the population.

Samloff (1969) showed that 8 bands of protease activity with an acid pH optimum can be resolved from different sections of gastric mucosal tissue. These bands are called Pg 1 through Pg 7, with Pg 1 having the most anodal mobility. The eighth, slowest zone is probably cathepsin. Pg 1 through 5 constitute one group of isozymes, while Pg 6 and Pg 7 make up a second group. Pg 2 through Pg 5 of the first group are consistently seen in urine, while Pg 1 is seen variably (Samloff and Townes, 1970a). The Pg 2–Pg 5 isozymes exhibit a polymorphism consisting of two phenotypes, A and B. In A, the Pg 5 is present, whereas in B, it is absent (Samloff and Townes, 1970b; Samloff *et al.*, 1973). Type B was estimated to occur in about 14% of U.S. Whites and Blacks, suggesting a gene frequency for Pg^A of 0.62 and about 0.38 for Pg^B. Townes and White (1974) said they had detected another phenotype in Blacks, called B^1. They also said that, in a sample of nearly 500 U.S. Blacks, about 22% were Pg B, while about 4% were Pg B^1. Bowen *et al.* (1972) presented evidence for another allele, Pg^C. Pg^C homozygotes had a strong Pg 4 band with a small amount of activity in Pg 3. In a sample of 424 Canadian schoolgirls, Pg^C occurred with a frequency of about 0.03. The genetics of the Pg system may actually be somewhat more complicated than the initial studies have suggested thus far (Weitkamp and Townes, 1975). Samloff and Liebman (1972) showed that Pg can be detected in semen, and that the seminal Pg is identical with Pg 6 and Pg 7. Antisera prepared against this

material, or against the Pg 6 and Pg 7 fractions of gastric mucosa do not cross react with the Pg 2 through Pg 5 fractions. It is quite likely that the two groups are under separate genetic control.

37.12 Uridine Monophosphate Kinase

Uridine monophosphate kinase (UMPK) catalyzes the phosphorylation of UMP by ATP to yield UDP and ADP. This enzyme belongs to the Enzyme Commission sub-subclass 2.7.4. UMPK is one of the newest polymorphic enzymes to be described in human red cells.

Giblett *et al.* (1974) found four electrophoretic phenotypes of the enzyme, which were called UMPK 1, 2-1, 2 and 3-1. Family studies indicated that these represented the expression of three codominant alleles at an autosomal locus, $UMPK^1$, $UMPK^2$ and $UMPK^3$. $UMPK^2$ occurs with a frequency of about 0.045 in U.S. Caucasians, 0.011 in U.S. Blacks and 0.071 in U.S. Orientals. The $UMPK^3$ allele is rare, except in the Cree Indians. The $UMPK^2$ isozyme has only about 1/3 the activity of the $UMPK^1$ product. There is a possibility that homozygosity for $UMPK^2$ may be associated with some defect in the immune response. The *UMPK* locus may have a linkage relationship with *Rh* or with *Sc* (Scianna blood group locus), but further study will be needed to be certain about it (Giblett *et al.*, 1975). Toyomasu *et al.* (1977) have described a fourth allele of *UMPK*, $UMPK^4$, which was detected as a 4-1 in a survey of 770 Japanese in Osaka. Teng *et al.* (1976) have purified the UMPK 1 and UMPK 2 enzymes and examined their properties. They were similar except for the greater thermolability of UMPK 2. Gallango *et al.* (1978) described the partial purification and characterization of the enzyme from an UMPK 3 homozygote. It differed in pH optimum and thermal stability, but had similar kinetic characteristics with the UMPK 1 and UMPK 2.

37.13 Diaphorases

"Diaphorase" is a trivial name for enzymes which catalyze the reduction of an acceptor by NADH or NADPH, or by both of them. These enzymes are classified in the 1.6 subclass by the Enzyme Commission. The sub-sub-class is dependent upon the nature of the acceptor. If the physiological acceptor is not known, the NADH-dependent enzymes are classified 1.6.99.3, while the NADPH-dependent enzymes are classified 1.6.99.1.

It is clear from the work of Fisher *et al.* (1977a) that the human diaphorases are determined by at least three separate genetic loci, which have been called DIA_1, DIA_2 and DIA_3. The products of the first two of these loci occur in red cells and in a number of tissues. The DIA_1 enzymes are NADH-dependent, while those of DIA_2 are NADPH-dependent. The products of DIA_3 occur in a number of tissues, but not in red cells. The DIA_3 locus is significantly polymorphic in human beings, and this fact was discovered by Caldwell *et al.* (1976) in spermatozoa. The isozymes determined by DIA_3 were, thus, first called 'sperm diaphorases' (SD).

37.13.1 DIA_1 and DIA_2

The enzyme determined by the locus now called DIA_1 was classically called NADH-methemoglobin reductase. Since methemoglobin does not bind oxygen, it is important to the well being of mature red cells and to the economy of the organism possessing them that methemoglobin concentrations not be allowed to reach very high levels. Pathologically excessive levels of methemoglobin in circulation characterize a condition known as methemoglobinemia. Many cases of congenital methemoglobinemia have been described, and it is now clear that the condition represents, in many cases, an inherited deficiency of NADH-dependent methemoglobin reductase activity. Mental retardation often accompanies the clinical picture seen in the condition as well. A number of variant types of NADH diaphorases are associated with the deficiency syndromes, some of which show altered electrophoretic mobility (Kaplan and Beutler, 1967; West *et al.*, 1967; Bloom and Zarkowsky, 1969; Hsieh and Jaffe, 1971). These have been named after the cities in which they were first observed. Leroux and Kaplan (1972) discovered that the NADH diaphorase occurs in many tissues besides red cells, including leucocytes, platelets, brain, muscle, liver and placenta. This finding suggested that the enzyme has some broader physiological significance than its methemoglobin reductase activity, since there is no hemoglobin in these other tissues. Hultquist and Passon (1971) presented experimental evidence that the NADH diaphorase is probably an NADH-cytochrome b_5 reductase, and that the reduction of methemoglobin in the red cell by NADH is mediated by cytochrome b_5. In this view, the enzyme catalyzes the reduction of oxidized cytochrome b_5 by NADH, and the reduction of methemoglobin by reduced cytochrome b_5 is spontaneous. These results help to provide a tidy explanation for the presence of the enzyme in nonerythroid tissues, and account for the origin of the current systematic name of the enzyme: 'cytochrome b_5 reductase'; NADH:ferricytochrome b_5 oxidoreductase; E.C. 1.6.2.2. Leroux *et al.* (1975) have studied the red cells and tissues of a number of victims of congenital methemoglobinemia, and have found that the enzyme defect is a generalized one, not restricted to the red cells. The fact that the enzyme occurs in brain tissue may help to explain why mental retardation is seen in connection with the deficiency syndromes (Leroux *et al.*, 1975). Leroux *et al.* (1977) have carried out further studies on the enzyme which suggests that, at least in terms of immunological identity, the red cell 'NADH methemoglobin reductase' and the 'cytochrome b_5 reductase' activities in red cells and tissues are the result of the same protein. A microsomal form of the enzyme from placental tissue, which could be solubilized by treatment with deoxycholate, was similar but not quite identical to the soluble forms of the enzyme. The subject of NADH diaphorase variation in the deficiency syndromes has been well reviewed by Scott (1968), Huennekens *et al.* (1968) and Jaffé (1969). Ross (1963) showed that cord blood contains significantly lower diaphorase activity than adult control blood, and said that

this was probably a major cause of methemoglobinemia in newborn infants. In the majority of cases, however, the condition is transient.

Red cell NADH diaphorse also exhibits rare inherited variants, which are not associated with methemoglobinemia. Brewer *et al.* (1967b) described a probable variant. In 1970, Detter *et al.* found two examples of a variant DIA among 378 donors. This variant enzyme was definitely inherited in an autosomal codominant fashion (Detter *et al.*, 1970a). In 1970, Hopkinson *et al.* screened over 2,700 people for electrophoretic DIA variants, and could identify six rare phenotypes in addition to the usual one, and these could be accounted for by the heterozygous expression of five rare DIA alleles, designated DIA^2 through DIA^6, where DIA^1 is the usual allele. About 1% of Europeans, Blacks and Asiatic Indians showed variant DIA phenotypes. Tariverdian *et al.* (1970a) found two variant phenotypes in four people out of 725 in Germany. They called their variant types '2-1' and '3-1'. Williams and Hopkinson (1975) screened a further 3,060 Australians, and found a new phenotype, called DIA 7-1, due to a seventh allele, DIA^7. They said that the '2-1' and '3-1' of Tariverdian *et al.* (1970a) probably corresponded to the 2-1 and 4-1 types of Hopkinson *et al.* (1970). Williams and Hopkinson (1975) knew of 12,608 people who had been phenotyped for red cell DIA, and only 103 were other than DIA 1. This number corresponds to a frequency of about 8.2/1,000 for all DIA variant phenotypes combined.

There was evidence from the work of Scott *et al.* (1965) that the red cell possessed an NADPH-dependent diaphorase, and that this was a separate enzyme activity from the NADH-dependent one. Kaplan and Beutler (1967) presented additional experimental evidence that the NADPH-dependent enzyme was indeed distinct. There are no reports of genetic variation in NADPH diaphorase, except for one family in which a deficiency of the enzyme was noted (Sass *et al.*, 1967). The deficiency was detected in a Black man, who had no hematological abnormalities and only a slight anemia. The condition was found in five other members of the family, however, and was clearly inherited. The fact that these deficient people were healthy lends strength to the belief that the majority of methemoglobin reductase activity in the red cell is attributable to 'NADH methemoglobin reductase', i.e., to the cytochrome b_5 reductase discussed in the foregoing paragraphs.

In 1977, Fisher *et al.* conducted an extensive study of the diaphorase activities of red cells and a number of different tissues. Their results (Fisher *et al.*, 1977a), coupled with the data from other studies that have already been discussed, indicated that the NADH-dependent and the NADPH-dependent diaphorases were under the control of separate genetic loci. The loci were designated DIA_1 and DIA_2. Thus, DIA_1 is responsible for the cytochrome b_5 reductase activity, which was called NADH methemoglobin reductase for a number of years. DIA_2 codes for the NADPH-dependent enzyme. They showed further, that a third kind of diaphorase found in human tissues, and discussed in section 37.13.2, is controlled by yet another genetic locus, which was designated DIA_3. The data of Fisher *et al.* (1977a) indicated that the DIA_1 isozymes had a MW of about 31,000, while those of DIA_2 were about 18,000 daltons. The DIA_2 enzyme was more thermostable than the DIA_1 product, and the two differed by 2 pH units in isoelectric point, DIA_2 enzyme being the more acidic.

Studies with human-rodent hybrid cell lines have resulted in the assignment of DIA_1 to chromosome 22 (Fisher *et al.*, 1977b).

37.13.2 DIA_3 (Sperm Diaphorase)

In 1976, Caldwell *et al.* discovered that an enzyme which had diaphorase activity, and which occurred in human spermatozoa but not in seminal plasma, exhibited three different electrophoretic patterns in 52 primarily Caucasian men. Population analysis strongly suggested that this polymorphism had an autosomal codominant genetic basis, and the pair of alleles throught to be responsible were designated SD^1 and SD^2 (for *S*perm *D*iaphorase). Family studies as such could not be done at the time. The phenotypes, which were detected on polyacrylamide gels, were called 1, 2-1 and 2, and were expressed in testicular tissue as well. The polymorphism was well distributed in the limited sample of people typed, SD^1 having a frequency of about 0.71.

Kühnl *et al.* (1977b) confirmed these findings in the Hessen population. SD was typed on thin agarose gels. They reported two additional phenotypes, which could be attributed to a third allele, SD^3, and the gene frequencies in 141 men were found to be $SD^1 = 0.76$, $SD^2 = 0.22$ and $SD^3 = 0.02$. Kühnl *et al.* (1977b) found that SD could be phenotyped in ovarian and oviduct tissues, where the polymorphism is also expressed. The enzyme is thus not restricted to male tissue, and the German workers said that 'sperm diaphorase' was probably not a good name for the enzyme.

Fisher *et al.* (1977a), in their extensive survey of diaphorases in many tissues, demonstrated that the 'sperm diaphorase' was not restricted even to gonadal tissues, but that it occurred in a number of human tissues. There was every indication from the data that this diaphorase was under the control of a distinct genetic locus, and they said that the most logical designation for the locus would be DIA_3. The alleles formerly designated SD^1, SD^2 and SD^3 thus become $DIA_3{}^1$, $DIA_3{}^2$ and $DIA_3{}^3$. The DIA_3 isozymes can oxidize both NADH and NADPH, and are not identical to a cytochrome b_5 reductase. DIA_3 enzymes were found to have a MW of about 31,000, similar in this respect to DIA_1 enzyme. The enzyme from $DIA_3{}^1$ homozygotes had an isoelectric point of 7.9. In 1979, Edwards *et al.* carried out studies on the biochemical properties of the DIA_3 isozymes. An improved starch gel electrophoresis system was described in this work. The isozymes determined by $DIA_3{}^1$ and $DIA_3{}^2$ were found to be quite similar in terms of thermostability, affinity for Blue Sepharose and slow anodal electrophoretic mobility in borate containing buffers, while the $DIA_3{}^3$ product differed markedly from the other two in

these respects. The gene frequencies for the three alleles in 346 English people were almost identical to those seen by Kühnl *et al.* (1977b) in Germany.

The phenotypic distribution of *DIA*$_3$ types make the system a potentially valuable genetic marker. It may be a particularly valuable marker in cases of sexual assault in which sufficient sperm cells could be recovered. This possibility was suggested by Caldwell *et al.* (1976) and by Gladkikh (1978). The latter investigator detected *DIA*$_3$ phenotypes in seminal stains, and the gene frequencies reported in his sample of 86 people in the U.S.S.R. were very similar to those observed by Caldwell *et al.* (1976), and by the European workers. Suyama *et al.* (1979) reported frequencies of *DIA*$_3^1$ = 0.84 and *DIA*$_3^2$ = 0.16 in a sample of 54 Japanese. Oepen *et al.* (1980) noted that *DIA*$_3$ isozymes could be determined in seminal stains up to 4 weeks old, and in some stains up to 6 weeks old. They discussed various technical problems with the typing systems as well. There were too few DIA$_3$ 1 types in their material (n = 32) as compared with the frequencies seen by other authors, and they said that 6 specimens could not be typed for the isoenzymes. Other investigators have reported *DIA*$_3^1$ frequencies ranging from 0.71 to 0.84 in various populations. Oepen *et al.* (1980) quoted Kopetz *et al.* (1979) as having typed 325 subjects for the polymorphism. Although *DIA*$_3^1$ frequency was about 0.8, a value similar to that found by others, Kopetz *et al.* (1979) apparently found 63 samples in their material which could not be typed. Oepen *et al.* (1980) found one DIA$_3$ 3-2 type, as well as 3 variants which had not previously been seen.

37.14 Phosphoglycolate Phosphatase

Phosphoglycolate phosphatase (PGP; 2-phosphoglycolate phosphohydrolase; E.C. 3.1.3.18) catalyzes the hydrolysis of 2-phosphoglycolate to yield orthophosphoric acid and glycolate. In 1978, Barker and Hopkinson found that human red cell PGP exhibited six electrophoretic phenotypes, which could be accounted for by three alleles operating at an autosomal locus, and called *PGP*^{1}, *PGP*^{2} and *PGP*^{3}. The phenotypes were designated PGP 1, 2-1, 2, 3-1, 3-2 and 3. The PGP polymorphism was found to be expressed in a variety of tissues besides red cells, including leucocytes, cultured fibroblasts, muscle, intestine, kidney, spleen, testis, ovary, lung and brain. An electrophoretic system was described which was suitable for PGP typing, in that it was relatively specific for this phosphatase activity. The enzymes were most active with phosphoglycolic acid as substrate, of the 33 organic phosphates tested. Some activity was demonstrable with 3-phosphoglycerol. Family studies confirmed the postulated mode of inheritance, and the gene frequencies in a random sample of Europeans were: *PGP*^{1} = 0.826, *PGP*^{2} = 0.129, and *PGP*^{3} = 0.045. As such, some 20% of Europeans are expected to be PGP 2-1, about 1% would be PGP 2 and about 7% would be PGP 3-1, the remainder of the phenotypes being relatively infrequent.

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Bibliographic Notes to References for Unit VI

§[1] *Forensic Serology News* [*Forensic Serol. News*] is an uncopyrighted newsletter publication for the informal exchange of information among forensic serologists in the U.S.A. It was published by the Criminal Justice Center, John Jay College of Criminal Justice, City University of New York from January 1975 through June 1976, at which time it was taken over by the Forensic Sciences Foundation, Inc., located in Rockville, MD until March, 1980, when it moved to Colorado Springs, CO. The newsletter was issued in six number per year from 1975 through 1977, and in four numbers per year thereafter. It has always been issued in one volume per year.

§[2] *Japanese Journal of Human Genetics* [*Jpn. J. Hum. Genet.*] has the Japanese title *Jinrui Idengaku Zasshi*

§[3] *Japanese Journal of Fertility and Sterility* [*Jpn. J. Fertil. Steril.*] has the Japanese title *Nippon Funin Gakkai Zasshi*

§[4] *Medical Journal of Osaka University* [*Med. J. Osaka Univ.*] has the Japanese title *Osaka Daigaku Igakubu*

UNIT VII.
HEMOGLOBIN, SERUM GROUP SYSTEMS, HLA AND OTHER GENETIC MARKERS

SECTION 38. HEMOGLOBIN

38.1 Introduction

Hemoglobin (Hb) is the major protein of human red cells, comprising about 95% of their dry weight. Adult human blood normally contains from about 4 million to 6.5 million red cells per mm^3 blood, the average figure being slightly higher for men. Hemoglobin itself is present in concentrations of about 14 to 16 g per 100 $m\ell$ blood. It is the oxygen transporting protein in higher animals; without a molecule having its properties, complex multicellular aerobic life as we know it would not be possible.

Hemoglobin is one of the most extensively studied of all proteins, and its literature fills many volumes. As noted in section 5.1, it acquired its present name over 100 years ago (Hoppe-Seyler, 1864). In forensic serology, hemoglobin is important in two principal contexts: (1) Blood is normally identified in questioned samples by procedures designed to demonstrate the presence of hemoglobin; and (2) hemoglobin exhibits a very large number of genetic variants, a few of which are comparatively common and conveniently detectable by electrophoretic and/or isoelectrofocusing procedures. Many of the reactions of hemoglobin, which form the basis of blood identification techniques, depend upon the heme moiety of the molecule. These were discussed in detail in Unit II, sections 4 through 7. Hemoglobin variants, which can serve as genetic markers, are discussed in this section. Hb F, which was discussed in section 8.3.1 as a marker for the blood of fetuses and young children, is also discussed here. Studies on hemoglobin variants and their detailed structures have contributed significantly to our current understanding of molecular genetics.

38.2 Hemoglobin Structure

The structure of hemoglobin can be described at several different levels. First, the molecule is a subunit protein, and may be described in terms of the polypeptide chains that make it up. Second, the detailed structure of each type of subunit polypeptide chain can be described in terms of amino acid sequence, and in terms of helical and nonhelical regions. Finally, the x-ray data have made possible a detailed description of the three-dimensional structure for some of the hemoglobins. Detailed structures will be discussed briefly in the sections below.

The hemoglobin molecule is tetrameric, consisting of four associated polypeptide chains held together by noncovalent forces. One heme group is associated with each polypeptide chain. Most hemoglobins consist of two α chains and two non-α chains. The non-α chains can be β, γ, δ, ϵ or ζ. Normal adult hemoglobin is designated Hb A, and consists of two α and two β chains. Its structure may thus be written: $\alpha_2\beta_2$. Similarly, Hb A_2, a minor adult hemoglobin, has the structure $\alpha_2\delta_2$. Normal fetal hemoglobin, Hb F, is $\alpha_2\gamma_2$, and so forth. Hemoglobins exist which contain only one kind of chain: Hb H is β_4, for example, and Hb Bart's is γ_4.

Jones (1961) pointed out that hemoglobin structural heterogeneity can be classified as follows: (1) Maturation heterogeneity, which refers to the fact that different hemoglobins are normally synthesized during different stages of development. There are embryonic, fetal and adult hemoglobins. (2) Minor hemoglobin heterogeneity, which refers to the presence of small amounts of structurally different but normal hemoglobins along with the major component characteristic of a particular stage of development; and (3) genetic heterogeneity, which refers to the various "abnormal" hemoglobin variants. Many of these are thought to be the result of point mutations, and with a few exceptions, variant hemoglobins are very rare. The excellent review of hemoglobins by Huisman (1969) was organized according to this classification of heterogeneity.

38.2.1 Normal adult hemoglobins

The structural studies on normal adult hemoglobins were prompted in part by the recognition that the sickle cell condition represented a discrete molecular alteration in the Hb molecule. Structural studies on normal adult and fetal, and variant hemoglobins were carried out simultaneously in an effort to relate the detailed structures to the genetics. Most of these studies occurred in conjunction with the development of present-day understandings of biochemical genetics (section 1.2.2), and have contributed importantly to them.

The major normal adult hemoglobin is called Hb A. It has been called Hb A_1, A_0 and A_{II} at different times (Holmquist and Schroeder, 1966a), but these latter usages are now discouraged (see in section 38.2.3.6). Hb A is a tetramer composed of two α and two β chains, and its structure is written $\alpha_2\beta_2$. Each polypeptide chain is associated with a heme group (Figures 4.4 and 4.5). Intact Hb A thus has 4 heme groups, and its MW is 64,450. The complete amino acid sequences of the α and β chains were worked out in the early 1960's, and are shown in Figure 38.1 (Braunitzer *et al.*, 1961; Konigsberg *et al.*, 1961). The sequencing studies have been well reviewed by Braunitzer *et al.* (1964). Part of the stability of protein molecules arises from the α-helical arrangement of the polypeptide chains (section 1.1.2.1). This structural feature was predicted by Pauling and Corey, and has since been found to occur widely in nature (Pauling, 1960). The helical structure of the polypeptide chains of Hb has been worked out using x-ray crystallographic and other techniques. The polypeptide chains consist of a series of helical regions which are periodically interrupted. These helical regions are indicated by upper case letters, as shown

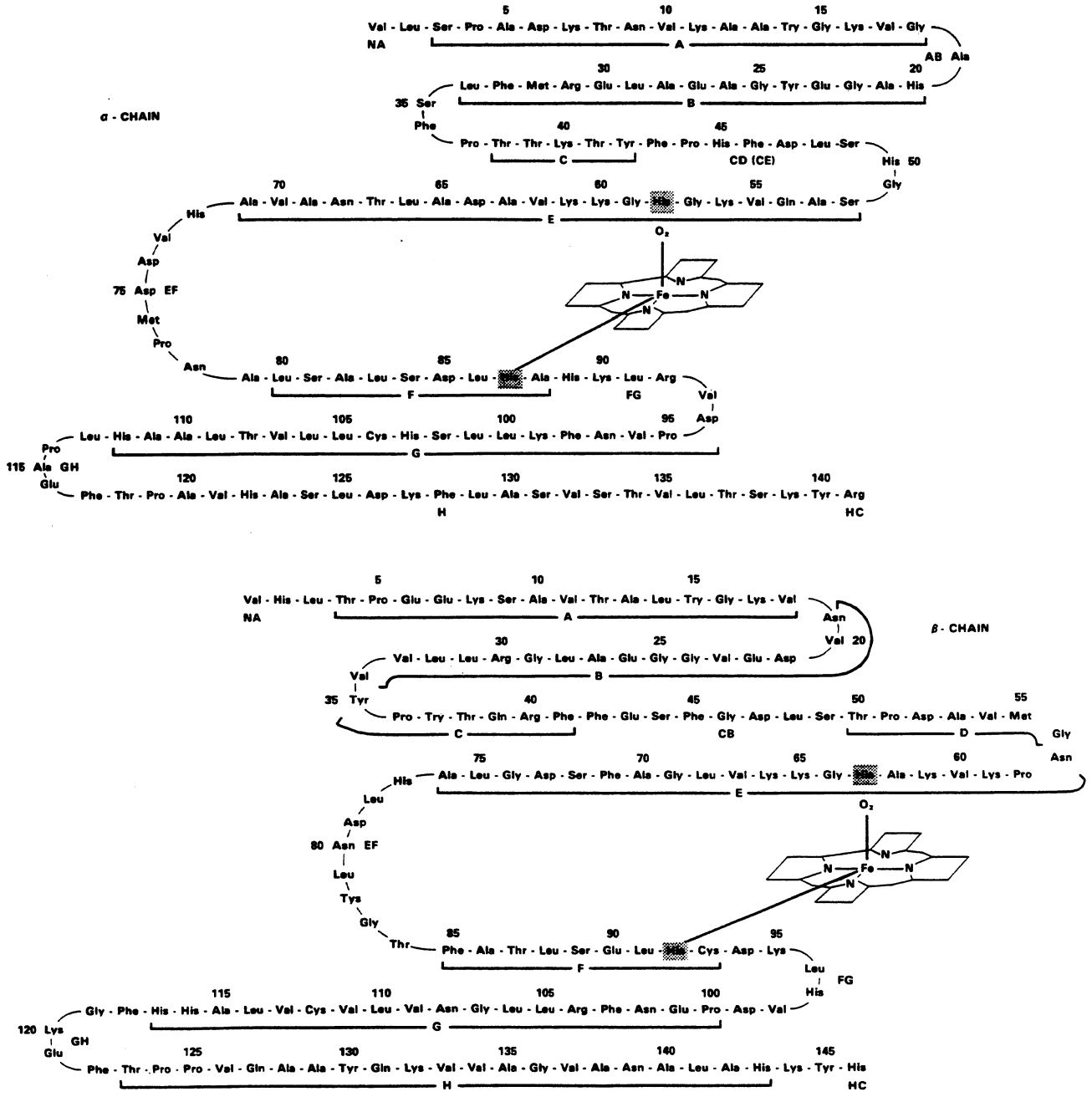


Figure 38.1 **α and β Chain Sequences in Hemoglobin.**

Helical regions are denoted by capital letters. The α 50 - 51 residues are denoted D6 - D7, D1 - D2, and CD8 - CD9 by different authors. There are no non-helical sequences BC and DE in α; since there is no helical sequence D, CD is followed by E. There are no nonhelical sequences AB, BC and DE in β. The proximal and distal histidines, which interact with heme, are boxed.

in Figure 38.1. The location of a particular amino acid residue in the chain may thus be indicated in two ways: first, all the amino acid residues are numbered sequentially from the N-terminal end of the chain (as is the usual convention); and second, the position of the amino acid in a particular helical region (numbering from N-terminal to C-terminal) may also be given. Transitional regions, which lie between two helical regions, are designated using two letters, the one representing the immediately preceding (N-terminal side) helical region, and the one representing the immediately following (C-terminal side) one. The N-terminal amino acids which precede the first (A) helical region are denoted "NA", while the C-terminal amino acids which follow the last (H) helical region are denoted "HC". A few examples of this nomenclature (see in Figure 38.1): (1) the N-terminal amino acid of the α chain, valine, can be called "1", or "NA 1"; (2) the Asn residue of the α chain is "68" or "E17"; (3) the 6th residue of the β chain is Glu, and is designated β6(A3); and (4) the Phe residue of the β chain at position 42 is β42(CD1). The helical designations are extremely useful in comparing different chains for structural homologies and differences. Present nomenclature recommendations call for the use of both the absolute position and the helical region position designations in specifying a particular residue (see in section 38.2.3.6). The exact location of the helical regions in the polypeptide, and the exact three-dimensional structure of the hemoglobin molecule have been determined for the most part by the x-ray crystallographic studies of Perutz, Kendrew and their collaborators in England. These studies actually began in the 1940's, but were interrupted by World War II. They were continued after the war, and were directed at determining the complete structures of human and animal hemoglobins and myoglobins. The solution of these structures represents one of the brightest moments in protein chemistry. The structure of horse Hb was obtained before that of the human molecule (Perutz, 1962 and 1965; Perutz *et al.*, 1960, 1964 and 1965; Muirhead and Perutz, 1963). In these papers as well as in many of the reviews cited in this section will be found drawings of the three-dimensional conformation of hemoglobin and photographs of the molecular models that were constructed based upon the measurements.

It has been known for many years that Hb A is not the only hemoglobin in adult red cells. Hb A_2 was observed by Kunkel and Wallenius in 1955. Chromatographic techniques have revealed the presence of several "minor" hemoglobins in adult blood. Huisman *et al.* (1958) detected two minor fractions in addition to A. Others detected a number of minor components, which were designated A_I, A_{III}, A_1, A_2, A_3 and so forth. Subfractions related to A_I were called A_{Ia} through A_{Ie} (Allen *et al.*, 1958; Clegg and Schroeder, 1959; Schnek and Schroeder, 1961; Jones and Schroeder, 1963; Atassi, 1964; Holmquist and Schroeder, 1964, 1966a and 1966b). The nomenclature of the components is quite complex, because different workers have used different designations, and because the number of minor hemoglobins seen depends on the separation technique employed. Hb A_2 is genetically distinct from Hb A. In 1961, Ingram and Stratton discovered that Hb A_2 is not made up of the same polypeptide chains as Hb A, consisting instead of α chains and δ chains. Hb A_2 has the subunit formula $\alpha_2\delta_2$. The sequence of the δ chain has been determined, and is shown in Figure 38.2. Hb A_2 represents about 2% of hemoglobins as a rule, but this proportion can be altered by pathological conditions. The other "minor" hemoglobins are not as well characterized in general, but are thought to result from post-synthetic alterations of the polypeptide chains, rather than from distinct genetic loci. Hb A_{Ic}, for example, appears to have hexose (probably glucose) condensed with the N-terminal amino acid of the β-chain (Holmquist and Schroeder, 1964, 1966a and 1966b; Lehman and Huntsman, 1974). Hb A_{Ic} comprises 5 to 7% of normal hemoglobins. Another minor hemoglobin may come about through the combination of Hb A with glutathione. It is now recommended that the minor hemoglobins be designated Hb A_2 ($\alpha_2\delta_2$) and Hb A_I (fast moving zone in electrophoresis at alkaline pH). Components of Hb A_I are Hb A_{Ia}, A_{Ib}, etc., and components of the subfractions are A_{Ia1}, A_{Ia2}, etc.

38.2.2 Normal embryonic and fetal hemoglobins

In 1867, Körber first described the differentiation of animal hemoglobins by the rate at which they denature in alkali. In addition, he found that fetal Hb was much more resistant to alkali denaturation than adult Hb. This property may still be used to distinguish between fetal and adult hemoglobins (section 8.3.1). The principal hemoglobin of cord blood at birth is called Hb F, normal fetal hemoglobin. Hb F is found in fetuses after about 10 weeks of gestation. Its level increases for a time until, at birth, it is found together with Hb A. After about 6 months, Hb F all but disappears. Hb F comprises only about 1% of normal adult blood hemoglobins (Chernoff, 1953b). Hb F can be distinguished from Hb A by differential sensitivity to alkali denaturation (Körber, 1867; Singer *et al.*, 1951), chromatographic techniques (Allen *et al.*, 1958; Huisman *et al.*, 1958), electrophoresis (Culliford, 1964; Huehns, 1968) or by immunological methods (Chernoff, 1953a and 1953b; Goodman and Campbell, 1953; Diacono and Castay, 1955). Hb F is made up of two α chains and two γ chains (Schroeder and Matsuda, 1958). Its tetrameric formula is $\alpha_2\gamma_2$. The α chains in Hb A and Hb F are identical (Schroeder *et al.*, 1963a). The amino acid sequence of the γ chain has been worked out (Schroeder *et al.*, 1963b) and is shown in Figure 38.2. It is now known that at least two slightly different γ chains are normally synthesized, and occur in Hb F (see below). There are minor components of Hb F, just as there are of Hb A (Allen *et al.*, 1958). Hb F_I, which can account for 10% of the fetal Hb, differs from Hb F in that the N-terminal residue of the γ chain is acetylated (Schroeder *et al.*, 1962). It was first thought that Hb F_I was $\alpha_2\gamma^F\gamma^N$, where γ^F was the usual Hb F γ chain, and γ^N was the N-acetyl-Gly. . . . . γ chain. It appeared that there was more to it, however (Huehns and Shooter, 1966), and with the finding of the different kinds of γ chains, the detailed

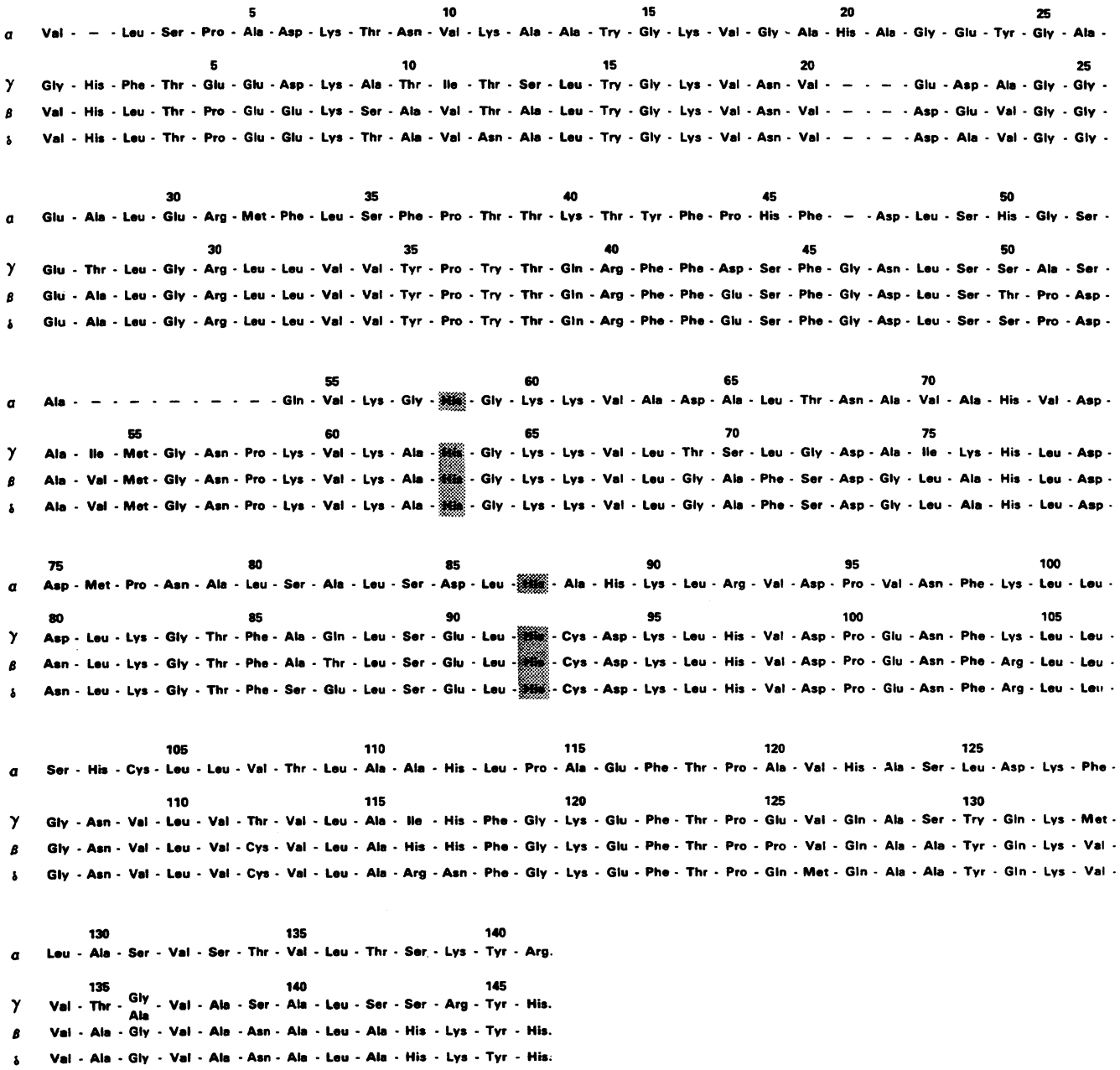


Figure 38.2 Sequences of the α, β, γ and δ Chains of Hemoglobin.

Sequences are arranged to show the maximum sequence homologies between chains. Large dashes represent 'Braunitzer gaps' (see text). The histidyl residues which interact with heme are half-toned. Not all authors place the gaps identically.

structure of Hb F_1 will undoubtedly be found to be more complex than originally thought.

The sequences of the α,β,γ and δ chains are shown together in Figure 38.2. One of the interesting features of the sequences, in view of what is now known about the genetics of Hb, is the similarity of sequence structure among the different chains. This similarity is called structural or chemical "homology". The structural homology can be maximized by introducing theoretical "gaps" into the chains at certain points. These are called "Braunitzer gaps", and they do not really exist, of course. Figure 38.2 has the Braunitzer gaps in order to illustrate the maximum chain homologies.

In 1954, Drescher and Künzer noticed that there seemed to be another hemoglobin in fetal blood that differed from Hb F. Its alkali denaturation curve was intermediate between that of Hb A and Hb F. Halbrecht and Klibanski (1956) confirmed another hemoglobin in very early fetuses by electrophoretic and alkali denaturation differences. Allison (1955) referred to this early hemoglobin as "primitive". In 1961, Huehns *et al.* found not one but two hemoglobins in early fetuses (less than 10 weeks of gestational age), which they called Hb Gower 1 and Hb Gower 2. These names are still used. Additional studies on these "embryonic" (as against "fetal") hemoglobins indicated that they contained a new polypeptide chain, designated ϵ. Hb Gower 2 had the structure $\alpha_2\epsilon_2$, while Gower 1 was thought to consist entirely of the new chain, i.e. ϵ_4 (Huehns *et al.*, 1964a and 1964b). In very young embryos, Hb Gower 1 is the dominant Hb (Hecht *et al.*, 1966), and as the embryo develops the proportion of Gower hemoglobins decreases as that of Hb F increases. In 1967, Capp *et al.* found a new hemoglobin in a baby girl in Portland, Oregon, who had been born with multiple abnormalities. This hemoglobin had two γ chains and two chains that were not yet identified, and was called Hb Portland 1. This Hb was soon found in normal infants as well (Hecht *et al.*, 1967), and is now believed to be a normal embryonic hemoglobin. The second kind of polypeptide chain in Hb Portland 1 was a new one, and was designated ζ (Capp *et al.*, 1970). Hb Portland 1 is thus $\gamma_2\zeta_2$, and it is now thought that Hb Gower 1 is $\zeta_2\epsilon_2$, instead of ϵ_4 as first thought. There are thus three embryonic hemoglobins, distinct from Hb F.

As noted briefly above, there is some further complexity in Hb F, which has the structure $\alpha_2\gamma_2$. Additional sequence studies on the γ chains revealed a rather startling finding: that Hb F normally contains two different kinds of γ chains (Schroeder *et al.*, 1968). These chains differ only at position 136, which may be occupied by Gly or by Ala. The resulting γ chains are designated $^{G}\gamma$ and $^{A}\gamma$, respectively. All the Hb F samples studied had both kinds of chains, from which had to be drawn the extraordinary genetic conclusion that there were at least two γ chain loci, and that they were nonallelic. There is now good evidence for the nonallelic γ chain loci, and the matter is discussed further in section 38.3. There is another complexity in γ chain structure. Ricco *et al.* (1976) found a Hb F with still a different γ chain. The new chain had Thr at position 75(E19) instead of Ileu, and was designated $^{T}\gamma$. Schroeder *et al.* (1979) believe that $^{T}\gamma$ is a product of a mutant $^{A}\gamma$ locus, and suggested that the chain be called $^{TA}\gamma$. A review of fetal and embryonic hemoglobins was given by Lorkin (1973). Recommendations have recently been made by an international body regarding standardized Hb F preparations (International Committee, 1979).

The tetrameric structural formulas of the normal adult, fetal and embryonic hemoglobins are summarized in Figure 38.3.

Adult

Hb A	$\alpha_2\beta_2$
Hb A_2	$\alpha_2\delta_2$

Fetal and Embryonic

Hb F	$\alpha_2\gamma_2$ ($\alpha_2{}^{G}\gamma{}^{A}\gamma$)
Hb Gower I	$\zeta_2\varepsilon_2$
Hb Gower II	$\alpha_2\varepsilon_2$
Hb Portland I	$\gamma_2\zeta_2$

Figure 38.3 Tetrameric Structure of Adult, Fetal and Embryonic Hemoglobins

38.2.3 Genetic variants of hemoglobin

38.2.3.1 Introduction. Obviously, the "normal" hemoglobins just discussed are genetically different, in that different genes are responsible for the synthesis of the different chains comprising them. The "genetic variant" hemoglobins, however, are those which exhibit structural differences from the "normal" hemoglobins. Hundreds of such variants are now known, and in most cases the exact structural difference is known. Many of the variants are very rare and can be explained by mutations. A few variant kinds of Hb reach polymorphic frequencies in certain populations, presumably because there is some selective advantage to their possessors. In some cases, the selective advantage can be explained, such as in the case of Hb S.

The first "abnormal" hemoglobin to be studied in detail was Hb S, or sickle cell hemoglobin. The recognition that sickle cell trait and sickle cell anemia represented the heterozygous and homozygous conditions of a variant gene, and the finding that Hb S differed from Hb A in a single amino acid, ushered in an era in human biochemical genetics. Hemoglobin thus became a model system for relating genetic variation to protein structure at the molecular level. Developments in protein separation methodology along with the solutions to the three-dimensional structure of hemoglobin have made it possible to find many variant hemoglobins, and to diagnose the differences in structure between normal and variant polypeptide chains. Since Hb A and Hb F are constructed from α, β and different γ chains, each coded for by different genetic loci, hemoglobin

variants are often categorized according to the chain in which the variation occurs, i.e. α chain variants, β chain variants and γ chain variants. The simplest type of Hb variants have a single amino acid substitution in one of the chains. There are variants which have two substitutions in the same chain. There are also more complex variants, such as those with extended chains, frameshift mutations, deleted residue chains and the so-called fusion hemoglobins.

38.2.3.2 α Chain variants. The α chain consists of 141 amino acids, and single amino acid substitution variants have now been found at many of the positions. Huisman (1969) noted that 40 possible variants had been reported, 22 of which had been fully characterized. Lehmann and Huntsman (1974) could list about 65 α chain variants in which the substitution had been characterized. In 1975, the International Hemoglobin Reference Center was established at the Medical College of Georgia in Augusta. Its purpose is to consolidate and organize all the information about Hb variants that appears in the literature, and which may be sent to the Center. A number of hemoglobin variants have several names, because some variants discovered in different laboratories and differently named have later turned out to be the same. The Center periodically communicates lists of variants in the new journal *Hemoglobin*. These lists are cited in this book as "IHIC Variant List, Year published", and the references are given under the same designation in the reference lists. By late 1976, the number of defined α chain variants was 73 (IHIC Variant List, 1976a), and is probably greater now. A few α chain variants (not selected for any particular reason) are shown in Table 38.1.

38.2.3.3 β Chain variants. The first variants of hemoglobin described were β chain substitution variants, and a description of the way the early variants were characterized is almost equivalent to a description of the early development of hemoglobin biochemical genetics. Not surprisingly, the first variants studied were those that are comparatively common. Because of their comparatively more frequent occurrence in populations, they are also the most important genetic markers from a forensic point of view.

Sickle cell hemoglobin is the "premiere" variant of Hb A. Many of the pioneering studies on Hb have been carried out on sickle hemoglobin (Hb S). The sickling of red cells from a patient was first noted by Dr. Herrick in Chicago, in 1910. Hahn and Gillespie (1927) studied the sickling behavior of red cells from affected people, and showed that the red cells could be induced to sickle *in vitro* under conditions of low oxygen concentrations, and that they would regain their shape if the O_2 level was brought back to normal. Detailed studies on the medical and physiological consequences of sickle cell trait and anemia have been carried out over the years by Diggs (Diggs and Ching, 1934; Diggs, 1956 and 1965), among others. The condition was recognized as being inherited, and largely restricted to Black populations, in 1934. Before the genetic details of sickle cell had been worked out, a government hospital worker in Rhodesia looked at the frequencies of "sickle" in two different population groups, and concluded that this characteristic could serve as a means of differentiating them anthropologically (Beet, 1946). In addition, he recognized that "sickle" was inherited in a simple Mendelian fashion, based upon studies of a large pedigree (Beet, 1949).

In 1949, Neel proposed a formal genetic scheme for the inheritance of sickle cell trait and disease. He regarded the sickle gene as a dominant characteristic. Very shortly thereafter, Pauling and his collaborators showed that Hb A and Hb S could be separated electrophoretically, and further that sickle cell trait people had about half Hb A and half Hb S, while sickle cell anemics had all Hb S. This finding indicated a simple codominant manner of inheritance in which both genes were expressed in heterozygotes. The presence of an S gene could thus be shown to produce a separate molecule, and Pauling *et al.* (1949) termed sickle cell anemia a "molecular disease". This new concept represented a different way of looking at numerous metabolic disease states, and hemoglobin served as the model system in its subsequent development (Itano, 1953). Sickle cell Hb was clearly different from normal adult Hb (Pauling *et al.*, 1949; Allison, 1957). In 1956, Ingram and his collaborators began publishing their studies on the exact chemical difference between Hb A and Hb S, the results of which opened up the present period of molecular genetic investigation. By a technique called peptide mapping, Ingram (1956) showed that Hb A and Hb S differed in a single peptide. Subsequent studies quickly demonstrated that Hb S differed from Hb A in a single amino acid residue (Ingram, 1957, 1958 and 1959; Hunt and Ingram, 1958a and 1958b). Hb S contained Val where Hb A had Glu. Hemoglobin C (Itano and Neel, 1950) was soon found to have a Glu→Lys substitution at the same position as the one in Hb S (Hunt and Ingram, 1960). Hemoglobin D was first described in Los Angeles, but reaches high frequencies in the Punjab. It is thus called Hb D Los Angeles, Hb D Punjab, and a variety of other names; it is often simply called Hb D, although there are other hemoglobins called "D", which are different. The comparatively common Hb D (Los Angeles or Punjab) has a Glu→Gln substitution at position 121 (Baglioni, 1962a). Hb D Ibadan, for example, is a β87 Thr→Lys variant, while Hb D Iran is β22 Glu→Gln. Hb E was found by Itano *et al.* (1954) using electrophoretic separations. It was shown to represent a Glu→Lys substitution (Hunt and Ingram, 1959 and 1961).

There are now known to be many β chain variants, and a sample of them is shown in Table 38.1. Huisman (1969) listed 45 variants. Lehmann and Huntsman (1974) showed over 120. The IHIC Variant Lists (1976b, 1977a and 1978) showed 138, 164 and 173 variants, respectively.

There are some hemoglobins that have a double substitution in the β chain. Hb C Harlem is a good example (Bookchin *et al.*, 1967). Its β chains are substituted 6Glu→Val and 73Asp→Asn. Moo-Penn *et al.* (1975) found a quite remarkable 35 year old Black man who was heterozygous for Hb S and Hb C Harlem.

38.2.3.4 γ Chain variants. There are fewer γ chain substitution mutants characterized than there are α or β ones.

Table 38.1 Some Hemoglobin Variants

Common Name	Synonyms	Scientific Designation	Reference
α Chain			
J Toronto		α 5 (A3) Ala — Asp	Crookston et al., 1965
Anantharaj		α 11 (A9) Lys — Glu	Pootrakul et al., 1975
I Philadelphia	I; I Texas; I Burlington	α 16 (A14) Lys — Glu	Beale and Lehmann, 1965
Fort Worth		α 27 (B8) Glu — Gly	Schneider et al., 1971
L Ferrara	Umi; Kokura; Michigan I; Michigan II; Yukuhashi II; L Gaslini	α 47 (CD5 or CE5) Asp — Gly	Bianco et al., 1963
Arya		α 47 (CD5 or CE5) Asp — Asn	Rahbar et al., 1975
Montgomery		α 48 (CD6 or CE6) Leu — Arg	Brimhall et al., 1975
Mexico	J Mexico; J Paris II; Uppsala	α 54 (E3) Gln — Glu	Jones et al., 1968; Fessas et al., 1969
J Rajappen		α 90 (FG2) Lys — Thr	Hyde et al., 1971
Chiapas		α 114 (GH2) Pro — Arg	Jones et al., 1968
β Chain			
S		β 6 (A3) Glu — Val	Ingram, 1957 and 1959
C		β 6 (A3) Glu — Lys	Hunt and Ingram, 1960
Saki		β 14 (A11) Leu — Pro	Beuzard et al., 1975
J Baltimore	J Trinidad; J Ireland; J Georgia	β 16 (A13) Gly — Asp	DeJong and Went, 1968
G Taipei		β 22 (B4) Glu — Gly	Blackwell et al., 1969
E		β 26 (B8) Glu — Lys	Hunt and Ingram, 1961
Alabama		β 39 (C5) Gln — Lys	Brimhall et al., 1975
Austin		β 40 (C6) Arg — Ser	Moo-Penn et al., 1977
Athens, Georgia	Waco	β 40 (C6) Arg — Lys	Moo-Penn et al., 1977
G Copenhagen		β 47 (CD6) Asp — Asn	Sick et al., 1967
J Kaohsiung	J Honolulu	β 59 (E3) Lys — Thr	Blackwell et al., 1971
J Cambridge	J Rambam	β 69 (E13) Gly — Asp	Sick et al., 1967
Atlanta		β 75 (E19) Leu — Pro	Hubbard et al., 1975
D	D Los Angeles; D Punjab; D Chicago; D North Carolina; D Portugal; Oak Ridge	β 121 (GH4) Glu — Gln	Baglioni, 1962a; DeJong and Went, 1968
Beograd		β 121 (GH4) Glu — Val	Efremov et al., 1973
γ Chain			
F Auckland		$^{G}\gamma$ 7 (A4) Asp — Asn	Carrell et al., 1974
F Kuala Lumpur		$^{A}\gamma$ 22 (B4) Asp — Gly	Lie-Injo et al., 1973
F Victoria Jubilee		$^{A}\gamma$ 80 (EF4) Asp — Tyr	Ahern et al., 1975
δ Chain			
A$_2$ Roosevelt		δ 20 (B2) Val — Glu	Rieder et al., 1976
A$_2$ Melbourne		δ 43 (CD2) Glu — Lys	Sharma et al., 1974
A$_2$ Indonesia		δ 69 (E13) Gly — Arg	Lie-Injo et al., 1971
A$_2$ Coburg		δ 116 (G18) Arg — His	Sharma et al., 1975
Deleted Residues			
Leiden		β 6 or 7 (A3 or A4) Glu missing	DeJong et al., 1968
Lyon		β 17-18 (A14-A15) Lys-Val missing	Cohen-Solal et al., 1974
Freiburg		β 23 (B5) Val missing	Jones et al., 1966
Extended Chains			
Constant Spring		α + 31C (142 Gln)	Clegg et al., 1971
Fusion Hemoglobins			
Lepore Hollandia		δ (1-22) β(50-146)	Barnabas and Muller, 1962
Lepore Baltimore		δ (1-50) β(86-146)	Ostertag and Smith, 1969

The finding that Hb F normally contains two different γ chains, $^{G}\gamma$ and $^{A}\gamma$ (section 38.2.2), means that the presence of either Gly or Ala at γ136 is normal. Variants at other positions thus have to be characterized at γ136 as well as at the "variant" position. Further, γ75 can be occupied by either Thr or by Ileu. It is now recommended (see in section 38.2.3.6) that these chains be separately designated: $^{A}\gamma^{I}$ = γ75Ile; 136Ala; $^{A}\gamma^{T}$ = γ75Thr; 136Ala; $^{G}\gamma^{I}$ = γ75Ile; 136Gly. There are now about 15 γ chain variants (IHIC Variant Lists, 1977b), and the subject has been recently reviewed by one of the pioneers in the field (Schroeder, 1977). A sample of γ chain variants is shown in Table 38.1.

38.2.3.5 Other variants and other hemoglobins. A number of variants of the δ chain have been found in Hb A_2. The IHIC Variant Lists (1977b) show 10, and a sample of them is included in Table 38.1. A few other types of variants are known. The majority of variants represent single amino acid substitutions; a few (like Hb C Harlem, mentioned in section 38.2.3.3) have a double substitution. Three other types of variants will be mentioned here: deletions, extended chains and fusion hemoglobins. Deletion variants are those which lack one or more amino acids found in the usual polypeptide chain. A few of them are shown in Table 38.1. Hb Freiburg is an example. It is missing the Val residue at β23, and is designated β23Val→0. The IHIC Variant Lists (1977b) show 10 deletion variants. Extended chain hemoglobins have more amino acid residues on the C-terminal end of the chain than are normally found. Hb Constant Spring (Clegg *et al.*, 1971), for example, has 172 residues in the α chain instead of 141. Some authors show it as "α141 31 additional residues" in α chain variant lists. There are 7 extended chain hemoglobins in the IHIC Variant Lists (1977b). The fusion hemoglobins are very interesting, and their existence has implications for the structure and arrangement of the β, γ and δ chain genes at the molecular level. In 1958, Gerald and Diamond found a most peculiar hemoglobin in Boston, which they called "Lepore". Characterization of the molecule (Baglioni, 1962b) showed that the non-α chains appeared to be made up of an N-terminal section of the δ chain attached to a C-terminal section of the β chain. The "break" occurs between what would be δ87 and β116, but because of the sequence homology of the chains (Figure 38.2), the exact location cannot be determined. This δ–β fusion chain was apparently made by a new gene having portions of δ and portions of β. There are other examples of these δ–β fusion mutants now (Table 38.1), and they are sometimes called "Lepore" hemoglobins. Individual examples are distinguished by their place of origin or discovery, such as Hb Lepore Boston, Hb Lepore Hollandia, etc. Huisman *et al.* (1972) found a hemoglobin, Hb Kenya, whose non-α chain is a γ–β fusion hybrid. The "break" is between γ81 and β86. The IHIC Variant Lists (1977b) show 7 fusion hemoglobins.

There are a few other hemoglobins which should probably not be considered "variants" strictly speaking, though they are abnormal. They are usually associated with anemias and other hematological disorders.

There is a class of anemia syndromes known as *thalassemias*. The term is derived from the ancient Greek word for "the Sea" (meaning the Mediterranean). It turns out to mean "sea in the blood", rather than Mediterranean anemia, which it was coined to denote (Lehmann and Huntsman, 1974). The usage has persisted in the literature, even though the thalassemia syndromes are not restricted to Mediterranean peoples. In effect, a thalassemia syndrome is characterized by an imbalance of α or β polypeptide chain production. α-Thalassemias are those in which there is underproduction of α chains, while β-thalassemias represent underproduction of β chains. β-Thalassemia is the classical kind of Mediterranean anemia (Cooley's anemia), and is the most important in terms of frequency of occurrence. It is controlled by a gene which behaves like an "allele" of the β chain structural gene. The terms "β thalassemia major" and "β thalassemia minor" indicate homozygosity and heterozygosity, respectively, for the gene, although clinicians may use these terms to indicate the severity of the clinical manifestations. In the α-thalassemias, there is an underproduction of α chains and a corresponding excess of β chains in adults and of γ chains in fetuses and children. Before any of this was clearly understood, two hemoglobins without α chains were described; Hb H is β_4 and Hb Bart's is γ_4. The latter was apparently named after St. Bartholomew's Hospital, where the baby in whom it was first seen was born (Lehmann and Huntsman, 1974). It is now known that Hb H and Hb Bart's arise by the same basic mechanism. There are two models for the inheritance of α-thalassemia. In the first, there are two allelic α-thalassemia genes: the severe classical α thalassemia 1 gene and an α thalassemia 2 gene. The four possible combinations give rise to a series of increasingly serious clinical conditions. The *αthal* 2 heterozygotes are least affected, while the *αthal* 1 homozygotes are stillborn in the last weeks of pregnancy. In the second model, one must accept that the α chain structural gene is duplicated (for which there is some evidence). Then, either one, two, three or all four α chain genes can be affected by thalassemia, again giving rise to the series of four conditions of increasing clinical severity (Kattamis and Lehmann, 1970). The full explanation for all of the thalassemia syndromes is somewhat more complex than has been indicated, and there is still more to be learned about them. Thalassemia syndromes have recently been reviewed by Bank (1978).

Methemoglobinemia simply means that met-Hb is present in the red cells at clinically significant levels. Met-Hb is hemoglobin in which the heme iron is in the Fe^{3+} state, and it cannot bind oxygen. Methemoglobinemia can come about in a number of ways, only one of which will be discussed here. As shown in Figure 38.1, the heme moiety of Hb is slung between two histidyl residues (His58 and His87 in the α chain, and His63 and His92 in the β). The histidyl-nitrogen of the proximal histidine (α87 and β92) is coordinated to the Fe^{2+} of heme, while the distal histidyl-N (α58 and β63) per-

mits space for the binding of molecular oxygen. This structure forces the iron to retain the ferrous state. A few hemoglobins have been found in which an amino acid substitution occurs at α58 or β63. These disrupt the stability of the heme-α chain or heme-β chain interaction, and allow methemoglobin to form in quantity. As a result, they are called the "M" hemoglobins. Substitutions at other positions in the chains can disrupt the heme-peptide chain stability too. Hb M Milwaukee (β98Val→Glu) causes the heme iron to take on the ferric state.

A final condition that will be mentioned is called "hereditary persistence of fetal hemoglobin" or HPFH. There are a number of anemias and other conditions that can lead to the presence of Hb F after infancy. In HPFH, however, there is continued production of Hb F into the post-infancy years because β and δ chain synthesis has not been "switched on". There are several possible genetic explanations for this phenomen, but it has been attributed to the presence of a "high F" gene. While this explanation is a convenient way of thinking about HPFH, it is probably too simple. Indeed, different examples of HPFH may have different explanations. The "high F" heterozygotes have 10% to 40% Hb F. Homozygotes who have been studied make no Hb A nor any A_2.

The material in section 38.2 has been reviewed on many occasions. The older literature is well reviewed by Braunitzer *et al.* (1964), Schroeder and Jones (1965) and by Huehns and Shooter (1965). More recently, there is Giblett (1969), Huisman (1969), Lehmann and Huntsman (1974), Bunn *et al.* (1977a) and Bank *et al.* (1980). The papers in the major N. Y. Academy of Sciences conference (Kitchin and Boyer, 1974) covered many aspects of hemoglobin. The so-called hemoglobinopathies are well reviewed by Bunn *et al.* (1977b) and by Huisman and Jonxis (1977). The latter book gives detailed procedures for the characterization of hemoglobin variants.

38.2.3.6 Hemoglobin nomenclature. Just as in many other genetic marker systems, the nomenclature of Hb variants has developed somewhat unsystematically. More recently, there have been efforts to systematize it. Some old names are so entrenched in the literature that they have been retained. With the IHIC now established (section 38.2.3.2), nomenclature standardization should prove to be considerably easier than previously.

In the early years of Hb variant research, capital letters were used to designate each new kind of hemoglobin that was found. Hb A was normal adult hemoglobin. The letter "B" was apparently skipped because sickle cell Hb had sometimes been called B. Everyone agreed on Hb S for sickle cell Hb, and soon after Hb S was characterized and electrophoresis came into widespread use as a screening procedure for hemoglobins, the commoner variants were quickly discovered. C, D and E were found not long after S had been characterized. Hb F has long stood for fetal hemoglobin. The letter designation nomenclature was formalized by Chernoff *et al.* (1953). In 1955, Allison suggested that *Hb* be the gene locus name for hemoglobin, but this usage is not followed because of the multiple polypeptide chain structural loci controlling hemoglobin. By the time the letter "Q" was reached, it was apparent that there were not going to be enough letters in the alphabet. Hemoglobins began to be named after places, hospitals or people. Some exotic names were proposed, such as 'Aida', 'Riverdale-Bronx' and 'Abraham Lincoln'. Some of the variants acquired many names, because they were discovered and rediscovered all over the world. It was not until the substitution or chemical alteration was clarified that the identities became apparent. In these cases, the name given by the first observer usually prevails.

A number of hemoglobin variants are distinguishable by electrophoresis in various buffer systems. New variants have sometimes been designated according to their similar electrophoretic mobility, or their similarity in other properties, to a known Hb, followed by the descriptive name. Thus the original Hb D [β121(GH4) Glu→Gln] is called Hb D Los Angeles, D Punjab, D Chicago, D North Carolina, etc. But there are also Hb D Bushman (β16 Gly→Arg), Hb D Iran (β22 Glu→Gln) and Hb D Ibadan (β87 Thr→Lys). Some of the variants have no capital letters in their designations: Hb Sawara (α6 Asp→Ala), Hb Winnipeg (α75 Asp→Thr), Hb Deer Lodge (β2 His→Arg), Hb Alabama (β39 Gln→Lys), etc.

The latest recommendations for standardizing the Hb variant nomenclature (Recommendations, 1979) cover a number of different aspects, some of which have been pointed out in the foregoing sections. For the major normal hemoglobins, Hb A ($\alpha_2\beta_2$) and Hb F ($\alpha_2\gamma_2$) are used. Designations such as A_O, A_{II}, F_O, and F_{II} are discouraged. The embryonic hemoglobins are Gower-I ($\zeta_2\epsilon_2$), Gower-II ($\alpha_2\epsilon_2$) and Portland-I ($\zeta_2\gamma_2$). The only letter designations recommended for the abnormal hemoglobins are Hb C, Hb E, Hb S and Hb H. The remainder should have the descriptive name following the letter, and many variants will have only a descriptive name. Previously, it was the practice to show the alteration as a superscript. Thus, Hb G Georgia was $\alpha_2{}^{95(G2)Pro\rightarrow Leu}\beta_2$, while Hb S was $\alpha_2\beta_2{}^{6(A3)Glu\rightarrow Val}$, and so forth. It is now recommended that only the variant chain, residue number (sequential and helical) and the amino acid substitution be shown, without the use of superscripts. Thus, Hb G Georgia = α95(G2)Pro→Leu and Hb S = β6 (A3)Glu→Val. The designation "Hb M" is retained for abnormal hemoglobins that have an increased tendency to methemoglobin formation, e.g. Hb M Boston = α58(E7) His→Lys. The deletion mutants should signify which residues are "missing", e.g. Hb Freiburg = β23(B5) missing. Fusion hemoglobins are designated so that the segments referring to the types of chains are identified, e.g. Hb Lepore-Boston = δ(1–87)β(115–146). The elongated variants are denoted by specifying the chain, the number of additional residues found at the carboxy-terminus (C), and the residue immediately following the normal C-terminus, (i.e. position 142 in α and 147 in β). Thus Hb Constant Spring = α + 31C (142Gln). Hb H is retained for β_4, and Hb Bart's for γ_4.

38.3 Biochemical Genetics of Hemoglobin

As has been noted, hemoglobin variants have provided a model system for human biochemical genetics. Now that the genetic code is known (Table 1.4), it is possible to speculate intelligently about the kinds of base sequence changes in DNA that could be responsible for the single amino acid substitution variants. Indeed, this kind of analysis can help in the understanding of the evolution of mutations in the human species (see, for example, Beale and Lehmann, 1965; Shaw *et al.*, 1977; and Vogel, 1969).

In addition, studies of the Hb variants have yielded much information about the molecular structure of the polypeptide structural genes themselves. It has been clear for some time that the α and β genes were on different chromosomes (Diesseroth *et al.*, 1976). It is now clear that the α gene is on chromosome 16, while the β and γ gene loci are on chromosome 11 (Diesseroth *et al.*, 1977 and 1978).

There is growing evidence that the α chain locus is duplicated in most people (Nute, 1974; Forget, 1979). It is also quite clear now that there are two γ gene loci per haploid set of chromosomes, one locus coding for the γ136Gly ($^{G}\gamma$) chain, and the other coding for the γ136Ala ($^{A}\gamma$) chain (Schroeder *et al.*, 1972; Schroeder and Huisman, 1974). This point was discussed in section 38.2.2. The chains can be present in different proportions in Hb F, suggesting further complexity at the genetic level (Huisman *et al.*, 1972). The so-called $^{T}\gamma$ chain, reported by Ricco *et al.* (1976) and discussed in section 38.2.2, has γ75Thr instead of γ75Ile. Schroeder *et al.* (1979) regard $^{T}\gamma$ as the product of a modified $^{A}\gamma$ locus and said that the chain should be called $^{TA}\gamma$. Thus, it appears that most individuals have a duplicate set of α genes on chromosome 16, and a series of closely linked γ, δ, and β genes on chromosome 11 (Schroeder and Huisman, 1974; Deisseroth *et al.*, 1977 and 1978). The organization of the chromosome 11 genes is thought to be $^{G}\gamma^{A}\gamma\delta\beta$ (Little *et al.*, 1979). This arrangement helps in understanding how control of the fetal-to-adult "switch" may come about at the level of DNA (Kabat, 1974), although the exact control mechanisms are not yet known (Forget, 1979). In addition, the Lepore type (fusion) hemoglobins and Hb Kenya (see in section 38.2.3.5) can be understood in terms of non-homologous crossovers between $\delta\beta$ regions (Lepore) or between $\gamma\beta$ regions (Kenya) because of misalignment of sister chromatids at meiosis.

Until recently, it was assumed that the information base sequence in DNA was colinear with that of the m-RNA, and that the m-RNA sequence was, in turn, colinear with the amino acid sequence of the protein (section 1.2.2). Recent fine structure studies have revealed the extraordinary fact, however, that the coding sequences of DNA are commonly interrupted by intervening sequences of DNA of variable length (introns) which are not represented in mature m-RNA. The sequences are transcribed initially into precursor m-RNA, and the m-RNA molecules then have these sequences removed enzymatically by a splicing process before the m-RNA is transported to the cytoplasm from the nucleus. Introns have been identified in the non-α chain coding regions of the human genome. The biochemical genetics of hemoglobin synthesis was recently reviewed by Forget (1979) and by Bank *et al.* (1980).

In his remarks opening the major hemoglobin conference at the New York Academy of Sciences in 1974 (Kitchin and Boyer, 1974), Prof. Motulsky summarized the importance of hemoglobin research to modern biochemical genetics (Motulsky, 1974):

> Hemoglobin research plays a role in human biochemical genetics similar to that of drosophila research in formal genetics and analogous to work with microorganisms in microbial genetics. Many fundamental concepts have become clarified by investigations on human hemoglobins.

38.4 Methods of Separating and Characterizing Hemoglobins

The most important method of separating normal and common variant hemoglobins is electrophoresis. Dozens of different electrophoretic procedures have been devised over the years for this purpose. Because some of the variant hemoglobins have clinically significant consequences, there have been a number of mass screening efforts in various populations. Apart from their medical value in identifying people who may require treatment, or who may be advised to seek genetic counseling, many variants have been identified through these studies. As has been noted, these variants have been characterized and are of considerable biochemical genetic significance. Population distributions of the more widely occurring variants are of anthropological and forensic interest as well. Although most hemoglobin variants are rare, it has been estimated that 1 in every 2,000 persons carries a detectable Hb variant (Motulsky, 1974).

The oldest electrophoretic procedures employed paper or starch blocks as support media. Procedures using agarose, CAM, and polyacrylamide gels have since been devised. Some workers still prefer paper electrophoresis (Lehmann and Huntsman, 1974). Most forensic serologists probably utilize cellulose acetate membranes or starch or agarose gels for Hb separation. Isoelectric focusing techniques have also been used to separate Hb variants.

Depending upon which hemoglobins are to be separated, different buffer systems are used. Hemoglobins which co-electrophorese in one system may separate in another. It is sometimes necessary, therefore, to run selected samples in more than one system to distinguish those types that have the same mobility in the first system. Three "basic" buffer systems are used for paper (and most other kinds of) electrophoresis, although dozens of minor variations have been introduced. These are a barbital buffer, pH 8.6–8.9, a Tris-EDTA-borate buffer, pH 8.6–8.9 and a phosphate buffer, pH 6.5. The alkaline buffers give good separations of Hb A, S and C. The Tris system resolves Hb A_2 much better than barbital. Hb A and Hb F are not well resolved in these buffers; neither are C and E, nor S and D. At pH 6.5,

all the hemoglobins migrate cathodically except Hb H. Paper electrophoresis is discussed in detail by Weiss (1968) and by Lehmann and Huntsman (1974).

Starch gel electrophoresis is somewhat more cumbersome and time-consuming than CAM electrophoresis. For many applications, barbital or Tris-EDTA-borate buffers in the pH 8.6–8.9 range are used. The relative electrophoretic mobilities of a number of hemoglobins in pH 8.6 buffers are indicated in Figure 38.4. Starch gel electrophoresis of hemoglobins has been reviewed by Huehns (1968), and cellulose acetate membrane techniques are given by Chin (1970). In 1957, Robinson *et al.* proposed using agarose gel electrophoresis in acidic (sodium citrate, pH 6) buffer for Hb separation. The system gave a good resolution of Hb A and Hb F, and additionally differentiates Hb S from D and Hb C from E. Relative electrophoretic mobilities of some hemoglobins in acid citrate buffered agarose are shown in Figure 38.5. Agarose gels have been used at alkaline pH as well (Lepp and Bluestein, 1978). Breen *et al.* (1968) reported improved resolution of hemoglobins on cellulose acetate with the Beckman Microzone system using Tris-glycine, pH 9.3, buffers. Jacobson and Vaughan (1977) reported a rapid starch gel procedure for mouse hemoglobins. Schneider and Hightower (1977) have studied the behavior of dozens of different hemoglobins on agarose gels using citric acid buffers at acid pH.

In 1977, Burdett and Whitehead used polyacrylamide gel isoelectrofocusing to separate hemoglobins in the pH 5–8.5 range. Hb A, A_2, S, F, D, C and E could be resolved in this system. In 1978, Bassett *et al.* applied PAGIF in the pH range 6 to 9 to the study of about 70 different Hb variants. They obtained good resolution of many of them, and said that the technique provided a good screening procedure if very thin gels were used in order to conserve the costly ampholines.

Electrophoresis is the method of choice for Hb screening in most laboratories. CAM methods are popular because they are so fast and require very small amounts of material if microtechniques are used. Identification of Hb A, A_2, S, F, D and E can be accomplished fairly readily by electrophoresis. It is necessary to use different electrophoretic techniques for certain of these. Samples running like Hb S at pH 8.6, for example, can be run in citrated agarose to see whether Hb D is present. Identification and characterization of other hemoglobins normally requires more sophisticated techniques. PAGIF is a good approach to Hb separation in laboratories equipped for it. If it is necessary to diagnose the amino acid replacement in a variant Hb, peptide "fingerprinting" and sequencing techniques must be used. Peptide fingerprinting is discussed very clearly by Lehmann and Huntsman (1974), as is the use of amino acid analyzers. Cohen-Solal *et al.* (1974) discussed a specialized sequencing procedure. Recently, Garver *et al.* (1977) have employed RIA techniques to the identification and quantitation of several variant hemoglobins. It is clearly necessary to have the monospecific antiserum in order to use this procedure, so it is useful for variants which have already been characterized. The antisera, however, are specific for the amino acid substitution in the chain, and are, therefore, extremely specific if a mixture of hemoglobins is being investigated.

38.5 Medicolegal Applications

38.5.1 Disputed parentage

No references to the application of Hb variants as such to parentage problems were found, and it does not appear that hemoglobin is widely used for this purpose. According to a recent survey of laboratories in this country (Polesky and Krause, 1977), about 25% of the 30 AABB Reference Laboratories could do Hb typing in the cases, and somewhat fewer did it routinely. About 17% of the other laboratories said that Hb typing was available, but less than 2% used it routinely. The main reason for using Hb typing would be detection of Hb S in cases involving Black subjects. The probability of excluding a falsely accused Black father on the basis of Hb S typing is about 4.5% (Chakraborty *et al.*, 1974). The value would be less for Hb C and Hb D, since they are less common. Hb E would be a useful parentage marker in certain Asian populations.

38.5.2 Hb Typing in bloodstains

For practical purposes, the hemoglobins that might be diagnosed in forensic cases are A, F, S and C. Hb D and E can be separated from Hb S and C under certain electrophoretic conditions. Investigations involving young children may call for Hb F differentiation. This may be achieved in a number of ways, including immunological, electrophoretic, and by alkali denaturation characteristics. This subject was discussed in section 8.3.1. It would, of course, be informative to find significant amounts of Hb F in an adult's blood, since this condition is comparatively rare. Hb F can be distinguished from Hb A in three ways: (1) differential alkali denaturation behavior; (2) immunologically; and (3) electrophoretically. The fact that Hb A is considerably more alkali-labile than Hb F was noted by Körber (1867), and studied in detail by Singer *et al.* (1951). This method of differentiation was applied to bloodstains by Culliford (1964). He followed the denaturation in the visible region. Watanabe (1969) said that the reaction could be followed in the UV. A description of the procedure is given in Culliford (1971), where he says that caution should be exercised in trying to differentiate Hb F by this method in bloodstains more than about a week old. The technique is also described in the most recent methods list from the London laboratory (MPFSL, 1978). Apparently, it is primarily a back-up for electrophoretic or immunological techniques.

Immunological procedures for the differentiation of Hb A and Hb F depend upon having comparatively specific anti-human Hb for the purpose. Efforts to prepare anti-Hb F have been going on for many years (Darrow *et al.*, 1940; Ikin *et al.*, 1953), and antisera to human Hb was first prepared by Klein (1904, 1905a and 1905b), following the work of Leblanc (1901) and Ide (1902) on animal hemoglobins. This subject was discussed in sections 7.1 and 8.3.1. Anti-Hb F procedures have been developed for bloodstains

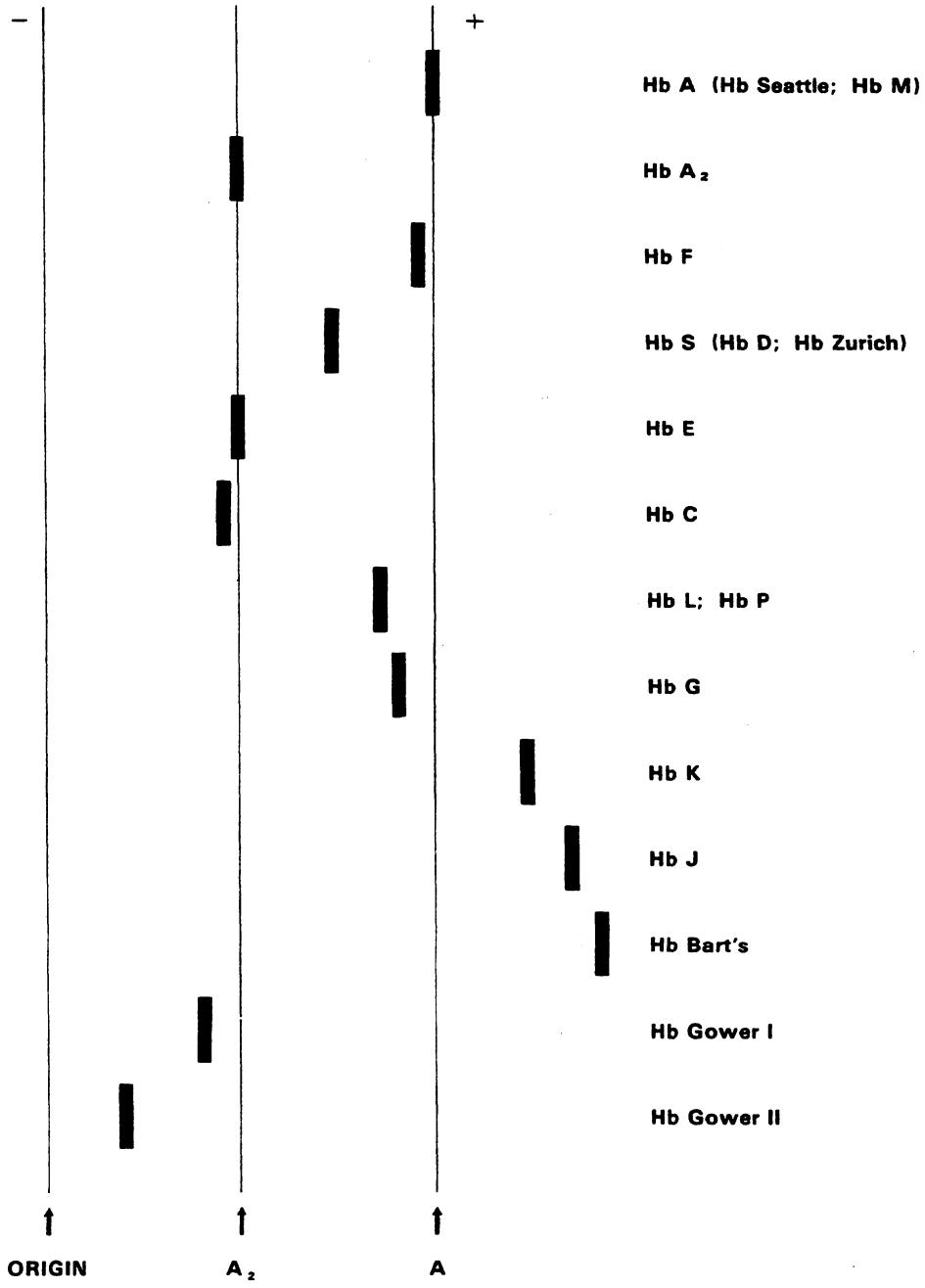


Figure 38.4 Relative Electrophoretic Mobilities of Some Hemoglobins at pH 8.6

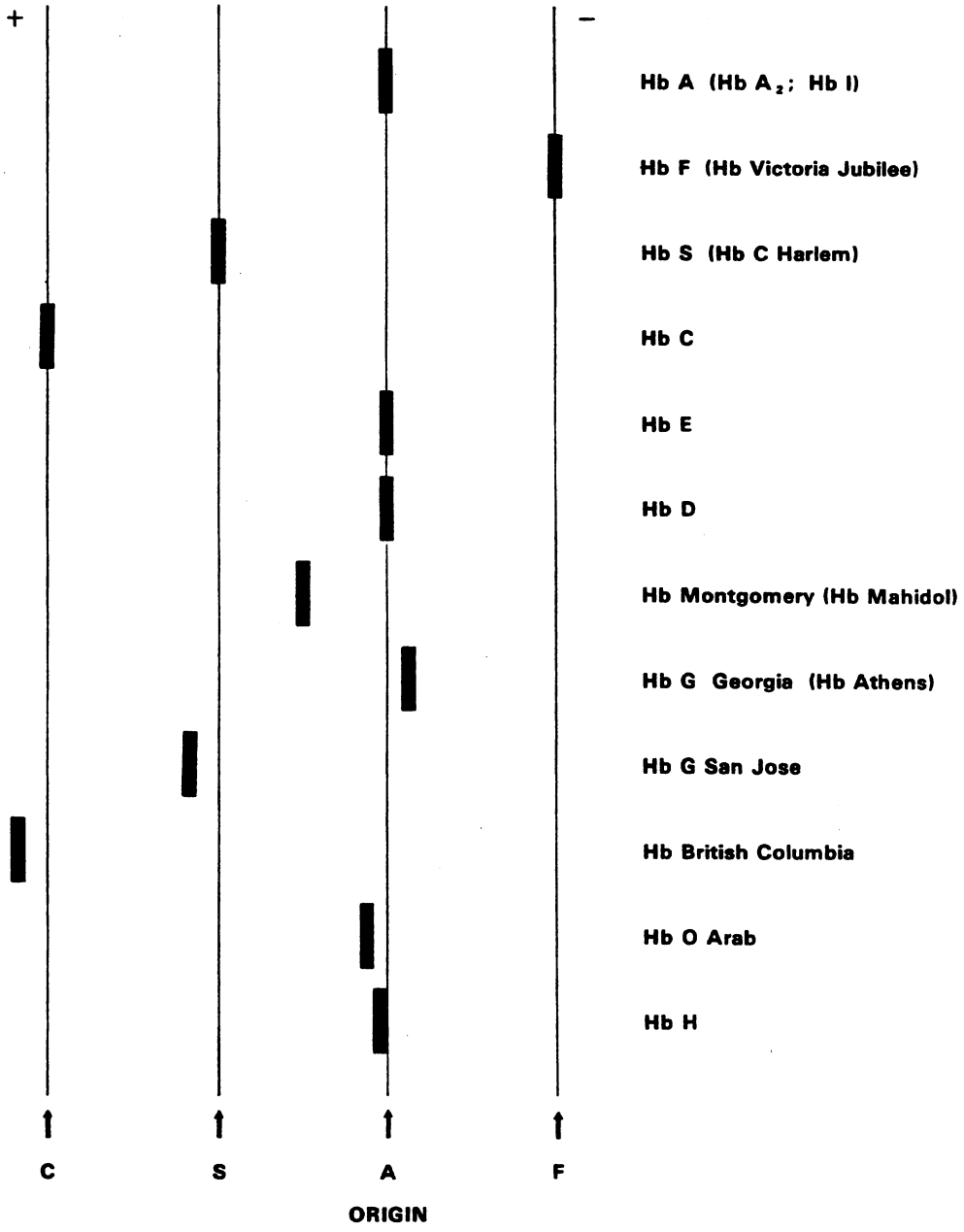


Figure 38.5 Relative Electrophoretic Mobilities of Some Hemoglobins of pH 6 in Agarose

using immunoelectrophoresis (Depieds *et al.*, 1960), Ouchterlony double diffusion, and crossed over electrophoresis (Baxter and Rees, 1974a; MPFSL, 1978; Wiggins and Wraxall, 1979).

Electrophoresis is the favored procedure for separating, and thereby identifying, not only Hb A and Hb F, but also C, S, D and E. Pollack *et al.* (1958) first suggested the application of Hb typing to bloodstain cases. They used paper electrophoresis, and could differentiate Hb A and Hb S in bloodstains on a robbery victim's clothing in a case in which the suspected assailant was Hb AS. They suggested that A and F might be separable as well. Hb A and F do not separate very well in the classical alkaline (pH 8.6–8.9) buffer systems. In 1972, Wraxall reported that good A and F separation could be obtained using barbital pH 8.6 cathodic tank and Tris–EDTA–borate pH 9.1 anodic tank buffers. This system was also applicable to A–S and A–C separations and is described in Culliford (1971). Wilkins and Oepen (1977) confirmed Wraxall's (1972) findings. In 1962, Huntsman and Lehmann applied paper electrophoresis to the separation of Hb A, S, C, D and E in bloodstains a few days old. Hb D and F could be differentiated by alkali denaturation, they said. Culliford (1964) used the discontinuous buffer system of Poulik (1957) to separate Hb A from S and C on Oxoid CAM. The procedure devised by Wraxall (1972) was adopted later (Culliford, 1971), and Sartorius CAM's were preferred. Recently, Wiggins (1978) applied the acid citrate agarose electrophoretic method to the differentiation of Hb S from D and Hb C from E in dried blood. Using this procedure, Hb can be run in the alkaline system (Wraxall, 1972) to distinguish A, F, S and C, and a series of rare variants (MPFSL, 1978). The Hb AS or S, and AC or C samples are then checked in the citric acid agarose system to distinguish S from D and C from E, and thus giving far better population discrimination when the rarer types are encountered. The way this technique works may be better appreciated in a general way by comparing Figures 38.4 and 38.5. Baxter and Rees (1974b) described Hb typing in conjunction with their routine Hp typing procedure (section 39), and said that A, S, C and D could be distinguished. Hb F could be presumptively identified as well.

Hb typing by electrophoresis is usually quite straightforward, provided the limitations of the techniques are recognized and suitable Hb standards are available. In 1979, Barnard and Grunbaum looked at a series of Hb A, AS and AC stains up to 28 days old, however, and noted that aqueous extracts of the bloodstains could exhibit altered electrophoretic mobility relative to the standard hemolysates. The effect was reversible by extracting the bloodstain with Cleland's reagent (DTT). Reversal of the observed effects by DTT strongly suggests that these alterations were based on sulfhydryl effects of some kind. Some years ago, Huisman *et al.* (1966) noted that Hb A, S and F showed similar effects upon 4° storage of hemolysates. Dialysis tended to prevent the effects, while reduced glutathione tended to enhance them. These workers thought that the storage effects could be understood in terms of mixed disulfide formation between glutathione and globin chains. These observations may be related to those of Barnard and Grunbaum (1979).

Most forensic science laboratories are not speciality hemoglobin laboratories. Differentiation of Hb A, F, S, C, D and E can be accomplished fairly readily. Beyond that, though, known standards are unlikely to be available. There are now hundreds of variants of Hb, and most of them are not readily distinguishable by electrophoresis. More complicated characterization techniques, such as peptide fingerprinting and sequence analysis, are beyond the scope of most forensic laboratories. In addition, fairly large samples are required which may not be available, and it is not completely clear that Hb isolated from dried blood is amenable to such analysis. Thus, while one might diagnose a sample as AC or AS or AD according to its electrophoretic mobility, there is a possibility that the sample is really a rare variant with a comparable mobility. While the majority of variants are indeed rare, it would not be surprising for a fairly busy casework laboratory that types Hb routinely to encounter one of them on occasion. According to Motulsky (1974) about 1 in 2,000 people has a detectable variant hemoglobin. If a rare variant were encountered, and could be identified (perhaps with the help of specialized Hb laboratory), it would be highly individualizing and informative.

38.6 Distribution of Common Hemoglobin Variants in U.S. Populations

The data are shown in Table 38.2. The most common variant hemoglobin is probably Hb S. The population genetics of Hb S, and by analogy to what is known of other Hb variants, suggest that the mutation causing S has occurred more than once. There is not much doubt that West Africa represents at least one point of origin. Hb S has apparently reached polymorphic proportions in areas of the world in which malaria was (or is) endemic. Heterozygotes (Hb AS) have an advantage against malaria, and this situation has been called "balanced polymorphism". The gene exists in other parts of Africa, in India and other parts of Asia, and in parts of Europe. The gene may now be seen in any population into which there have been any significant migrations from high S regions. In this country, Hb S is largely, but not exclusively, restricted to Blacks of African origin. Hb S may be seen in Asians, and it is occasionally seen in Whites (e.g. Crane *et al.*, 1977). It is not clear from most of the reports of Hb S in Whites whether the hemoglobin has been rigorously identified as the β6Glu→Val molecule, or whether a different variant which looks like S electrophoretically could be present. It is, of course, possible that Hb S that is β6Glu→Val arises periodically in a family by spontaneous mutation. Hb D was first described in Los Angeles, but occurs in significant frequencies in certain Asian Indians. It is also seen in Pakistan, Iran and Afghanistan. It is seen in Europeans, particularly those from countries which had close ties with India, and it has been noted that Hb D was most common in this country in people who were partly of English origin. Hb C was

detected in American Blacks. It is present in West Africa, but almost absent in East Africa. It is also seen in Europeans upon occasion. There is recent evidence (Kan and Dozy, 1980) that the S and C mutations arose independently, and that Hb AS arose by several independent mutational events in different places. This conclusion has been reached by studying the relationship of Hb S and C to a polymorphism for a restriction endonuclease recognition site on DNA, adjacent to the β chain structural gene. Hb E is principally an Asian type, being found at appreciable frequencies in Burma and Indochina. It is not common in India nor in modern Chinese. Lehmann and Huntsman (1974) provide a thorough discussion of the world population distributions of various hemoglobins. The best and almost only comprehensive reference source to population frequencies is Livingstone (1967).

Table 38.2 Distribution of Common Hb Phenotypes in U.S. Populations

Population	Number	Phenotypes — Number (Percent) A	AS	S	AD	D	AC	C	Reference
CAUCASIAN									
Baltimore, MD	500	500 (100)							Smith and Conley, 1953
Ann Arbor, MI	72						(0)		Neel, 1954
Houston, TX	350	350 (100)							Haynie et al., 1957
Durham, NC ("non-Black")	734	732 (99.7)	1 (0.14)		1 (0.14)				Chernoff and Welchselbaum, 1958
Southern Louisiana (children)	140	139 (99.3)	1 (0.7)						Moffitt and McDowell, 1959
Memphis, TN area (autopsy)	1,250	1,250 (100)							McCormick, 1960
St. Louis, MO (infants)	90	90 (100)							Minnich et al., 1962
Baltimore, MD (infants)	180	180 (100)							Weatherall, 1963
Mississippi	1,045	1,044 (99.9)		1 (0.1)					Thompson et al., 1964
California	6,004	(99.8)	(0.2)				(0)		Grunbaum et al., 1978
Detroit, MI	503	503 (100)							Stolorow et al., 1979
California	1,040	1,040 (100)							Grunbaum et al., 1980
NEGRO									
Baltimore, MD	500 ★	449 (89.8)	36 (7.2)	5 (1)			9 (1.8)		Smith and Conley, 1953
Ann Arbor, MI	209	(98.6)					(1.4)		Neel, 1954
St. Louis, MO	1,020	896 (87.8)	94 (6.3)		4 (0.4)		26 (2.5)		Chernoff, 1956
Galveston, TX (patients)	1,550	1,369 (88.3)	141 (9.1)	4 (0.3)			35 (2.3)	1 (<0.1)	Schneider, 1956
Houston, TX	400 ★	351 (87.8)	36 (9)	5 (1.3)			6 (1.5)	1 (0.25)	Haynie et al., 1957
Durham, NC	390	338 (86.7)	33 (8.5)		1 (0.25)		13 (3.3)		Chernoff and Welchselbaum, 1958
Southern Louisiana (children)	564 ☆	479 (84.9)	47 (8.3)	18 (3.2)			10 (1.8)		Moffitt and McDowell, 1959
Puerto Rico	602 ✪	561 (93.2)	29 (4.8)	2 (0.3)			7 (1.2)	1 (0.2)	Suarez et al., 1959
Philadelphia, PA (patients)	1,000 ✪	895 (89.5)	74 (7.4)	3 (0.3)	1 (0.1)		23 (2.3)		Myerson et al., 1959
Baltimore, MD	400 □				4 (0.4)				Marder and Conley 1959
Memphis, TN area (autopsy)	2,800 ■	2,459 (87.8)	254 (9.1)	19 (0.7)	1 (<0.1)		60 (2.1)		McCormick, 1960

Table 38.2 (Cont'd.)

Population	Number	Phenotypes — Number (Percent) A	AS	S	AD	D	AC	C	Reference
Washington, D.C. (tuberculous)	310	282 (91)	28 (9)						Ryan et al., 1960
St. Louis, MO (infants)	449◇	359 (79.9)	47 (10.5)		2 (0.4)		9 (2)		Minnich et al., 1962
Wash. D.C. (pregnant women)									
Study group	524	490 (93.5)	25 (4.8)	1 (0.2)			8 (1.5)		Jenkins and Clark, 1962
Control group	304	283 (93.1)	11 (3.6)				10 (3.3)		
Baltimore, MD (infants)	900◆	784 (87.1)	67 (7.4)				26 (2.9)		Weatherall, 1963
Maryland	681	625 (91.8)	44 (6.5)				12 (1.8)		Boyer et al., 1963
Southeast Georgia	237	214 (90.3)	19 (8)				4 (1.7)		Cooper et al., 1963
Gainesville, FL (pregnant women)	944	869 (92.1)	65 (6.9)	1 (0.1)			9 (0.95)		Cotter and Prystowsky, 1963
Mississippi	1,310	1,100 (84)	114 (8.7)	37 (2.8)	14 (1)		38 (2.9)	7 (0.5)	Thompson et al., 1964
Southern Louisiana (tuberculous)	220	211 (95.9)					9 (4.1)		Coulter, 1965
Alabama	249,089●	220,405 (88.5)	21,423 (8.6)	574 (0.2)			6,074 (2.4)	102 (0.04)	Schneider et al., 1976
California	1,025○	(89.3)	(8.6)				(1.8)		Grunbaum et al., 1978
Detroit, MI	504▲	452 (89.7)	37 (7.3)				14 (2.8)		Stolorow et al., 1979
California	792△	716 (90.4)	54 (6.8)				18 (2.3)		Grunbaum et al., 1980
HISPANIC									
Puerto Rico ("White")	1,487	1,486 (99.9)	1 (0.06)						Suarez et al., 1959
California, (Chicano/Amerindian)	1,596●	(99.6)	(0.1)				(0.2)		Grunbaum et al., 1978
California (Mexican)	1,569○	1,561 (99.5)	3 (0.2)				3 (0.2)		Grunbaum et al., 1980
ASIAN									
California/Hawaii	3,053●	(99.9)							Grunbaum et al., 1978
California/Hawaii	1,451○	1,448 (99.8)	1 (0.06)						Grunbaum et al., 1980

★ one SC
☆ ten SC
✪ two SC
□ those classified as AS by electrophoretic screening were tested further
■ four SC and three other
◇ 32 other
◆ 23 other
• 149 of the "S" were S/β+ thal, 73 were S/high F; 329 were S/C, 11 were S/other and 7 were C/other; 164 were rare variants
○ 0.4% were rare
▲ one rare
△ four rare
● 0.1% rare
○ two rare

SECTION 39. INTRODUCTION TO SERUM (PLASMA) PROTEINS

Various polymorphic serum protein (serum group) systems will be considered in subsequent sections (40 through 45) of this unit, and a brief introduction to serum proteins is appropriately included here. Some of the enzymatic activities considered in Unit VI occur in serum, and they can be considered "serum proteins" as well. Any polymorphic system which exhibits enzymatic activity has been included in the previous unit of this book, however. The serum proteins and serum group systems included in Unit VII do not possess known *in vivo* enzymatic activities, but are defined by a number of other properties (often immunological, serological or electrophoretic). Some of these proteins are not yet understood in terms of physiological function. The terms "serum protein" and "plasma protein" are used interchangeably in what follows.

The development of various, reproducible protein separation techniques which complement one another in terms of resolution and specificity, has enabled investigators to separate, classify and characterize many of the proteins of human plasma (as well as thousands of other proteins). Human plasma possesses a large number and variety of proteins, some of which are known to be under the control of polymorphic genetic loci. A number of the polymorphic serum protein systems may be utilized in forensic and medicolegal investigations.

The nomenclature of plasma proteins is rather complex and cumbersome, because it incorporates a number of historical classification designations along with the refinements that have been introduced as separation techniques improved and the proteins have been better characterized. There has never been a coordinated international effort to arrive at a truly systematic nomenclature, such as was done for the enzymes by the Enzyme Commission (see section 1.1.3.2). Plasma proteins were first classified according to their solubility in water and salt solutions, then grouped into classes according to electrophoretic mobility at a restricted pH, defined in terms of cold ethanol solubility, and eventually resolved by various kinds of electrophoresis, immunoelectrophoresis and electro-focusing techniques into various components, not all of which have yet been identified with a particular function. An older review of serum proteins, primarily of historical interest, may be found in Howe (1925). As happens repeatedly, some of the proteins have been identified in a number of different laboratories, and have been given a variety of names. In most cases, one of the names gains widespread acceptance. The nomenclature of certain classes of plasma proteins (such as the immunoglobulins) has been standardized by international agreement. Some effort has been made to arrive at a uniform nomenclature for the genetic variants of serum proteins, and this has been partially successful. This matter is considered in subsequent sections in connection with particular systems.

The question of what actually constitutes a serum (plasma) protein is not that easy to answer. Some criteria can be established, but there are always proteins that are difficult to classify, in part because they are poorly characterized and/or have no known function. Putnam (1975a) distinguished between the "true" serum proteins, and what were called "passenger proteins". The latter may be found in the serum at certain times, or even all the time, but they traverse the serum in transit from one site to another as the result of a particular physiological condition (e.g. pregnancy), as the result of disease, or because they are directly involved in transport processes. No classification system can easily accommodate the number and variety of plasma proteins now known. There are some protein systems in plasma whose members act together in a concerted manner, such as the complement system, the lipoproteins, the coagulation proteins, the protease inhibitors and the immunoglobulins. There are about 35 to 50 proteins generally recognized as true plasma proteins; but there are probably 100 or more other proteins which can be found in plasma, but have not been well characterized.

Putnam (1975a) presented several criteria for classifying a protein as a "plasma protein": (1) The protein must be present in plasma after the neonatal period; (2) Synthesis must take place in the liver or the reticuloendothelial system; (3) The primary function (if known) must be mediated in the vascular system, rather than in the target system; (4) The protein should be actively secreted into the bloodstream, and not be there as a result of tissue damage or capillary permeability; (5) The concentration should be higher in blood than in other fluids, except in the specialized cells which synthesize the protein; (6) It should have an appreciable half-life in plasma, and not be transitory; (7) Genetic polymorphism (if exhibited) should not be traceable to tissues of origin, as is the case with a number of the enzyme systems (see in Unit VI); and (8) A true plasma protein is not derived by proteolytic cleavage or catabolism of other plasma proteins, as for example the Fab and Fc fragments of immunoglobulins; however, true precursors, and their active forms, e.g. plasminogen and plasmin, should both be considered plasma proteins.

It is difficult to arrive at a criterion based upon minimal concentration in plasma, because the plasma proteins exhibit a very wide range of concentrations. Albumin is present in concentrations of about 5 g/100 mℓ, while the immunoglobulin IgE occurs to the extent of about 5×10^{-7} g/100mℓ. On the basis of concentration in normal

plasma, one can arbitrarily establish four classes of serum proteins (Putnam, 1975a): Predominant proteins, such as albumin and IgG (1–5 g/100 mℓ); Other major proteins (100–1000 mg/100 mℓ); Minor proteins (10–100 mg/100 mℓ); and Trace proteins (< 10 mg/100 mℓ). The lower the concentration of a protein, the more sensitive must be a technique used to measure its presence, and the less likely that normal concentration ranges have been established. Some of the proteins occurring at very low normal concentrations have been studied because their levels are greatly elevated for various reasons in certain individuals. A list of some of the better characterized serum proteins, and some of their properties, is given in Table 39.1. The properties of many of these proteins have been reviewed by Putnam (1975b) and by Cooper (1978).

The serum proteins which exhibit genetic variation at polymorphic levels constitute an important class of genetic markers. They are discussed in more detail in the sections which follow. A review of the properties of a number of polymorphic serum proteins was given by Gitlin and Gitlin (1975). Genetic variation in a protein may be reflected in the protein's structure itself, or in the amount of the protein that is present. Both structural and quantitative variation may have a number of different genetic bases. The principles of biochemical genetics that were outlined in section 1.2.2, that were seen to operate in the case of isoenzyme variation (Unit VI), and about which a great deal has been learned from the study of Hb variants (section 38), are fully applicable to plasma proteins. Alterations, additions or deletions of bases in the coding sequence for a protein may lead to an altered structure for that protein which is detectable and recognizable. The alteration can be a single amino acid substitution, or a much more drastic one. Deletions or additions of bases in the coding sequence can cause a shift in the "reading frame", and alter the structure of the protein beyond the point of occurrence. The amino acid sequence may be different, or the polypeptide chain can be shortened if a nonsense or termination codon was introduced. Unequal crossovers between genes can result in polypeptide chains much shorter or longer than is usual. Quantitative changes are often genetically controlled as well, although the mechanisms may be indirect. Alterations in genes controlling products responsible for conversion of one protein to another, alterations in operator or regulator genes which control structural genes, or alterations in the production of m-RNA can all give rise to various quantitative variations. The number and variety of genetic variants known for a particular system are dependent upon the likelihood with which they will come to someone's attention, and upon the ability of the various techniques to discriminate them. Subtle, or even not so subtle, variations which have no clinical manifestations or implications are less likely to be noticed than those which do have. Similarly, even in cases where various populations are screened for variants or abnormalities of a certain protein, the technique being used is unlikely to be able to detect every kind of variant. Electrophoresis, for example, readily detects variants in which the net charge of the protein is altered. But the substitution of one uncharged, straight chain amino acid for another, for example, could easily escape electrophoretic detection. A one base change in a gene leading to a GUU codon instead of a GCU codon would lead to a Ala→Val substitution in the polypeptide chain for example.

The development of many of the methods used for the study of serum proteins was discussed in section 2. In many ways, the nomenclature of the serum proteins has grown up and developed along with the methods used to study them. In the last century, the terms "albumin" and "globulin" were coined to represent serum protein fractions that were soluble and insoluble, respectively, in water. With the development of salt fractionation techniques, different "globulins" were distinguished (euglobulins; pseudoglobulins). An account of the many studies conducted, and the terms that were introduced, may be read in Pedersen (1945). Development of moving boundary electrophoresis (Tiselius, 1930) by Tiselius and his collaborators was an important step. Using the technique, four distinct fractions could be distinguished in horse serum (Tiselius, 1937), and these were named α, β and γ globulin components. The fourth fraction was albumin. Development of analytical ultracentrifugation, and its application to the study of serum proteins (see Pedersen, 1945), was also an important step in the effort to characterize the various components more completely. Subsequent electrohoretic investigations led to subdivision of the original serum protein fractions: α_1, α_2, β_1, β_2, γ_1, γ_2. The subscript notation is traditional, but it is equally acceptable today to write α1, α2, β1, etc., or α-1, α-2, β-1, etc. (Putnam, 1975a). These electrophoretic characterizations of serum proteins are still used to place many of them into generic classes, even though it is recognized that any number of structurally and functionally diverse proteins may be under a single electrophoretic peak. Development of zone electrophoretic, immunoelectrophoretic and isoelectrofocusing procedures, as well as immunofixation detection techniques and their variations, have accelerated the flow of new information about various serum proteins. Zone electrophoresis was first performed on paper as a supporting medium (Cremer and Tiselius, 1950; Kunkel and Tiselius, 1951), and later on starch gels (Kunkel and Slater, 1952). Starch gel electrophoresis was significantly refined by the investigations of Smithies and collaborators (Smithies, 1955a, 1955b, 1959a, 1959b; Poulik and Smithies, 1958), with the characterization of several serum proteins occurring along the way (see in subsequent sections). Resolution could be enhanced by carrying out electrophoresis in two dimensions (Smithies and Poulik, 1956). Electrophoresis may be carried out on cellulose acetate foils (Kohn, 1957 and 1958), agarose gels (Gordon *et al.*, 1949), and polyacrylamide gels (Raymond and Weintraub, 1959) as well. The sensitive and high-resolution polyacylamide disc gel electrophoresis technique was introduced by Ornstein (1964) and Davis (1964). Immunoelectrophoresis is likewise an important and sensitive technique for the separation and characterization of serum proteins (see in Grabar and

Table 39.1 Properties Of Some Serum (Plasma) Proteins

Name of Protein	Synonyms	Symbol	Molecular Weight	Electrophoretic Mobility pH 8.6 Barbital (μ = 0.1)
Prealbumin	D_1-protein Prealbumin I Thyroxine-binding prealbumin (TBPA)	PA	55,000	7.6
Albumin	Serum albumin	Alb	68,300	5.9
α_1-acid glycoprotein	orosomucoid α_1-seromucoid α_1-globulin	α_1-S	44,000	5.2
α_1T-glycoprotein	Tryptophan-poor α_1-glycoprotein	α_1T	≅ 60,000	
Transcortin		TC	55,700	
α_1-antitrypsin	α_1-3.5S glycoprotein α_1-glycoprotein α_1-seromucoide de Schultze α_{1A}-globulin α_{1B}-globulin	α_1-AT (PI)	54,000	5.4
α_1-antichymotrypsin	α_1X-glycoprotein	α_1X	68,000	
α_1B-glycoprotein	easily precipitable α_1-glycoprotein	α_1B	50,000	
Zn-α_2-glycoprotein		Znα_2	41,000	4.2
Thyroxine-binding globulin		TBG	58,000	
Antithrombin III	α_1-antithrombin	ATIII	≅ 65,000	
Gc-globulin	Group specific component Gc-factor postalbumins 2 + 3	Gc	50,800	
C1s component	C'1 esterase	C1s	86,000	
Inter-α-trypsin inhibitor		IαI	≅ 160,000	
Retinol binding protein		RBP	21,000	
α_2HS-glycoprotein	Ba-α_2-glycoprotein α_{2Z}-globulin α_{2HS}-mucoid postalbumin 3	α_2-HS	49,000	4.2
C1 inactivator	α_2-neuraminoglycoprotein C'1 esterase inhibitor	C1-Ina	104,000	
3.8S histidine rich α_2-glycoprotein		HRG	58,500	
C9 component		C9	79,000	
Haptoglobin	seromucoide α_2	Hp	100,000 (1-1)	4.5
Ceruloplasmin	α_2-IV Metalloseromucoide α	Cp	151,000	4.6
Serum cholinesterase	Pseudocholinesterase	PCE ChE E_1	348,000	3.1
α_2-macroglobulin	Sα_2-globulin seromucoide α_2 de Schultze	α_2M	725,000	4.2
Plasminogen	Profibrinolysin	Pmg (Pg;PLG)	81,000	3.7
Hemopexin	Heme-binding β-globulin Seromucoide β_{1A} Seromucoid β_{1B} β_{1B}-globulin β_1-haptoglobin (β_{1H})	Hpx	57,000	3.1
Transferrin	Siderophilin β_1-metal combining globulin β_{1S}-globulin	Tf	76,500	3.1
C2 component		C2	206,000	
C3 proactivator	glycine-rich β-globulin	C3PA	≅ 80,000	
C1r component		C1r	160,000	
C5 component	β_1F-globulin	C5	180,000	
C3 component		C3	185,000	
C4 component	β_1E-globulin	C4	206,000	
β_2-glycoprotein-I	β_{2X}-globulin β_2-mucoid	β_2I	40,000	1.6
C6 component		C6	95,000	
C7 component		C7	100,000	
IgA	γA immunoglobulin γ_{1A}-globulin γ_{2A}-globulin	IgA	160,000	2.1
C8 component		C8	153,000	
Fibrin stabilizing factor	Coagulation factor XIII	FXIII	340,000	
IgM	γM immunoglobulin γ_1M-globulin γ_1-macroglobulin 19S γ-globulin β_{2M}-globulin	IgM	1,000,000	2.1
IgG	γG immunoglobulin 7S γ-globulin γ-globulin γ_{SS}-globulin γ_2-globulin	IgG	160,000	1.2
C reactive protein		CRP	135,000	
Porperdin			220,000	
C1q component	11S component	C1q	400,000	
Lysozyme	muramidase		≅ 15,000	
α_1-lipoprotein	high density lipoprotein			
β-lipoprotein	low-density lipoprotein			
Fibrinogen	coagulation factor I		341,000	2.1

Burtin, 1964). The quantitative versions of immunoelectrophoresis (such as rocket electrophoresis and crossed immunoelectrophoresis) yield additional information about a mixture of serum proteins (see Axelsen *et al.*, 1973). One of the problems with many serum proteins is specific detection. Methods making use of specific antibodies are among the most useful for this purpose, and electrophoretic separation may be combined with an overlay detection system that incorporates a specific antibody. The technique is usually called immunofixation electrophoresis (Alper and Johnson, 1969); it was originally devised by Wilson (1964), who called it "direct electrophoresis". Immunofixation electrophoresis is used for the electrophoretic typing of several polymorphic serum protein systems of interest in forensic science. The relationship between various serum proteins as they appear after separation by various forms of electrophoresis and immunoelectrophoresis is shown in Figure 39.1.

Individual polymorphic serum protein systems are discussed in subsequent sections of Unit VII. Attempts to diagnose individual differences in "serum protein profiles" are discussed in Unit VIII.

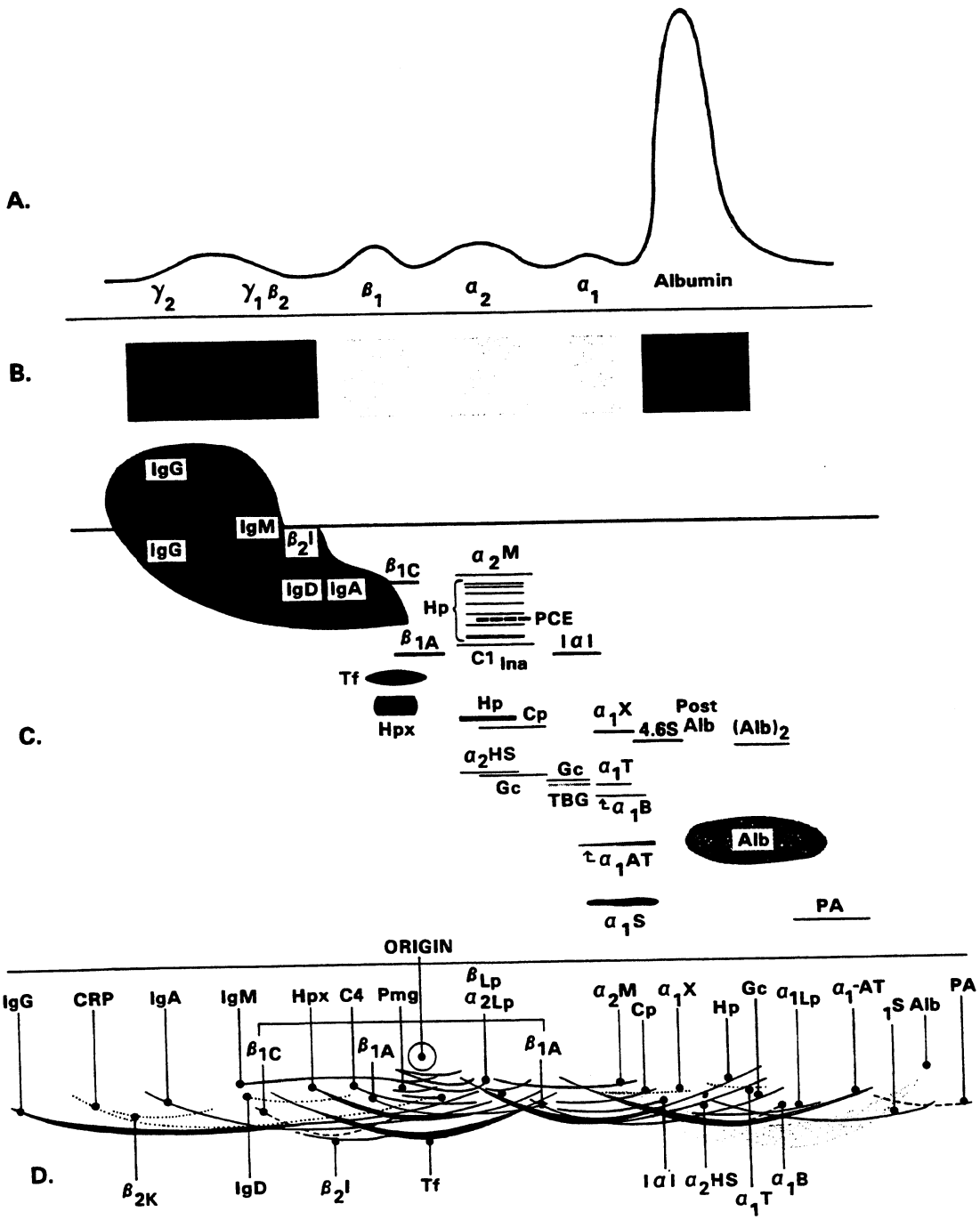


Figure 39.1 Plasma (Serum) Protein Profiles by Various Techniques

A. Moving Boundary Electrophoresis (pH 8.6)
B. Paper Electrophoresis
C. Two-dimensional Starch Gel Electrophoresis
D. Immunoelectrophoresis

SECTION 40. HAPTOGLOBIN

40.1 Recognition of Haptoglobin (Hp)

In 1938, Polonovski and Jayle found a protein in serum which bound hemoglobin. They had noticed that the addition of serum enhanced the peroxidase activity of Hb (section 6 introduction). The Hb binding protein was further characterized, found to be an α_2 glycoprotein, and given the name "haptoglobin", from a Greek root meaning "to bind" (Polonovski and Jayle, 1940). They had considered calling the protein "prosaptoglobin", but had settled on the now familiar name. "Haptoglobin" is not a single molecule, but a group of closely related ones, and it is proper to speak of "the haptoglobins". The multiplicity of molecular forms is now known to be based on the control of Hp synthesis by a polymorphic genetic locus, as a result of which one may refer to the "haptoglobin system". Determination of serum haptoglobin levels was found to be a clinically useful and significant measurement, and standardized procedures based on Hb binding and peroxidase activity (Jayle, 1951; Connell and Smithies, 1959) and using quantitative immunochemical techniques (Kluthe *et al.*, 1965) were developed. It is now clear that the quantitative immunochemical methods are not as straightforward as was first thought (Valette *et al.*, 1979), in that the results obtained are dependent on the Hp phenotype. This behavior can probably be explained by immunodeterminant differences in the Hp subunits (see below). The extensive studies of the Paris group on haptoglobins were well summarized by Jayle and Moretti (1962).

40.2 Haptoglobin Physiology

Many studies have been conducted on haptoglobin physiology and function (Jayle, 1956; Kirk, 1968a; Giblett, 1968 and 1969; Javid, 1967b; Pintera, 1971), and not every recorded observation has been satisfactorily accounted for as yet. The liver is probably the site of haptoglobin synthesis, and the amount of Hp produced is subject to many influences. It can vary considerably in different physiological and pathological states. There is some variation in the normal level of Hp, values being of the order of 40 to 180 mg/100 mℓ, expressed in terms of Hb binding capacity. The quantity of Hb bound by Hp is related to the Hp type (see below). Haptoglobin may be catabolized as free Hp, or in complex with Hb. More is known about the latter route. Free hemoglobin released into circulation, is immediately complexed with Hp, and the complex is removed by the reticuloendothelial system. The half-life of the complex in circulation is a function of its concentration (Noyes and Garby, 1967), the clearance rate tending to be more exponential at lower concentrations of complex, and more linear at the higher ones.

Free hemoglobin in plasma can pass across the renal glomerulus, whereas Hb complexed with Hp cannot (Allison and ap Rees, 1957). As a result, hemoglobin is cleared from plasma (at least in rabbits) significantly faster than is Hb–Hp complex (Murray *et al.*, 1961). However, Hb is found to be cleared more quickly even in nephrectomized animals. In 1958, Allison suggested that a principal function of Hp was to prevent the loss of Hb (and, hence, the loss of iron) across the renal glomerulus. Both free Hb and Hb–Hp complex are catabolized primarily in the liver (Keene and Jandl, 1965; Murray *et al.*, 1961). If free Hb is injected into the bloodstream, it is rapidly complexed by haptoglobin, and the complex is cleared from circulation, thus temporarily depleting the plasma of (the complexed) Hp (Noyes and Garby, 1967; Laurell and Nyman, 1957). In order to observe hemoglobinuria, the level of free Hb in circulation must exceed the level of Hp available for complex formation (Laurell and Nyman, 1957). Hemoglobinuric patients are found to have little or no plasma haptoglobin (Allison and ap Rees, 1957). Free Hb may get into the circulation in significant amounts as a result of hemolytic episodes. If an amount of free Hb sufficient to bind all the Hp is injected into circulation, Hp levels fall to undetectable levels within about 24 hrs (Laurell and Nyman, 1957). Normal Hp levels will return within a few days following total depletion. Haptoglobin is synthesized to the extent of about 30–50% of the intravascular pool each day (Noyes and Garby, 1967). There is no evidence that haptoglobin depletion causes an increase in the rate of Hp synthesis by a "feedback" mechanism. The effectiveness of haptoglobin in conserving hemoglobin (and, thus, iron) has been questioned on several accounts (Giblett, 1969), and the physiological role of haptoglobin is undoubtedly more complicated (Pintera, 1971).

Recently, Prof. Dr. Prokop and his collaborators have observed that human haptoglobins can behave as antibodies against streptococci possessing the T4 antigen. This effect varies according to the Hp phenotype, type 2-1 and 2-2 Hp's behaving as high titered complete antibodies, while 1-1 derived Hp acts like a "blocking" antibody (Köhler *et al.*, 1978; Prokop and Köhler, 1979; Prokop, 1979). Homologous animal haptoglobins exhibit the effect as well. These observations are particularly interesting in view of the structural homology between the short polypeptide chains of Hp and the light chains of the immunoglobulins (see below in 40.3.6).

40.3 Genetics and Biochemistry of Haptoglobins

40.3.1 Genetic variation in haptoglobin

Haptoglobin was well studied electrophoretically, both as free Hp and complexed with Hb, before the polymorphism

was fully recognized (Wieme, 1953; Jayle *et al.*, 1952; Tuttle, 1955a and 1955b). In 1947, Jayle and Gillard noticed that there appeared to be more than one kind of haptoglobin present in plasma on the basis of ammonium sulfate fractionation, which was part of the Hp purification procedure. In 1955, Smithies found the genetically controlled variation in haptoglobin by starch gel electrophoresis. These studies were the same ones in which the now familiar zone electrophoresis technique on starch gels was elaborated (section 2.3.4). Three different starch gel electrophoretic patterns of serum proteins could be observed, the differences lying in those proteins between the so-called "fast" and "slow" α_2 globulins. Complex changes occurred in the banding patterns depending upon the amount of Hb present. The proteins all bound hemoglobin, and were easier to type in its presence (Smithies, 1955a). The different "groups" were originally called I, II_A and II_B, and were well distributed even in a small sample of sera from different individuals. It was suggested (Smithies, 1955b) that these differences had a genetic basis. Studies on eighteen families were consistent with a straightforward genetic model in which "group I" and "group II_B" were homozygotes for each of a pair of codominant alleles, and "group II_A" was the heterozygote (Smithies and Walker, 1955). It was quickly realized that the proteins exhibiting this variation were identical to the hemoglobin-binding proteins of Polonovski and Jayle (1938), Wieme (1953), Tuttle (1955a), and others, namely haptoglobin (Smithies and Walker, 1956). Accordingly, it was suggested that this family of proteins be designated the "haptoglobin system", that the genetic locus be called *Hp*, and the two alleles Hp^1 and Hp^2. "Group I" was Hp 1-1 (Hp^1Hp^1), "II_A" was Hp 2-1 (Hp^2Hp^1) and "II_B" was Hp 2-2 (Hp^2Hp^2). These features of this serum group system were quickly confirmed (Moretti *et al.*, 1957; Sutton *et al.*, 1956), and the proposed mode of inheritance has been widely confirmed by family and population studies (e.g. Galatius-Jensen, 1956, 1958a and 1960; Prokop *et al.*, 1961; Kirk, 1968a and 1971). It was also clear that there were significant differences between the Hp allele frequencies in ethnically distinct populations (Sutton *et al.*, 1956; Allison *et al.*, 1958; Giblett, 1959), and that some Black African populations presented certain complexities in their Hp, not seen up to that time in Europeans (Allison *et al.*, 1958; Giblett, 1959).

40.3.2 Additional genetic variation at the Hp^1 locus—Haptoglobin "subtypes"

In 1962, Connell *et al.* subjected partially purified haptoglobins, representing the three common (1-1, 2-1 and 2-2) phenotypes, to electrophoresis after reductive cleavage in the presence of mercaptoethanol. Two classes of products were obtained, one of which appeared to be common to all Hp molecules, and which did not migrate in starch gels at acidic pH unless 8M urea was present. However, the other products migrated differently according to the phenotype. This latter, called the "hp 1" product, showed two different patterns, fast (F) and slow (S). This behavior was attributed to the presence of two Hp^1 alleles, Hp^{1F} and Hp^{1S}. With three alleles, Hp^{1F}, Hp^{1S} and Hp^2, six phenotypes could be observed (provided appropriate electrophoretic conditions were employed): Hp 2-2, Hp 2-1F, Hp 2-1S, Hp 1F-1F, Hp 1F-1S and Hp 1S-1S. Family studies by Smithies *et al.* (1962a) indicated that this genetic model was correct, and a survey of a small number of Europeans (from Toronto and Madison, WI) indicated that the frequencies of Hp^{1F} and Hp^{1S} were about 0.16 and 0.24, respectively, assuming Hp^2 to be about 0.6. This genetic hypothesis has been confirmed (Shim and Bearn, 1964a; Ehnholm, 1969; Fagerhol and Jacobsen, 1969). Fagerhol and Jacobsen (1969) used a different discontinuous buffer system for the resolution of the Hp 1 subtypes, and originally called them "E", "K" and "R", but said that they were equivalent to Hp 1F-1F, 1F-1S and 1S-1S, respectively. Electrophoretic patterns of the three common Hp phenotyhpes as seen on starch gels at pH 8.6, and those of the α polypeptides in the subtypes under denaturing conditions, are shown in Figure 40.1. The biochemical genetics of haptoglobin types and subtypes is discussed further below.

40.3.3 Other Hp variants and Hp 0

A number of variants of haptoglobin have been found, and the genetic explanation for some of them is not as clear as it is for others. The variants are described in this section. The genetic basis for their occurrence is considered later in connection with the structure and biosynthesis of the haptoglobins. Giblett (1969) usefully classified the Hp variant phenotypes as "quantitative" or "qualitative".

40.3.3.1 Quantitative variants. The majority of quantitative variants are modifications of the 2-1 phenotype. The Hp 2-1(mod) (Giblett, 1959), or Hp 2-1M (Connell and Smithies, 1959) phenotype shows a spectrum of patterns, ranging from a high concentration of the slower moving polymers (see below) with no visible fast Hp 1 band, to a high concentration of the fast band with only one of the members of the polymeric series visible (Giblett, 1969). Hp 2-1M (where "M" and "mod" indicate "modified") occurs at appreciable frequencies in the Black population (some 10% of American Blacks), but only occasionally in Europeans. Harris *et al.* (1960) described Hp 2-1M in a White family, and said that the pedigree indicated either a Hp^{2M} gene at the *Hp* locus, or modifier gene at another locus. If the Hp^{2M} gene was present, it gave a Hp 2-1M pattern in Hp^1Hp^{2M} people, but a 2-2 pattern indistinguishable from the usual one in Hp^2Hp^{2M} people. Giblett and Steinberg (1960) examined about 500 sera from American Blacks in 92 families, and found about 15% of a sample of unrelated individuals to be Hp 2-1M. They thought that the phenotype was a manifestation of an Hp^{2M} allele. Sutton and Karp (1964) thought that Hp 2-1M could be divided into four classes, called b, c, d and e, on the basis of the shift toward the faster moving bands. They believed that there were two alleles, Hp^{2cd} and Hp^{2e}, controlling the 2-1M phenotypes. Parker and Bearn (1963) offered an alternative explanation based on variation at a regulator gene locus,

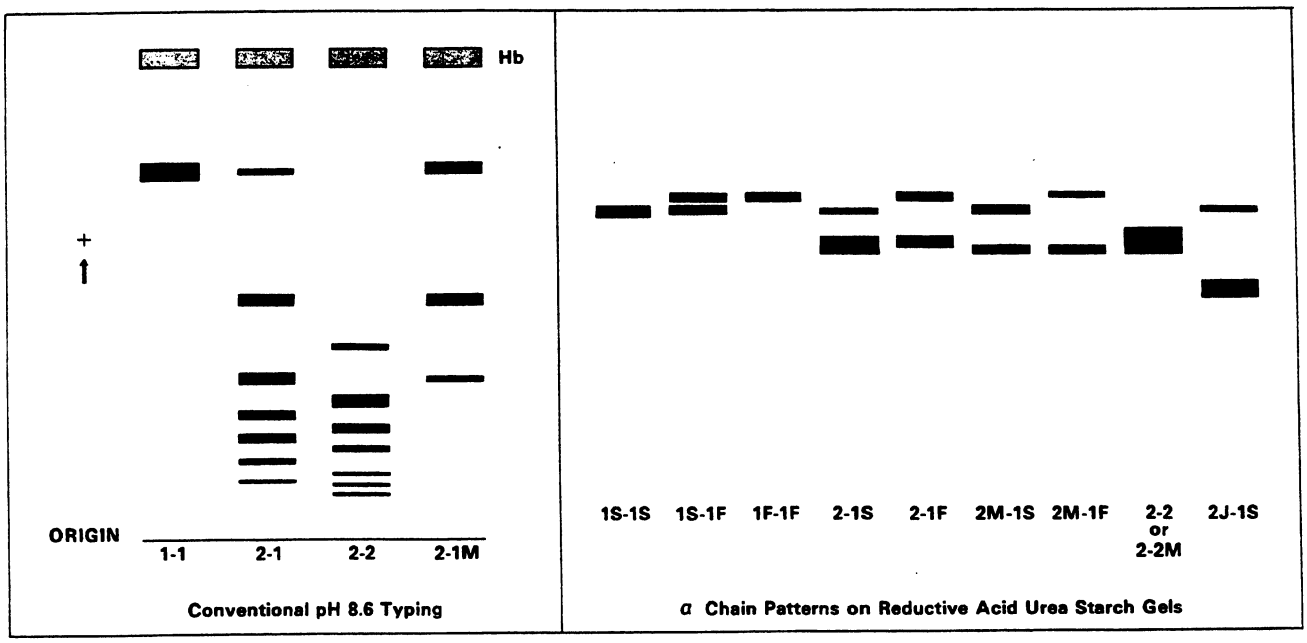


Figure 40.1 Electrophoretic Patterns of Hp Types and Subtypes

close to but not identical with the *Hp* locus. The genetic basis for Hp 2-1M is apparently still not completely understood.

Galatius-Jensen (1958b) found a phenotype which represented quantitative variability in the Hp^1 product. It was called 'Hp Carlberg', usually written Hp Ca. It has been observed in other populations as well (Giblett, 1964; Nance and Smithies, 1964; Harris *et al.*, 1959). The electrophoretic pattern resembles that of a mixture of Hp 2-2 and 2-1 in variable proportions. Subtyping shows reduced Hp^1 product in relation to Hp^2 product, although the ratio can vary even within the same family. Sutton (1965) has suggested that Hp Ca may be the result of genetic mosaicism, different cell populations in the same person producing Hp 2-2 and Hp 2-1. Hp Ca is inherited, however, and if mosaicism is the explanation, then an inherited tendency toward cellular somatic mosaicism would have to be postulated (Giblett, 1969).

Two other variants, described by Giblett (1964), are Hp 2-1(trans) and Hp 2-1(Haw). The 2-1(trans) exhibits a pattern that shows an increase in the faster moving components and a proportionate decrease in the slower ones. This shift is not as pronounced as in Hp 2-1M, and gives the appearance of a 'transitional' phase in going from an Hp 2-1 to an Hp 2-1M pattern (hence, the name 'trans'). Hp 2-1 (Haw) was so named because it was found in the serum of an Hawaiian subject. The pattern is similar to 2-1M in having a heavy concentration of the fastest bands, but differs in that there is no associated increase in the intensity of the second band nor a relative decrease in the intensities of the third and fourth bands.

One of the more puzzling kinds of quantitative variation is represented by the phenotype "Hp 0", which denotes anhaptoglobinemia or hypohaptoglobinemia. Failure to detect Hp by most of the electrophoretic methods used does not necessarily mean that it is completely absent; it usually means that the amount of Hp present is less than 15–20 mg/100 mℓ expressed as Hb-binding capacity (Giblett, 1969).

In 1958, Allison *et al.* tested 99 Nigerian sera for Hp and found that a little over 32% of them had no detectable activity. The finding was further discussed by Allison in 1958. Sutton *et al.* (1956) saw no Hp in a small sample of bloods from the Ivory Coast. Giblett (1959) found that about 4% of about 400 Black people from Seattle were Hp 0. Hp 0 is much more common as the result of matings involving an Hp 2-1M or Hp 0 parent, and it is rare if the parents are Hp 1-1 (Giblett and Steinberg, 1960; Sutton and Karp, 1964). The $Hp^{2M}Hp^2$ genotype, when expressed, cannot be distinguished from Hp^2Hp^2. The offspring of Hp 2-1M × 2-1M matings are found to be Hp 1-1, Hp 2-1M or Hp 0. It appears that $Hp^{2M}Hp^{2M}$ people are usually (or always) Hp 0. Black people who are phenotypically Hp 0 are often $Hp^{2M}Hp^1$ or $Hp^{2M}Hp^2$ (Giblett and Steinberg, 1960), although they can have apparently normal genotypes (Hp^2Hp^2 or Hp^2Hp^1) as well. These genotypical interpretations were given on the assumption, of course, that there really is an Hp^{2M} allele. Parker and Bearn (1963) proposed that Black populations carried a mutant in a regulator gene, and that Hp 0 could represent homozygosity for this mutant allele. There is evidence that such a gene may occur in Caucasian populations as well, albeit at very low frequencies (Murray *et al.*, 1966). In spite of the difficulties associated

with proving regulator gene hypotheses, they do appear more attractive than the original one based solely on Hp^{2M}. It is quite likely that the factors involved in determining Hp 2-1M and Hp Ca are applicable to Hp 0. There is some evidence for the existence of a very rare Hp^0 allele (Harris *et al.*, 1958a; Matsunaga, 1962; Schwerd and Sander, 1967). In these families, a homozygous Hp 1-1 or 2-2 parent had one or more children who appeared to be homozygous for the other allele. In some cases, Hp^0Hp^1 and Hp^0Hp^2 combinations are expressed like Hp^1Hp^1 or Hp^2Hp^2, respectively, but in other cases the phenotype is Hp 0.

Giblett (1969) noted that, in view of the fact that an Hp 1 phenotype could be the result of Hp^1Hp^1 or Hp^1Hp^0 genotypes, and similarly for the Hp 2 phenotype, the phenotypic nomenclature should reflect this fact. For some reason, homozygous haptoglobin types are more often expressed as "1-1" or "2-2", rather than just "1" or "2". For most other systems, phenotypic nomenclature denotes what is observed rather than implying a genotype. Giblett said that this latter practice should be followed with haptoglobin as well, and the suggestion is a good one. In summary, it appears that there is more than one genetic explanation for Hp 0, and that the one based on an operator or regulator gene mutation is not completely developed as yet.

It is important to note, before leaving the subject of anhaptoglobinemia, that haptoglobin genotypes are not fully expressed in a majority of fetuses and newborns. Many are, therefore, "anhaptoglobinemic", but this condition is transitory and reflects the rate at which the *Hp* genes become active. Detectable haptoglobin is usually found in about 10-15% of cord blood and newborn sera (Rausen *et al.*, 1961), but this percentage increases rapidly until it reaches nearly adult levels by about 4 months age (Hauge *et al.*, 1970). Few infants have less than 30 mg/100 mℓ haptoglobin at 6 months age (Bergstrand *et al.*, 1961). The Hp type expressed in cord blood and newborn serum reflects the genotype of the infant, and not that of the mother (Hirschfeld and Lunell, 1962; Siniscalco *et al.*, 1963). Hirschfeld and Lunell (1962) found an Hp 2-1 mother with a pair of twins (who had been stillborn), and the twins had Hp 1 and Hp 2 phenotypes. The data of Siniscalco *et al.* (1963) suggested that the onset of Hp synthesis in infants might depend on the maternal genotype, while Hauge *et al.* (1970) noted that the Hp 2 phenotype tended to develop more slowly than the others.

40.3.3.2 Qualitative variants. These variants contain components which are not present in the common phenotypes, and they are all very rare. They may be further classified as α chain variants or β chain variants, since it is now clear that the two kinds of polypeptide chains are coded for by different and independent genetic loci (see below). The best known rare variant is Hp 'Johnson', originally observed in a Black woman and her daughter in Seattle by Dr. Giblett. It reveals one or the other of the 1S or 1F α peptides, and a much slower, heavily-staining α chain (Smithies *et al.*, 1962a; Giblett and Brooks, 1963). The polypeptide made by the 'Johnson' allele was originally called hp 2Jα, but Giblett (1969) suggested that it should perhaps be called 'α^J' or 'α^3' (the latter, since it may be the result of a partial gene triplication—see in 40.3.6). When Hp^J is heterozygous with Hp^2, the haptoglobin synthesis is greatly reduced, and it is only possible in the occasional specimen to determine the electrophoretic pattern (Giblett, 1969). Isolated examples of Hp 1-J have been seen in such widely disparate populations as American Blacks, Kurdish Jewish, Australian aborigine and European (Smithies *et al.*, 1962b). Mukherjee and Das (1970) described a 2-1J in a Bengali Hindu, and Höglund *et al.* (1970) found 7 Hp 1-J types among 15,601 Swedish adults. Minor differences in the patterns are consistent with independent origin of the genes according to the unequal crossing over hypothesis (see below), although the samples were not freshly drawn when compared. In 1966, Giblett *et al.* described two more structural variants, which were the result of a new allele Hp^B. Both 1-B and 2-B phenotypes were observed. The α polypeptide conditioned by Hp^B migrates between hp α^2 and hp α^{1S}. Hp 1-B and 2-B are referred to as the "Ba" types by Giblett (1969). It is possible too, she said, that these represent β chain variations. Renwick and Marshall (1966) described the Hp 2-1D phenotype, thought to be the result of an allele Hp^{1D} paired with Hp^2. In the presence of saturating amounts of Hb (which is the way Hp is usually typed), Hp 2-1D and Hp 2-1 are not distinguishable. But under "subtyping" conditions, a band which runs faster than 1F is revealed (representing the α^{1D} peptide). The "D" stood for "dashing" in the name.

The so-called 'Marburg' phenotypes were originally seen in a German family (Aly *et al.*, 1962), and have been extensively studied by Cleve and Deicher (1965) and Weerts *et al.* (1965). All the electrophoretic components of Hp 2-1Mb demonstrated atypical immunological reactions with certain anti-Hp sera. The antigenic determinant called "B" (see below), which is on the β chain and is normally blocked in the Hp-Hb complex, still reacted even when saturating amounts of hemoglobin were present. Subtyping revealed no unusual α polypeptide chains, and it was concluded that this variant represented a β chain mutation. Bowman and Cleve (1967) have shown that the 'fingerprint' of the β chain of Hp Mb is different from the usual one. Another phenotype, called Hp 2-1 Bellevue, was described by Javid (1967a). It had immunological properties resembling those of the 'Marburg' haptoglobins, but the electrophoretic pattern differed. Electrophoresis under subtyping conditions suggested that the phenotype represented heterozygosity for a β chain variant. The 35 year old Black propositus had three sons who had apparently inherited the variant gene.

Other variants have been described which may represent β chain mutations. In 1964, Robson *et al.* described five new phenotypes called Hp 1-P, 2-P, 1-H, 2-H and 2-L. The electrophoretic behavior of the components of these haptoglobins differed in the presence and absence of hemoglobin, suggesting that something was unusual about their ability to complex with Hb. The alleles thought to be responsible for these types were called Hp^P, Hp^H and Hp^L. A similar

variant was reported by Giblett (1964). Hp Ab, as it was called, was found in a woman in Boston by Dr. Irving Umansky. The α polypeptides in these phenotypes migrated like 1F, 1S and 2 α chains usually do. The low pH of the gels used for subtyping might prevent separation of the variant chain (if it were there) by protonating carboxyl groups (Nance and Smithies, 1964). Amino acid residues which were neutral or equally charged could also be involved. Shim *et al.* (1965) said that the P and L variants might be additional mutants at the β chain locus.

The structure of haptoglobin is discussed below, but the molecule is known to consist of α and β chains, coded for by different loci. The so-called *Hp* locus is the α chain locus, and most of the variants, including the three common types representing the polymorphism, are α chain variations. Some β chain variants are now known, however, and it would be useful to distinguish between the structural loci in the nomenclature. Javid (1967a) showed that the α and β loci are not closely linked, and suggested Bp as a symbol for the β chain locus. The common allele would be Bp^A and Bp^B would represent the allele seen in Hp 2-1 Bellevue. Giblett (1969) suggested, however, that it would be preferable to call the α chain locus Hp_α and the β chain locus Hp_β, and this seems like the best idea. The electrophoretic patterns exhibited by some of the Hp variants are shown in Figure 40.2. A useful table of many of the variants was given by Kirk (1968a), and is reproduced in Pintera (1971) and in Putnam (1975c).

40.3.4 Structure of the haptoglobins

Efforts to purify and characterize haptoglobin have been undertaken by many workers, since the first work by Jayle and his collaborators as summarized by Jayle and Moretti (1962). One of the first purification schemes was devised by Jayle and Boussier (1954). Refinements were developed by Guinand *et al.* (1956), Jayle *et al.* (1956), Moretti *et al.* (1958) and Herman-Boussier *et al.* (1960). Haptoglobin has been isolated from serum, from ascitic fluid in some patients, and from the urine of certain patients who excrete it if it is of the 1-1 type. Purification methods for haptoglobin are discussed in some detail in Pintera (1971) and in Putnam (1975c).

The monomeric unit of human haptoglobin (represented by the molecule found in type 1-1 people) is composed of four polypeptide chains, two α chains and two β chains, connected by disulfide bridges (Shim and Bearn, 1964b; Malachy and Dixon, 1973a and 1973b; Malachy *et al.*, 1973). The general structure of the molecule (Figure 40.3) is reminiscent of the structure of the IgG molecule (Figure 1.41). The chain can be dissociated by reductive cleavage with mercaptoethanol in the presence of 8M urea (Smithies *et al.*, 1966; Connell *et al.*, 1966). The Hp 1-1 molecule is the only one, from among the three common types, that appears to be homogeneous. It migrates as a single band in starch gel electrophoresis, whether complexed with Hb or not, and exhibits a single ultracentrifuge peak. The 2-1 and 2-2 types contain more than one protein species, which can be observed in the ultracentrifuge, or by starch gel electrophoresis (Bearn and Franklin, 1958 and 1959), and which differ to some extent immunologically (Korngold, 1963; Eichmann *et al.*, 1966). The multiplicity of bands seen upon starch gels with 2-1 and 2-2 types is familiar to anyone who has typed haptoglobin. These bands are now known to represent a series of polymers, which are, however, very stable,

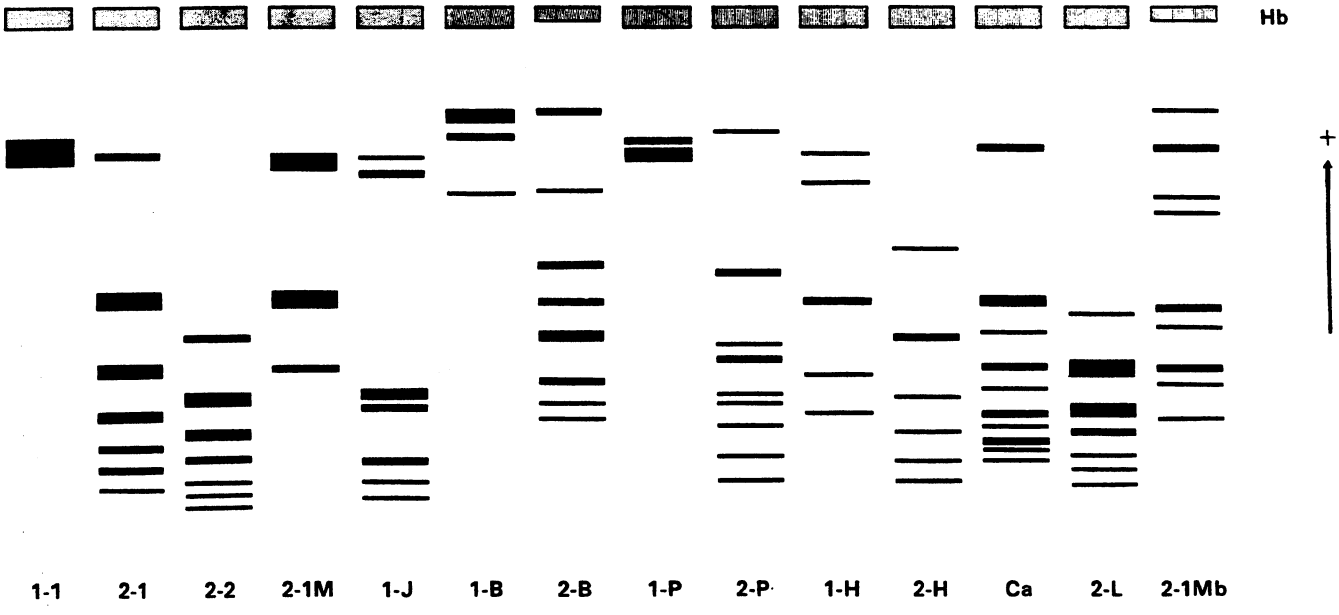


Figure 40.2 Electrophoretic Patterns of Some Haptoglobin Variants

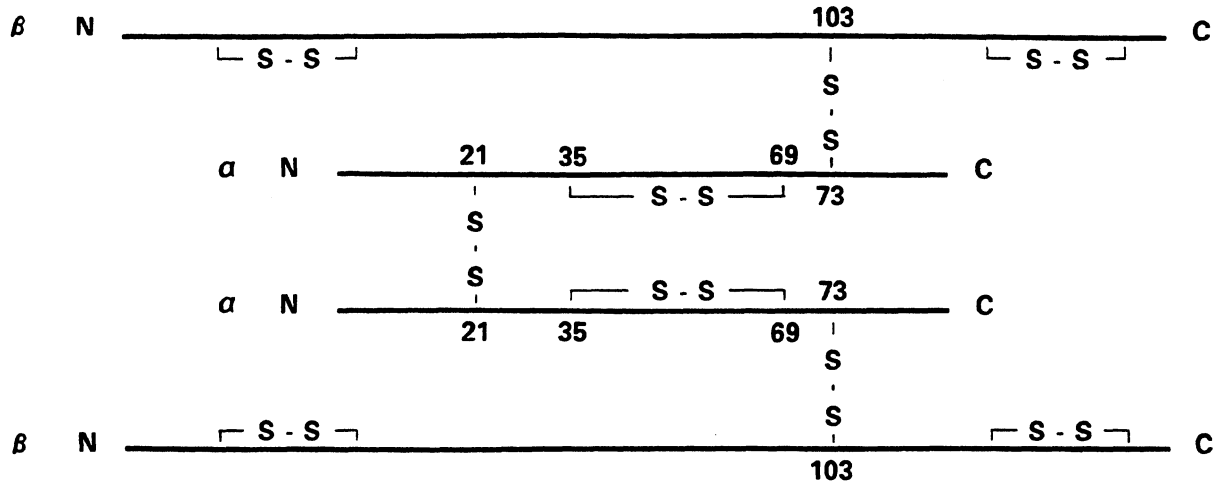


Figure 40.3 Diagrammatic Structure of Monomeric Haptoglobin

and not artifacts (Connell and Smithies, 1959). The characteristic 2-1 pattern cannot be produced by the mixing of 1-1 and 2-2 sera. The nature of the individual components in the 2-2 polymer series turned out to be rather complex, and was finally worked out by Fuller *et al.* in 1973. Until that time, there was disagreement as to the number of α and β chains in the polymers, and whether each successively larger member of the series represented the addition of a β chain, a half molecule (essentially $\alpha\beta$) or an entire monomer. Fuller *et al.* (1973) isolated several discrete polymers, and could show that each consisted of α^2 (the α chain coded for by the Hp^2 allele) and β chains in a 1:1 ratio. Members of the series differed from one another by an average MW increment of 54,500 daltons, approximately equivalent to one α^2 chain (17,300) and one β chain (40,000). Reductive cleavage experiments established that the "$\alpha^2\beta$" units were joined by disulfide bridges to form the polymers, and the polymers corresponded to a series $\alpha_n\beta_n$, n = 3 to 8. Javid (1964) carried out experiments on the differences between the 2-1 and 2-2 patterns, confirming the thinking of Allison (1959) and Parker and Bearn (1963) that α^1 chains (the α chain product of the Hp^1 allele) are incorporated into the polymers formed by the α chain products of Hp^2 (α^2 is the higher MW chain, and is responsible for the polymerization phenomenon) in the 2-1 type. The Hp 1-1 molecule is thus $(\alpha^1\beta)_2$ while the 2-2 haptoglobins consist of a series of molecules $(\alpha^2\beta)_n$, n = 1,2,3. . . . . Type 2-1 haptoglobins are expected to be a series of polymers of the kind $(\alpha^1\beta)_m$—$(\alpha^2\beta)_n$.

The nature of the Hp·Hb complex has been widely studied using a variety of methods, and these are reviewed by Putnam (1975c). The exact nature of the binding has not been completely worked out, and this is still an active area of investigation. Haptoglobin combines stoichiometrically with hemoglobin A to form a very stable complex, the binding being so tight that the complex formation reaction is considered irreversible. The heme moiety of Hb is unimportant in the linkage, though Hp combines with oxyhemoglobin, and not with deoxyhemoglobin. The oxygen equilibrium of the complexed Hb is greatly altered (Nagel and Gibson, 1971). The combination is not species-specific, and human Hp can combine with animal hemoglobins. Hp can likewise combine with Hb F and with some of the abnormal hemoglobins (see in section 38). Bearn and Franklin (1958) complexed Hp with Hb C in order to cause the complex to have a slower electrohoretic mobility than it would have had with Hb A. The α chains of Hb are essential for binding to haptoglobin, though they bind more weakly than intact Hb, but much more strongly than isolated Hb β chains. Human Hb α chains will bind animal haptoglobins, and Terpstra and Smith (1976) have, for example, studied Hb α chain binding to porcine Hp. There is recent evidence (Kazim and Atassi, 1980), however, that Hp may in fact bind Hb β chains quite strongly under certain conditions, but that special assay techniques are required to demonstrate the interaction. Laurell (1960) suggested that the binding of Hp to Hb occurs through the $\alpha\beta$ dimer of hemoglobin. The fully saturated Hp 1-1 (the simplest haptoglobin) is, in this view, bound to two halves of an Hb molecule rather than to one intact molecule, and there is considerable experimental evidence that this view is correct. The data of Nagel and Gibson (1971) suggested that Hp possesses four binding sites, two for each $\alpha\beta$ Hb dimer. One pair of sites in Hp binds a Hb $\alpha\beta$ dimer, and thereby induces an allosteric change in Hp creating a second site for an Hb $\alpha\beta$ dimer. The exact nature of the binding regions in the two molecules, and of the amino acids actually involved, is not completely known. Most of the evidence is indirect, and based upon various molecular probe studies, e.g. Russo and Chen (1976), Osada *et al.* (1978), Katnik and Dobryszycka (1978) and Hwang and Greer (1979). Conformational changes in Hp are clearly

involved, and Hevér (1977) has shown that the reduction of Hb binding capacity by the haptoglobin in heated serum varies with Hp type and subtype.

40.3.5 Subunit and polypeptide chain structure

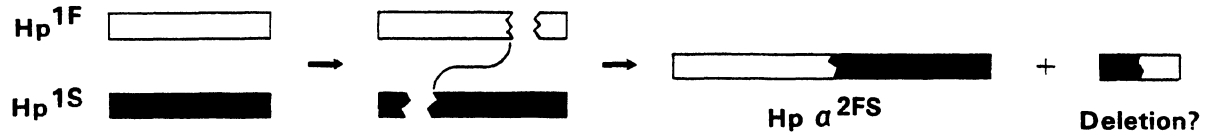
Haptoglobin resembles the immunoglobulins in subunit structure, as noted above. There are two α (light) chains and two β (heavy) chains (Fig. 40.3). There are a number of different α chains, because it is the α chain structural locus which exhibits most of the genetic variation except for a few rare variants of the β chain. The three major Hp types can be divided into six subtypes on the basis of the electrophoretic behavior of their polypeptide chains following reductive cleavage in urea (Connell *et al.*, 1962). Electrophoresis under these conditions reveals that there are only two polypeptide chains in Hp, α and β, in spite of the multiplicity of bands seen in starch gels at pH 8.6 (explained above). Only the α chain patterns are of interest in electrophoretic subtyping (Fig. 40.1), since all six types have common β chains (Cleve *et al.*, 1967) which migrate very slowly in acid-urea starch gels. It has become more or less conventional to designate haptoglobins with the symbol "Hp", the polypeptide chains as "hp", and the genes as *Hp*. The α chain structural locus should really be designated Hp_α, as suggested by Giblett (1969), to distinguish it from Hp_β, now that β chain variants have been found. The α chains have been designated in a number of different ways by various workers, and these usages can be quite confusing to non-haptoglobin specialists. There are, essentially, three kinds of α chains, produced by the $Hp_\alpha{}^{1S}$, $Hp_\alpha{}^{1F}$ and $Hp_\alpha{}^{2}$ genes. The $Hp_\alpha{}^{1S}$ locus produces hp α^{1S}, which has also been called "hp 1Sα"; similarly, $Hp_\alpha{}^{1F}$ produces α^{1F}, and $Hp_\alpha{}^{2}$ produces α^2, which have also been designated in the alternative ways shown for α^{1S}.

The classical Hp 1-1 phenotype can have haptoglobin with hp α^{1F} chains, with α^{1S} chains, or with equal quantities of both. The subtype phenotypes (and genotypes) are then, respectively, Hp 1F-1F ($Hp^{1F}Hp^{1F}$), Hp 1S-1S ($Hp^{1S}Hp^{1S}$) and Hp 1F-1S ($Hp^{1F}Hp^{1S}$). The classical 2-1 phenotype may be subtyped into Hp 2-1F or Hp 2-1S, which contain hp α^2, and either hp α^{1F} or hp α^{1S} chains, but not both. Hp 2-2 contains hp α^2 chains (although there may well be molecular variants of α^2 chains—see further below).

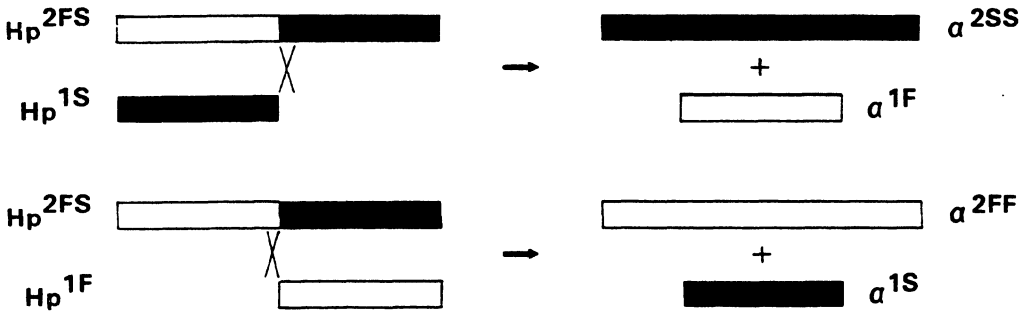
The primary structure of the Hp constituent polypeptide chains has been determined, and the α chain sequences have been most informative from a genetic point of view. The sequence work on the α chains may be found in the papers of Black and Dixon (1968 and 1970), Black *et al.* (1970), Malachy and Dixon (1973a and 1973b) and Malachy *et al.* (1973). The α^{1S} and α^{1F} chains contain 84 amino acid residues, and are identical except that α^{1S} has Glu at position 54 where α^{1F} has Lys. The α^2 chain is almost twice as long as the α^1 chains, containing 143 amino acid residues. But what is most extraordinary about the hp α^2 chain sequence is that it consists of the first 71 and the last 72 amino acid residues of hp α^1, joined together to form a 143 residue chain. The Asp13–Ala71 of α^1 is repeated in hp α^2 as Asp72–Ala130. One of the repeated sequences is from α^{1S} and the other is from α^{1F} (which can be discerned because of the Glu/Lys difference mentioned above). The hp α^2 polypeptide chain represents the first example of a partial gene duplication fully documented by amino acid sequence analysis. The point is discussed more fully in the following section. The hp α chains contain Cys at α21 and form an α21–α21 inter-α chain disulfide bridge. There is an α35–α69 intra-α chain disulfide linkage, and the Cys forming the α-chain half of the interchain disulfide bridge to the β chain is at α73. The sequence of the β chain has been almost completely worked out (Barnett *et al.*, 1972; Kurosky *et al.*, 1976), and the α chain is attached to it through β103 Cys. All the carbohydrate in haptoglobin appears to be associated with the β chain, and is probably attached (at least in part) through β23 Asn (Kurosky *et al.*, 1976).

40.3.6 Biochemical genetics

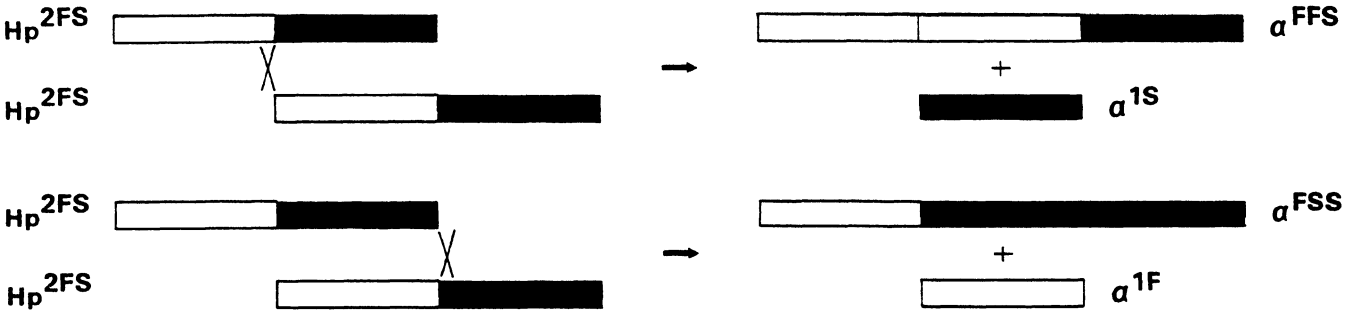
Soon after the observation of the haptoglobin polymorphism, it became clear that the monomeric molecule was of the $\alpha_2\beta_2$ structure, and that the α chain locus was responsible for the genetic variation. Early peptide analyses of the α^{1S}, α^{1F} and α^2 chains indicated considerable similarity between α^{1S} and α^{1F}, but a great difference in size between them and the α^2. The peptide analysis suggested to Smithies *et al.* (1962b) that the α^2 chain was almost a duplicate of the α^1 chains, and that α^2 had arisen because of a partial gene duplication of the α^1 genes caused by a homologous but unequal crossover at the Hp_α locus. This event gives rise to an α chain which contains most of α^{1F} and most of α^{1S} in the same polypeptide chain, namely α^2, and for this reason the α^2 chain can be designated α^{2FS}. The way in which the partial gene duplication may have occurred is shown in Figure 40.4A. During meiosis in an Hp 2-1 individual, when homologous chromosomes pair with one another, the α^1 gene on the one chromosome (whether it is α^{1S} or α^{1F}) will find itself unable to pair with the almost twice as long α^2 gene; however, because of the structural homology, the α^1 can readily pair with one or the other half of α^2 (the duplicated half). Therefore, as Smithies (1964) discussed in detail, once the partial gene duplication has occurred, the possibility for further genetic variation is increased. Because perfect synapsis is impossible between either α^1 gene and the α^{2FS} gene in a 2-1 individual, the α^{1S} or α^{1F} genes will pair with a segment of the α^{2FS} gene, as shown in Fig. 40.4B. Crossovers in such heterozygous synapses can then lead to different α^2 chains, namely α^{2FF}, α^{2SS} and α^{2SF}. In a similar way, because of the duplicative chain homology, displaced synapsing could occur in homozygous 2-2 individuals (Fig. 40.4C). Here, part of one α^{2FS} gene pairs with an (almost) homologous part of the other α^{2FS} gene (the "F" and "S" parts differ in a single amino acid). A crossover under these circumstances could lead to two varieties of triplicated genes, giving α^{FFS} or α^{FSS} chains. It is believed that Hp Johnson contains just such a chain, and these are thought to have arisen independently in the various different populations in which they have been



A. Possible Mechanism for Formation of the Hp^{2}_{α} Gene



B. Unequal Crossing Over in Hp 2-1 Heterozygotes



C. Mechanism of Partial Triplication by Displaced Synapse in Hp 2-2 Homozygotes

Figure 40.4 Diagrammatic Representation of Possible Unequal Crossovers in Hp α Chains (modified from those in Giblett, 1968 and 1969).

observed. Such genetic events are not expected to be common, particularly in the Hp 2-2 case, where completely homologous pairing is possible, and it is not particularly surprising, therefore, that 'Johnson' phenotypes are quite rare. Giblett (1968 and 1969) has suggested that if the triplication hypothesis is confirmed for Hp 'Johnson', then the resulting α chain should be called α^3. Nance and Smithies (1963) subtyped many sera and found α chains in some with migration rates that could well correspond to α^{2FF} or α^{2SS}. These genes are apparently infrequent, however (Shim and Bearn, 1964a). While the nature of many of these "probable" Hp α chains will eventually be determined by sequence analysis, there are considerable technical difficulties associated with telling the difference between chains like α^{FS} and α^{SF}, for example. Haptoglobin biochemical genetics is discussed in Giblett (1969), Harris (1975) and Putnam (1975c).

Another point of some interest is the structural homology between the Hp polypeptide chains and those of other proteins. The α chains bear structural homology to the light (κ or λ) chains of IgG. On this basis, it was expected that the Hp β chain would show structural homology with the IgG heavy chains, but this expectation has not been fulfilled. Instead, an unexpected homology was found between Hp β and the corresponding regions of the so-called chymotrypsin family of serine proteases. This matter is further discussed by Putnam (1975c) and by Kurosky *et al.* (1976).

40.4 Medicolegal Applications

40.4.1 Disputed parentage

The Hp system is applied in cases of disputed paternity in many laboratories. The use of the system is discussed extensively by Galatius-Jensen (1956, 1958a, 1960 and 1962), Baitsch (1961), Giblett (1963) and Prokop and Bundschuh (1963). The probability of excluding a falsely accused father in Western European populations is about 18%, placing it among the better systems for this purpose. In the U.S. population, the chances of excluding a falsely accused father are about 18% for both White and Black people according to Chakraborty *et al.* (1974), and about 18% for Whites and 15% for Blacks according to Dykes and Polesky (1978). The system is not widely employed in U.S. laboratories, however (Polesky and Krause, 1977), but the percentage of laboratories using Hp routinely is higher among AABB Reference Laboratories than among other laboratories.

The most serious problems that could arise in the application of Hp to disputed paternity cases would involve the apparently very rare silent allele *Hp*^{0}. Many newborns and infants are temporarily anhaptoglobinemic, and their sera cannot be typed until they are a little bit older. In addition, there are occasional anhaptoglobinemic adults (section 40.3.3.1). These people would simply not give a typing result. There may be more than one genetic basis for anhaptoglobinemia in older children and adults, and the condition is comparatively more common in people of Black African origin than in Europeans. The Hp 2-1M phenotype, which is also more frequent in people of Black African origin, appears to be related in some way to the anhaptoglobinemia phenomenon, and could, if encountered, lead to difficulty in the interpretation of the inheritance patterns. Most laboratories utilize conventional Hp typing in these cases, under which circumstances the three common phenotypes can be detected. The discriminating power of the system would be increased by the application of *Hp*^{1} subtyping.

40.4.2 Haptoglobin typing in dried bloodstains

A number of different methods have been used for the phenotyping of Hp in bloodstains. Early studies on the use of starch gel techniques similar to those used for serum typing were only partially successful (Dürwald, 1961 and 1963; Falk and Bundschuh, 1963), although some stains could be typed. Prokop and Bundschuh (1963) pointed out that the results to be expected from stains depended on the substratum upon which the blood is deposited because of the differences in extractability of the serum. Culliford (1963) obtained fair, but not completely satisfactory results with bloodstains using starch gels and the discontinuous buffer system of Poulik (1957).

In 1966, Culliford and Wraxall described an immunoelectrophoretic technique for Hp phenotyping in bloodstains which was found to be more reliable than starch gel procedures. Immunoelectrophoretic methods had been applied to Hp typing in serum by Hirschfeld (1959c) and by Fine and Battistini (1960). Hirschfeld (1968a) summarized the studies on the immunoelectrophoretic method. In this procedure, serum or bloodstain extract is electrophoresed in agar gel at about pH 8.6, after which a trough is cut in the gel and filled with an anti-Hp serum. After a suitable diffusion period, precipitin arcs develop, and those representing Hp 1, 2-1 and 2 phenotypes can be distinguished by their somewhat different positions (electrophoretic mobilities). Bargagna and Cave Bondi (1968) confirmed the usefulness of Culliford and Wraxall's (1966) procedure in bloodstain typing. They typed stains up to 69 days old. Whitehead and Morris (1969) presented a modification of the method which they said gave precipitin arcs that were easier to interpret in terms of phenotype. Katnik and Dobryszycka (1977) have carried out immunochemical studies on Hp in an effort to find out which amino acid residues in the molecule are involved in the antigenic determinants. The results indicated that tyrosyl residues are not essential. The immunochemical studies of Shim *et al.* (1965), in which anti-β and anti-α^2 chain sera were used, showed that the former reacts only with free Hp but not with Hp–Hb complex, while the latter reacted with either. The reason for the difference is that the β chain contains the Hb binding sites. The immunoelectrophoretic technique for bloodstain Hp typing is described in Culliford (1971) as well.

Electrophoresis is the preferred technique for Hp grouping. For whatever reasons, the starch gel methods that are usually used for serum typing are not very satisfactory for bloodstain typing (Dürwald, 1961 and 1963; Falk and Bundschuh, 1963; Culliford, 1963 and 1971; Turowska, 1969;

Hilgermann, 1972b). As a result, other electrophoretic support media have been employed for blood stains. Polyacrylamide gels appear to have proved most satisfactory. In 1971, Culliford described a reliable procedure for Hp typing in stains based on the use of polyacrylamide gel gradients. These were prepared in slab form using a gradient former, and the polyacrylamide concentration varied from about 5 to about 30%. More recently, gels of this kind have become commercially available. Earlier applications of horizontal nongradient polyacrylamide gel electrophoresis to Hp stain typing were not particularly successful (Gervais and Viescou, 1965), but Felix *et al.* (1977) reported satisfactory results using such a technique. Polyacrylamide disc gel electrophoresis has also been successfully used for bloodstain typing (Castilla *et al.*, 1972; Hilgermann, 1972a and 1972b). Hilgermann (1972b) could type stains up to 4 weeks old by polyacrylamide disc gel electrophoresis, and said that the technique was much better than starch gel or immunoelectrophoresis. The gradient gel procedure is fully described in MPFSL (1978). It has been used in connection with the Microzone electrophoresis system (Grunbaum, 1975), and Baxter and Rees (1974b) reported that Hp phenotypes and several Hb variants could be determined simultaneously in this way. Wraxall and Stolorow (1978) described a procedure using horizontal nongradient polyacrylamide gels which is fully satisfactory for typing bloodstains. Hp can be rapidly typed in serum samples on agarose gels (Rafowicz and Lavergne, 1974).

Haptoglobin is normally typed by electrophoretic separation of Hp–Hb complexes, and the separated proteins are detected through their peroxidase activity (which the Hb still possesses). Many of the reagents used for presumptive (catalytic) blood identification tests, based on the peroxidase activity of hemoglobin (section 6), have been employed for the detection of Hp–Hb complexes in gels following electrophoresis as well. Benzidine was often preferred until its use was discontinued because of its carcinogenicity. Owen *et al.* (1958) tested a series of oxidizable catalytic substrates, including guaiacol, leucomalachite green, amidopyridine, p-anisidine, benzidine, o-tolidine and o-dianisidine. They preferred o-dianisidine, which was also recommended by Compton *et al.* (1976). Queen and Peacock (1966) liked guaiacol; Burdett (1977) found leucomalachite green to be preferable in comparison with several noncarcinogenic compounds including phenolphthalin, diphenylamine, 2,6-dichlorophenol indophenol, N,N′-diethyl-p-phenylenediamine sulfate and 2,2′-diazo-di-(3-ethylbenzthiazoline-6-sulfonic acid). Singh (1967) recommended initial benzidine-peroxide staining, followed by an Amido Black 10B staining step to give clearer, more permanent bands.

Many investigators have noticed that older bloodstains become difficult to type for Hp. Until fairly recently, it was seldom possible to type bloodstains more than a few weeks to a few months old. Older stains tended to give streaky, overstained, and frequently unreadable patterns. One of the reasons apparently has to do with the fact that hemoglobin undergoes degradation in bloodstains, and the Hb degradation products are extracted from the stain along with the Hp. Various methods have been proposed to get around this difficulty. A related issue is the extractability of Hp itself in older bloodstains. It is common knowledge that older bloodstains do not yield up their constituent proteins to aqueous extraction media as readily as do fresher ones, and there is no reason for supposing that haptoglobin is an exception in this respect. Thus, one might fail to obtain readable typing results from a bloodstain for a variety of different reasons, such as (1) the sample could have been an Hp 0, or some other weakly expressed phenotype to begin with; (2) Sufficient Hp was not extracted to be detectable by the methods being used; (3) Degradation of the haptoglobin protein may have occurred; or (4) Hemoglobin degradation products may interfere with the electrophoretic typing. The first three of these problems could apply to any plasma protein or isoenzyme genetic marker. Studies by Nikolenko (1972 and 1975) in the U.S.S.R. and by Shaler *et al.* (1977) in this country indicate that procedures designed to better extract the Hp from bloodstains greatly improve the results with older specimens. Nikolenko's (1972 and 1975) results indicate that the addition of urea to the extraction buffers allows typing of somewhat older stains, presumably because haptoglobin is more efficiently extracted. The details of his work must be left to those with a better reading knowledge of Russian. Shaler *et al.* (1977) extracted bloodstains with buffer alone, and with buffers containing the detergents sodium dodecyl sulfate (SDS), Triton-X-100 and Tween-20, at pH's of 4.6 and 8.4. The quantity of Hp extracted was determined by "rocket electrophoresis" (section 2.4.3.1). At low pH, Triton and buffer extractions were about equally effective with stains of comparable age, while at higher pH, Triton and Tween were more effective. More important than the detergent effect, however, was the observation that the amount of immunologically detectable Hp extracted increased significantly (as much as 133%) with 24 hrs extraction as compared with 6 hrs. Extraction with detergent at low pH, and with buffer at high pH, was essentially complete at 24 hrs. Stains from 44 to 202 days old were tested with and without Triton in the extraction buffer, and the differences were not significant. Extraction at pH 8.4 was far superior to that at pH 4.6 with older stains, however. The common practice of soaking stains for a matter of minutes in electrophoretic gel buffer prior to electrophoresis is not, therefore, the best approach if one wants to type Hp in an older stain.

Improvements in bloodstain typing results have also been observed if the Hb and its apparent degradation products are separated from the stain extract prior to electrophoresis. Fresh Hb A must, of course, be added back to the sample before insertion into the typing gel. Gazaway (1976) separated the Hb material from the Hp by an agar gel electrophoresis procedure in cylindrical tubes. Conditions were employed in which the unwanted hemoglobin material migrated into the gel while Hp did not. A simpler procedure has recently been described by Stolorow and Wraxall (1979). The stain extract is subjected to a simple $CHCl_3$ extraction

in a test tube, and the aqueous layer removed for electrophoretic typing. Chloroform extraction is an old procedure for this purpose, dating back to the early studies of Formanek (1900) and of Krüger (1901). This procedure is applicable to the preparation of samples for electrophoresis by any desired typing method, and it is possible to type bloodstains some months older in this way than has routinely been possible.

A rather different approach involving specific immunoprecipitation of Hp from bloodstain extracts was reported by Blake and Sensabaugh (1978b). Subsequent typing in SDS-containing polyacylamide gels was possible in bloodstains up to 18 months old.

Haptoglobin cannot be detected in saliva or in semen (Blake, 1976; Blake and Sensabaugh, 1976 and 1978a; Schwerd and Fehrer, 1979). If the protein is present, the concentrations are extremely low, and the system is not applicable as a genetic marker in these fluids. In seminal plasma, Blake and Sensabaugh (1978a) could have detected Hp at concentrations of $\geqq 5.6\ \mu g/m\ell$, and they were unable to do so.

Chun and Sensabaugh (1979) reported preliminary but promising results on the possibility of subtyping Hp in blood stains. Immunoprecipitation and absorption procedures were employed for the isolation of the Hp from stain extracts. The discrimination index for the Hp system would be improved significantly if the Hp 1 subtypes could be discriminated.

Reviews of many aspects of the haptoglobin system may be found in Harris *et al.* (1959), Laurell (1960), Galatius-Jensen (1960 and 1962), Jayle and Moretti (1962), Giblett (1963, 1968 and 1969), Prokop and Bundschuh (1963), Javid (1967b), Kirk (1968a), Prokop and Uhlenbruck (1969), Pintera (1971), Braun (1972), Harris (1975) and Putnam (1975c).

40.5 Distribution of Hp Phenotypes in U.S. Populations

The data are shown in Table 40.1. The worldwide distribution of Hp phenotypes was discussed by Walter and Steegmüller (1969), and extensive tables may be found in Mourant *et al.* (1976). The highest Hp^1 frequency in major ethnic groups is found in Australian aborigines, and the lowest is found in Lapps. Walter and Steegmüller (1969) had data on almost 145,000 Caucasians. Hp^1 in European Caucasians was 0.386, and somewhat lower in those of non-European derivation (0.254). Hp 2-1M was seen at frequencies greater than 1% only in Negroes, but non-European Caucasians had a frequency of 0.5%. Hp 0 occurred in 4.5% of Negroes, and in 1–2% of non-European Caucasians, Mongoloids, Australian aborigines and Lapps. European Caucasians showed 0.5% Hp 0. Hp^{1S} seems to be more common than Hp^{1F} in most populations.

Table 40.1 Distribution of Hp Phenotypes in U.S. Populations

Population	Total	Frequency - Number (Percent) 1	2-1	2-1M	2	0	Note	Reference
CAUCASIAN								
Ann Arbor, MI	68	9(13.2)	40(58.8)		19(27.9)			Sutton et al., 1959
Michigan and Illinois	161	23(14.3)	77(47.8)		61(37.9)			Bayani-Siosin et al., 1962
Seattle, WA	409	54(13.2)	206(50.3)	0	149(36.4)		1	Giblett and Brooks, 1963
Southeastern GA	145	27(18.6)	64(44.1)	0	52(35.9)	2(1.4)		Cooper et al., 1963
Maryland	192	24(12.5)	85(44.3)		83(43.2)			Queen and Peacock, 1966
Chicago, IL	101	18(17.8)	51(50.5)		32(31.7)			Shih and Hsia, 1969
Tecumseh, MI	7,655	1,307(17.07)	3,734(48.78)	11(0.0014)	2,575(33.64)	22(0.0029)	2	Schreffler et al., 1971
Orange County, CA	185	(16)	(48)		(36)		3	Fitzpatrick et al., 1976
Bexar County, TX	200	(17)	(50)		(33)			Ganaway and Lux, 1978
Pittsburgh, PA	1,263	185(14.6)	555(43.9)	26(2.1)	495(39.2)	2(0.16)		Hagins et al., 1978
California	274	(14.6)	(49.3)		(36.1)			Grunbaum et al., 1978
Detroit, MI	503	69(13.72)	213(42.35)	0	220(43.74)		4	Stolorow et al., 1979
Miami, FL	366	58(15.8)	161(44)		141(38.5)	6(1.6)	4	Stuver, 1979
Los Angeles, CA	311	62(19.9)	149(47.9)		100(32.2)		4	Siglar, 1979
California	860	154(17.9)	418(48.6)		288(33.5)			Grunbaum et al., 1980
NEGRO								
Seattle, WA	406	(26.4)	(38.2)	(9.8)	(21.4)	(4.2)		Giblett, 1959
Ann Arbor, MI	48	17(35.4)	17(35.4)		9(18.8)	5(10.4)		Sutton et al., 1959
Seattle, WA and Cleveland OH	178	45(25.3)	70(39.3)	27(15.2)	31(17.4)	5(2.8)		Giblett and Steinberg, 1960
New York, NY	100	(40)	(38.9)	(0.08)	(21.1)	(0.02)		Parker and Bearn, 1961
Seattle, WA	1,657	472(28.5)	641(38.7)	181(10.9)	307(18.5)	56(3.4)	5	Giblett and Brooks, 1963
Southeastern GA	167	48(28.7)	61(36.5)	13(7.8)	42(25.1)	3(1.8)		Cooper et al., 1963
Chicago, IL	101	30(29.7)	47(46.5)		24(23.8)			Shih and Hsia, 1969
Bexar County, TX	200	(35)	(49)		(17)			Ganaway and Lux, 1978
Pittsburgh, PA	721	206(28.6)	293(40.6)	66(9.2)	148(20.5)	8(1.1)		Hagins et al., 1978
California	124	(33.9)	(41.9)		(21)		6	Grunbaum et al., 1978
Detroit, MI	504	142(28.17)	204(40.48)	59(11.71)	84(16.67)	11(2.18)	4,7	Stolorow et al., 1979
Miami, FL	346	120(34.7)	164(47.4)		40(11.6)	22(6.4)	4	Stuver, 1979
Los Angeles, CA	130	47(36.2)	58(44.6)		25(19.2)		4	Siglar, 1979
California	463	135(29.2)	236(51)		89(19.2)		8	Grunbaum et al., 1980
HISPANIC								
Bexar County, TX	200	(26)	(51)		(24)			Ganaway and Lux, 1978
California	161	(21.7)	(55.9)		(22.4)		9	Grunbaum et al., 1978
Miami, FL	360	75(20.8)	179(49.7)		97(26.9)	9(2.5)	4	Stuver, 1979
Los Angeles, CA	145	43(29.7)	67(46.2)		35(24.1)		4	Siglar, 1979
California	775	218(28.1)	393(50.7)		163(21)		10	Grunbaum et al., 1980
OTHER								
U.S. Japanese	23	2(8.7)	10(43.5)		11(47.8)			Harris et al., 1959
New York, NY Chinese	118	(14.8)	(38.3)		(47)	(0.03)		Parker and Bearn, 1961
Seattle, WA Oriental	494	34(6.9)	190(38.4)	0	270(54.7)	0	11	Giblett and Brooks, 1963
New York, NY Chinese	113	16(14.2)	45(39.8)		52(46)		12	Shim and Bearn, 1964a
California and Hawaii Asian	376	(7.7)	(37.8)		(53.5)		13	Grunbaum et al., 1978
California and Hawaii Asian	1,105	148(13.4)	444(40.1)		512(46.3)		14	Grunbaum et al., 1980

1. 66 Hp 1 and 2-1 were subtyped:
   4(6.1)1S-1; 5(7.6)1S-1F; 36(54.5)2-1S; 21(31.8)2-1F
2. 4 were HpCa and 2 were 'Johnson'
3. Approximately 15% of the sample were Hispanic (by surname)
4. And see Shaler (1978)
5. 222 Hp1 and 2-1 were subtyped:
   26(11.7)1S-1S; 20(9.0)1F-1F; 49(22.1)1S-1F; 61(27.5)2-1S;
   55(24.8)2-1F; 6(2.7)2M-1S; 5(2.3)2M-1F
6. 3.2% were "rare"
7. 4(0.79) were "rare"
8. 3 were "rare"
9. "Chicano/Amerindian" population
10. "Mexican" population; 1 was "rare"
11. 80 Hp1 and 2-1 were subtyped:
    14(17.5)1S-1S; 1(1.3)1S-1F; 64(80)2-1S; 1(1.3)2-1F
12. Subtyping:
    all 16 1-1 were 1S-1S; all 45 2-1 were 2-1S
13. 1.1% were "rare"
14. 1 was "rare"

SECTION 41. GROUP SPECIFIC COMPONENT

41.1 Recognition—Genetic Variation

Group specific component (Gc) was first recognized because of its polymorphism. Hirschfeld (1959b) found that normal human sera subjected to immunoelectrophoretic analysis showed qualitative differences in proteins in the α_2 globulin region. Sera could be classified into three groups, originally called "1", "2" and "3". These differences were not attributable to the Hp polymorphism, and those seen in human sera were analogous to qualitative variation seen in rabbit sera when the latter were tested by immunoelectrophoresis with a horse anti-rabbit serum (Hirschfeld, 1959a). A preliminary report on the variation in human sera was given at a meeting late in 1958 (Hirschfeld, 1960). The type "1" and "3" human sera showed single immunoprecipitin arcs, while type "2" showed both. The type "2" pattern was indistinguishable from that given by an equal mixture of type "1" and type "3" sera, and the patterns were invariant in a given person over the course of time. Family, and mother-child pair studies (Hirschfeld *et al.*, 1960) indicated that this was an inherited variation in which types 1 and 3 were homozygous, and type 2 was the heterozygote. All the observatons could be explained by a pair of alleles, and it was suggested (Hirschfeld and Beckman, 1960) that the system be called the 'group specific component', and the genetic locus controlling its expression be called *Gc*. The alleles were designated Gc^1 and Gc^2, and the system was independent of Hp, Gm, Tf and pseudocholinesterase. Types "1" and "3" thus became Gc 1-1 and Gc 2-2, respectively, and type "2" was Gc 2-1, by analogy to the nomenclature that had been developed for haptoglobin. Family and population studies soon established that the two allele hypothesis of inheritance was correct (Hirschfeld *et al.*, 1960; Nerstrøm, 1963a and 1963b; Reinskou and Mohr, 1962; Mansa *et al.*, 1963; Bütler *et al.*, 1963; Reinskou, 1965a; Seppälä *et al.*, 1967; Suyama and Uchida, 1969), and that the gene frequencies differed in racially and ethnically distinct populations (Hirschfeld and Beckman, 1961; Cleve and Bearn, 1961a and 1961b; Baitsch *et al.*, 1963; Blumberg *et al.*, 1964a; Hummel *et al.*, 1970). The earlier studies on the Gc polymorphism were well reviewed by Hirschfeld (1962).

The Gc types are detectable electrophoretically as well. In 1959, Smithies had noticed that there were differences in the migration patterns of proteins in the "post albumin" region, and thought that these might have a genetic basis (Smithies, 1959a and 1959b). In 1962, Schultze *et al.* (1962a) showed clearly that the Gc types were determinable by starch gel electrophoresis. The following year, it became clear that the Gc types and the "post albumin" variations were identical, as sera were examined both by immunoelectrophoresis and by starch gel electrophoresis (Arfors and Beckman, 1963). Gc phenotypes have been determined under a number of different conditions on starch gels (Parker *et al.*, 1963; Bearn *et al.*, 1964a) and on polyacrylamide gels (Kitchin and Bearn, 1966). Under some electrophoretic conditions, heterogeneity in the homozygous types was noticed. It was thought that this might indicate something about the subunit structure of the molecule. The matter is discussed further in a subsequent section. Azen *et al.* (1969) separated the Gc proteins on starch at high voltages, and said that better resolution was obtained, but that an additional component appeared in the 1 and 2-1 types. Gc phenotypes can be determined by a number of variations and combinations of electrophoresis and immunoelectrophoresis, as is discussed further below.

41.2 Further Gc Phenotypes

Hirschfeld (1962) found two Gc types that did not correspond to the common phenotypes. One, from the serum of an African Negro, appeared to represent Gc 1 and a faster moving component called "Y"; thus Gc 1-Y. The other, from a Caucasian serum, had a component that was faster than 2, but slower than 1, and it was called "Gc X". Parker *et al.* (1963) also reported a fast variant in a Black subject, and one of intermediate mobility in a Caucasian. Family studies could not be carried out in either of these cases to prove that the variants were reflections of other alleles at the *Gc* locus, however. The 'Caucasian' variant of Parker *et al.* (1963) is sometimes called 'Gc Caucasian' or 'Gc Cau', and it is probably the same as 'Gc X' but the two have never been directly compared (Johnson *et al.*, 1975). Cleve *et al.* (1963a) found several additional phenotypes that could be accounted for by two additional variant alleles. The first was seen in Chippewa Indians, and was called 'Gc Chippewa' or 'Gc Chip'. Gc Chip-1 was hard to distinguish by immunoelectrophoresis, but was distinctive by starch gel electrophoresis. It was due to an allele designated Gc^{Chip}. Evidence of the second allele was detected in Australian aborigine sera, which was called Gc^{Ab}. All three phenotypes, Gc Ab-1, Gc Ab-2 and Gc Ab-Ab, were observed. The Gc Ab band was faster than Gc 1 or Gc Chip, and was best detected in polyacrylamide gels (Kitchin and Bearn, 1966). No differences could be detected by Kitchin and Bearn (1966) between Gc Ab, Gc Y and another variant originally described in Greenland Eskimos by Persson and Tingsgaard (1965), either by immunoelectrophoresis or polyacrylamide gel electrophoresis, and these were thought to be the same. In a direct comparison on thin layer agarose gels, followed by immunofixation detection, however, Johnson *et al.* (1975) found that Gc Ab/Y was different

from the Eskimo variant, which they called 'Gc Esk'. In addition, 'Gc Esk' could not be distinguished from 'Gc D' (see below), and so 'Eskimo' and 'Darmstadt' may be identical. Reinskou (1965b) found another pair of variant types in Norwegian sera, which could be designated 'Norw-1' and 'Norw-2'. He did not care for the descriptive nomenclature that had developed, though, and suggested that the gene responsible for the 'Norw' phenotypes be called Gc^{1C}, and the phenotypes 1-1C ('Norw-1') and 2-1C ('Norw-2'). The 'Norw' and 'Chip' phenotypes appear to be characterized by altered proportions of the heterogeneous Gc 1-1 proteins. Gc Chip has an increased anodal portion, and Gc Norw an increased cathodal one. The "C" in the Gc^{1C} designation stands for "cathodal", and Reinskou (1965b) said that Gc^{Chip} should be designated Gc^{1A}, where "A" stands for "anodal". The Norw variants are apprently quite rare. A second example was reported by Rittner and Dahr (1969). Other variants that have been reported are: Gc^{Z}, observed as 1-Z and 2-Z (Hennig and Hoppe, 1965; Cleve *et al.*, 1966); Gc Bangkok or Gc^{Bkk}, seen as Gc 1-Bangkok (Rucknagel *et al.*, 1968); Gc Darmstadt, or Gc^{D}, seen as D-1 and D-2 (Cleve *et al.*, 1970); Gc Wien, or Gc^{W}, seen as Gc W-1 and W-2 in 8 members of a family in Vienna (Wien) (Speiser *et al.*, 1972); Gc Japanese or Gc^{J}, seen as Gc J-2 (Omoto *et al.*, 1972); Gc Opava, or Gc^{Op}, seen as Gc Op-1 and Op-2 (Cleve, 1973; Vavrusa and Cleve, 1974); Gc Boston, or Gc^{B} (if genetic transmission can be proven), seen as B-2 (Johnson *et al.*, 1975); and Gc Toulouse, or Gc^{T}, seen as Gc T-2 (Constans *et al.*, 1978).

Cleve (1973) reviewed all the known variants to date, and could classify them into four major categories: (1) Molecular variants, with electrophoretic mobility different from Gc 1 and Gc 2. The faster ones are Gc Ab, Gc D, Gc Wien and Gc Japanese; Gc Opava migrates in between Gc 1 and Gc 2; and the slower ones are Gc Bangkok and Gc Z. (2) This group included Gc Chippewa and Gc Norway variants, which, as noted above, seem to represent altered proportions of heterogeneous bands of Gc 1-1; (3) This group consisted of 'subgroups' of the Gc^{1} allele, as disclosed by a particular antiserum, raised in sheep against Gc 2-2 (Ruoslahti, 1965). Type 1 sera could be classified as 'reacting' or 'nonreacting', and the 'reactor' characteristic was inherited in the manner of a Mendelian dominant. (4) The fourth group comprised the silent alleles of *Gc*. These are apparently very rare. Henningsen (1966) reported the possibility of Gc^{0} in a family with peculiar Gc inheritance. Prokop and Uhlenbruck (1969) reported a family in which a Gc 2 father had a Gc 1 daughter, who, in turn, had a Gc 2 son. There was no question about the maternal relationship of the daughter to her son, and the easiest explanation was that the father was $Gc^{2}Gc^{0}$, his daughter $Gc^{1}Gc^{0}$, and her son $Gc^{2}Gc^{0}$. Patscheider and Dirnhofer (1979) described an extraordinary sibship in which Gc^{0} and ESD^{0} were apparently segregating together. A child in the third generation was believed to be $Gc^{1}Gc^{0}$, $ESD^{1}ESD^{0}$, and the family study indicated that the Gc^{0} had been inherited from the maternal grandfather, and the ESD^{0} from the maternal grandmother. The child's mother was also, thus, a double heterozygote for the silent alleles.

This classification of the Gc phenotypes by Cleve (1973) would have to be revised somewhat in view of the finding that there are two Gc^{1} alleles, whose products are revealed by isoelectric focusing (section 41.3). In addition, the best technique for typing most of the Gc variants seems to be thin layer agarose electrophoresis, followed by immunofixation detection (Johnson *et al.*, 1975). Their studies clarified some of the relationships between the rare variants. Patterns of some of the Gc types as seen using this technique are shown in Figure 41.1.

The majority of the Gc variant phenotypes are rare. Gc Chip reaches polymorphic frequencies in the Indians after whom it was named. Gc^{Ab}, giving rise to the Gc Ab phenotypes (likely equivalent to 'Gc Y' in Black populations), occurs in appreciable frequencies in the aborigines,

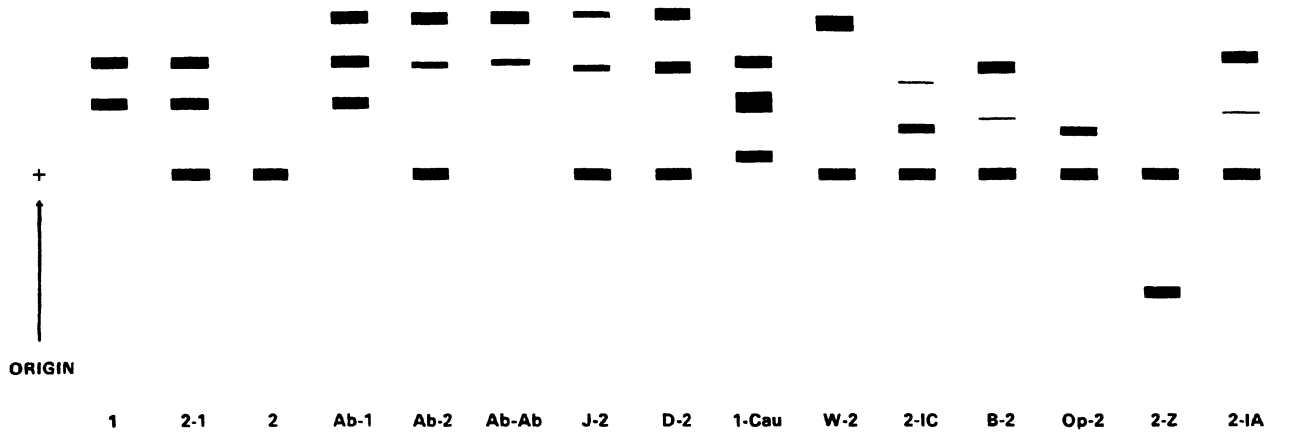


Figure 41.1 Electrophoretic Patterns of Some Gc Phenotypes (after immunofixation agarose gel electrophoresis, pH 8.6). Identities and probable identities: Gc Ab = GcY; Gc X = GcCau; Gc 2-1A = Gc 2-Norw; Gc 2-1A = Gc 2-Chip.

and is occasionally seen in Black people. There is little population data, but Gc^{Ab} had a frequency of 0.015 in 492 Black Americans from Pennsylvania and Georgia (Kueppers and Harpel, 1979). Gc Ab phenotypes are seen in various populations in Oceania as well (Kirk *et al.*, 1963).

41.3 Additional Genetic Variation at the Gc^1 Locus—The Gc "Subtypes"

In 1977, Constans and Viau found that "subtypes" of Gc 1 could be distinguished by polyacrylamide gel isoelectric focusing (PAGIF), and detection of the Gc bands by immunofixation. The "fast" and "slow" bands of Gc^1 were designated "F" and "S", and accounted for by the alleles Gc^{1F} and Gc^{1S}. These Gc alleles, along with Gc^2 give rise to the phenotypes 1F, 1F–1S, 1S, 2–1F, 2–1S and 2. The Gc^{1F} frequency was comparatively low in a Pyrenean valley population, but about 0.58 in a central African pygmy group. These observations, and the proposed genetic explanation for them, have been amply confirmed (Constans *et al.*, 1978; Kühnl *et al.*, 1978; Ishimoto *et al.*, 1979; Hoste, 1979; Kueppers and Harpel, 1979). The Gc^{1F} and Gc^{1S} frequencies in Hessen were 0.125 and 0.603, respectively (Kühnl *et al.*, 1978), while in a Belgian population, they were 0.167 and 0.543 (Hoste, 1979). Constans *et al.* (1978) extended their original studies, and found two additional variants of Gc. One, found in an African pygmy population, exhibits a band in about the same position as the second band of a 1–1 on polyacrylamide gel electrophoresis. It was a variant of Gc 2, and was called '2a' (for 'anodal'). It was inherited, and the responsible allele was designated Gc^{2a}. Both 2a–1 and 2a–2 phenotyes were observed, and the former can be further classified as 2a–1F or 2a–1S by isoelectrofocusing. The other variant was found in a tribe of South American Indians from Bolivia. It was called 'Gc Am–1' and was indistinguishable from Gc Ab–1 by electrophoresis, but it was assumed that Gc Am types reflect a new allele, Gc^{Am}. Ishimoto *et al.* (1979) subtyped Gc in Japanese populations, and found that the Gc J phenotypes could be further subdivided into a series of phenotypes representing combinations of Gc^{Ja}, Gc^{Jb}, Gc^{Jc} and Gc^{Jd} with Gc^{1S}, Gc^{1F} or Gc^2. They found some further variants as well, which were called Gc Ja, Jb, Jc, Jd, Ok and Mie. In a note attached to the reprint, Dr. Ishimoto noted that these variants were equivalent to the Gc 1A2, 1A3, 1A9, 1A8, 1C2 and 1C4, respectively, of Dr. Constans. This latter nomenclature resulted from an international conference on Gc variants and nomenclature, held in Paris in 1978 (Constans and Cleve, 1979). Correspondence between old and new nomenclature for about 30 known variants was given, along with representations of the electrofocusing patterns. Briefly, the "A" and "C" designations mean "anodal" and "cathodal", and variants are then classified according to mobility. For example, Gc Ab is now Gc 1A1, and the phenotype that would previously have been called Gc 'Ab–1F' is now Gc '1A1–1F'. The paper must be read by those interested in Gc typing. Equivalences are shown in Table 41.1.

41.4 Methods of Phenotyping Gc

A number of procedures have been employed to distinguish the Gc phenotypes in serum. The three common phenotypes, 1, 2–1 and 2, can be diagnosed with any of them in experienced hands. Some of the rarer variant types are more difficult to discriminate by certain methods, and easier using other ones, and this has been one of the difficulties in the unequivocal classification of the variants observed in different laboratories using different methods. The original procedure, immunoelectrophoresis, serves well in many laboratories (see in Hirschfeld, 1968a). Interpretation of the patterns is made considerably easier if specific anti-Gc serum is used rather than anti-human serum. Both starch and polyacrylamide gel electrophoresis have been used for typing Gc, as discussed above. A polyacrylamide disc gel procedure was devised by Raunio *et al.* (1966). Polyacrylamide gel electrophoresis can be employed on a preparative

Table 41.1 Gc Variant Nomenclature Equivalents

New Gc Nomenclature ★	Old Designations
1S	1S
1F	1F
2	2
1A1	Ab;Y;☆
1A2	Or;◇ J; OMS 25;◇ Ja●
1A3	OMS 6;◇ OMS 22;◇ N; Jb●
1A4	Igloo; Y;●◇ Eskimo
1A5	V^a◇
1A6	1C◇
1A7	Op_1
1A8	Am1;◇ TK2;◇ OMS 10;◇ Jd●
1A9	Am2; TK1;◇ OMS 14;◇ Jc●
1C1	T;
1C2	OMS 1;◇ Ok●
1C3	1B; V^1◇
1C4	OMS 21;◇ Mie●
1C5	Bagnère◇
1C6	1D1◇
1C7	1D2;◇ Op_2
1C8	1E
1C9	SI
1C10	1G◇
2A1	2C◇
2A2	Op_1
2A3	2A
2A4	2Y
2A5	V^2;◇ 2B
2A6	Wien
2C1	2D◇
2C2	Z

★ According to Constans and Cleve (1979); only one gene product is shown; only variants actually compared by isoelectric focusing are shown

☆ The "Y" seen in Black populations is thought to be equivalent to "Ab"

● Seen in Japanese ● According to Ishimoto et al., (1979)

◇ Familial transmission not clearly established

scale (Simons and Bearn, 1967). Prokop (1963) described a kind of two-dimensional immunoelectrophoretic procedure suitable for Gc. Electrophoresis was performed as usual, but then two antibody troughs were cut in parallel on either side of the sample position, and electrophoresis performed at 90° to the original direction. This procedure was much faster than classical immunoelectrophoresis, in which the antibodies are allowed to diffuse into the gel. The antigen-antibody crossed electrophoresis procedure of Laurell (1965), which is described in detail by Weeke (1973), has also been used for Gc typing (see in section 2.4.3.2). Sutcliffe and Brock (1973) used this technique, and it has also been found useful in comparing Gc variants. In 1969, Alper and Johnson described a technique called immunofixation electrophoresis (section 2.4.2); the technique is further discussed by Ritchie and Smith (1976). Here, the separated components are allowed to react with specific antibody in the gel, after which the gel is washed free of excess protein, and the specific complexes stained. This technique has been used extensively for the detection of Gc types following electrophoresis (Johnson *et al.*, 1975; Martin and Kopietz, 1976; Grunbaum and Zajac, 1977; Wraxall and Stolorow, 1978). Immunofixation may also be used to detect Gc following isoelectric focusing. Most of the techniques that have been found suitable for bloodstain typing utilize some form of immunofixation detection scheme (see below). Hoste (1979) detected the Gc bands on polyacrylamide gels after isoelectrofocusing by a simple non-immune sulfosalicylic acid staining procedure.

41.5 Physiological and Biochemical Studies on Gc

41.5.1 The function and properties of Gc protein

For years, Gc proteins were a genetic curiosity, the system being well studied as a genetic marker, but the protein constituents of it having no known physiological function. It has been known for many years that serum contains some sort of Vitamin D binding protein, and that this protein is an α-globulin (Thomas *et al.*, 1959). Belsey *et al.* (1974) found that rat and chick, in addition to human, sera all have this protein, and that it binds 25-hydroxy-Vitamin D preferentially. The Vitamin D binding protein was isolated from human serum and partially purified by Imawari *et al.* (1976). Daiger *et al.* (1974 and 1975a) then observed polymorphism in the human serum Vitamin D binding protein, and quickly realized that they had 'rediscovered' the Gc polymorphism. Their studies indicated that Gc and Vitamin D binding proteins are identical. Bouillon *et al.* (1976) rapidly confirmed the identity using an extensively purified preparation. They said that the protein should be renamed "transcalciferin". Haddad and Walgate (1976) purified the serum Vitamin D binding protein, and likewise showed that it was immunologically identical to Gc. Cleve and Patutschnick (1977) used autoradiography to show that the components of the common Gc phenotypes, as well as those of many of the variants, bind Vitamin D_3. No differences in Vitamin D_3 binding were detected among the variant types. The MW of the purer preparations of Gc is in the neighborhood of 52,000 (Imawari *et al.*, 1976) to 58,000 (Bouillon *et al.*, 1976).

There have been various reports of the Gc level in normal serum. Published values range from about 0.28 to about 0.75 mg per mℓ of serum. The higher values are those of Kitchin and Bearn (1965) using quantitative immunoprecipitation, and the lowest values were obtained by radial immunodiffusion measurements (Kueppers and Harpel, 1979), who used a commercial standard serum for calibration. There have been suggestions, too, that the normal Gc content of serum differs according to phenotype, but this variation was not seen by Kitchin and Bearn (1965) nor by Kueppers and Harpel (1979).

41.5.2 Biochemical studies

The amino acid composition of Gc has been studied by Cleve *et al.* (1963b), Bowman and Bearn (1965) and Bowman (1967 and 1969) among others. The fast and slow bands of 1-1 and 2-2 are all very similar, and the Asp and Glu content is quite high. The molecule contains carbohydrate but little is known about how it is arranged. Schultze *et al.* (1962b) found the equivalent of 7 hexose, 5 acetylhexosamine and 1 fucose per 51,000 MW, but no sialic acid. The data of van Baelen *et al.* (1978), however, strongly suggest that the Gc 1 molecule must contain sialic acid.

Bowman (1969) proposed a molecular model for Gc, based on end group analysis, peptide maps and other chemical data. The model is further discussed in the review by Putnam (1977) as well. The molecule is a dimer, according to her proposal, which can be made up of three different kinds of chains controlled by three separate genetic loci. The chain called δ is common to fast bands of both 1-1 and 2-2. In 1-1, δ is combined with α^1 to give $\alpha^1\delta$ which is characteristic of 1-1. In 2-2, δ is paired with an α^2 chain to give the $\alpha^2\delta$ characteristic of 2-2. A β chain combines with α^1 to give the $\alpha^1\beta$ slow band of 1-1. Van Baelen *et al.* (1978) carried out studies on the Gc proteins as separated by isoelectrofocusing. Gc 2-2 contained a single protein, where Gc 1-1 had two bands with lower pI. The 2-1 contained all three. An anodal shift was observed after incubation of the apo-Gc with excess 25-hydroxy-Vitamin D. Sialidase treatment had no effect on Gc 2-2, but the faster Gc 1-1 band focuses in the position of the slower band after treatment, strongly indicating N-acetyl neuraminic acid in the Gc 1-1 fast band.

Reviews of the Gc polymorphism may be found in Cleve and Bearn (1962), Bearn *et al.* (1964b), Reinskou (1968a), Giblett (1969), Prokop and Uhlenbruck (1969) and Putnam (1977).

41.6 Medicolegal Applications

41.6.1 Disputed parentage

The Gc polymorphism has been used in disputed paternity cases in a number of laboratories for quite some time (Hirschfeld and Heiken, 1963; Nerstrøm, 1963a; Ritter,

1963; Marek *et al.*, 1963; Bütler *et al.*, 1963; Holzhausen *et al.*, 1964; Reinskou, 1966b). The probability of excluding a falsely accused father of Western European origin is about 15–16% and the system is considered reliable (Reinskou, 1966a). In U.S. populations, the exclusion probabilities are about 16% for falsely accused White fathers, and about 7–8% for falsely accused Black fathers (Chakraborty *et al.*, 1974; Dykes and Polesky, 1978). The system is apparently employed only rarely in disputed parentage cases in this country (Polesky and Krause, 1977). The exclusion probabilities given above are based on the detection of the three common phenotypes. Serum phenotyping is generally straightforward. The possibility of rare or silent alleles, though they are not often encountered, must be kept in mind. If isoelectrofocusing equipment is available and the Gc^1 subtypes can be determined, the exclusion probability is increased significantly. Kühnl *et al.* (1978) indicated that the figure would be 24% in the Frankfurt population; Hoste (1979) said it would be almost 32% in Belgium.

41.6.2 Gc phenotyping in bloodstains

The earlier investigators used immunoelectrophoresis for Gc typing, since this technique was the only one available for several years. A number of efforts were made to type Gc in bloodstains by this procedure, but most of them were not very successful. More recent applications of electrophoresis and immunofixation detection techniques have been more productive.

In 1963, Nerstrøm and Skafte Jensen reported that only the very freshest bloodstains could be typed for Gc using immunoelectrophoretic procedures. Peculiarly, serum stains could be grouped for several weeks. Vogt (1963) in Prof. Dr. Prokop's institute had similar results, although he could type stains up to about 10 days old made on glass plates. Heifer and Bolkenius (1966) found, however, that Gc seemed to be a little more stable in stains made on glass than in those on other substrata. There seem always to have been some difficulties with older bloods, or sera that have been transported over great distances (as is sometimes done in population studies), when immunoelectrophoresis is used for the Gc typing. Problems associated with stored, older sera were discovered by Nerstrøm (1963b) and Persson and Tingsgård (1966). There is a distinctive decrease in the quality of the precipitin arcs in these samples. Nerstrøm (1963c and 1964) was intrigued by the fact that the Gc types in serum stains were more stable than in whole bloodstains, and that old, hemolyzed blood gave typing problems that separated serum did not. The lysed cells were thought to have an adverse effect on the Gc proteins, and studies showed that lysed leucocyte and thrombocyte material did disturb the Gc pattern. A similar effect could be induced with proteolytic enzymes and products from yeast. Heifer and Bolkenius (1966) were able to type most of a series of bloods kept stored for two years, and about half the post mortem samples they attempted. They too found that serum stains could be grouped a little longer than whole blood stains on linen. Similar observations were made by Brzecka and Mikulewicz (1966). They said that a major difficulty was in concentrating the protein in bloodstain extracts to a sufficient extent that the Gc types were detectable. Gc could be typed in fairly fresh post mortem blood, but older hemolyzed samples were unsatisfactory. Tumanov and Il'ina (1974) utilized an immunoelectrophoretic technique, but with a monospecific anti-Gc serum. Stains on a variety of substrata could be typed for up to 18 months time, a considerable improvement compared with the older procedures.

In 1975, Wraxall reported briefly on efforts to apply the antigen antibody crossover electrophoresis technique to bloodstains. Apparently, many of the problems encountered in immunoelectrophoretic techniques are alleviated by the use of electrophoretic separations followed by immunofixation detection. Johnson *et al.* (1975) said that they had no difficulty typing aged, contaminated or jaundiced sera in this way, in contrast to the difficulties reported previously when immunoelectrophoretic techniques were being used. They preferred agarose gels for immunofixation electrophoresis over polyacrylamide or starch gel media. Martin and Kopietz (1976) reported that immunofixation electrophoresis in agarose had helped to clarify the phenotypes of some samples which were originally typed by immunoelectrophoresis. One serum typed as a 2-1 in agarose, but had looked like a "1-variant" of some kind by immunoelectrophoresis. Similarly, a specimen thought to be a possible 2-W by its immunoelectrophoretic behavior turned out to type as a Gc 2-Ab in agarose. Immunofixation may be used in conjunction with cellulose acetate membrane electrophoresis, as well as with electrophoresis in gel media. Grunbaum and Zajac (1977) described such a procedure, which needed only 20 minutes of electrohoresis to achieve separation. This technique could be used to type bloodstains up to 2 months old (Zajac and Grunbaum, 1978). A chloroform extraction technique was used to remove the hemoglobin degradation products from the bloodstain extract prior to electrophoresis. Wraxall and Stolorow (1978) described an immunofixation agarose electrophoresis typing procedure, which is fully applicable to bloodstain typing as well. The procedure is currently used in several laboratories that we know of, and yields satisfactory results on stains up to a number of months old.

Gc is not detectable in semen by radial immunodiffusion techniques which would detect it in 2.5% solutions of serum (Blake, 1976; Blake and Sensabaugh, 1976 and 1978a). If the serum level of Gc is taken to be 800 $\mu g/m\ell$, therefore, Gc would have to be present in concentrations of less than about 20 $\mu g/m\ell$ to go undetected. The serum concentration of Gc may actually be lower than 800 $\mu g/m\ell$ (section 41.5.1). The Gc system is not, therefore, a useful genetic marker in semen. Gc cannot be detected in healthy urine by immunoelectrophoretic analysis, but it can sometimes be determined in pathological (proteinuric) specimens (Nielsen *et al.*, 1963). Thus, while it is highly unlikely that Gc could be detected in urine stains, it is equally unlikely that urine contamination would interfere with Gc bloodstain typing.

41.7 Distribution of Gc Phenotypes in U.S. Populations

The data is shown in Table 41.2. The worldwide distribution of the common Gc^1 and Gc^2 alleles was discussed by Cleve (1973) and Walter and Steegmüller (1969). Gc^2 is about 0.25 to 0.30 in most European populations, and quite a bit lower in most Black African populations (of the order of 0.1). In most Asians, the frequencies are closer to those seen in Europeans. Gc^1 subtyping is comparatively recent, and there is not very much data as yet. It appears, however, that 75–85% of European Gc^1 genes are Gc^{1S}. In Black populations, Gc^{1S} is a much lower fraction of Gc^1 alleles, perhaps 15–25%. In Japanese populations, the fraction is of the order of 35%.

Table 41.2 Distribution of Gc Phenotypes in U.S. Populations

Population	Total	Frequency — Number (Percent) 1	2-1	2	Note	Reference
CAUCASIAN						
New York, NY	122	63(51.6)	49(40.2)	10(8.2)	1	Cleve and Bearn, 1961a
New York, NY	86	48(55.8)	32(37.2)	6(6.97)		Cleve and Bearn, 1961b
Southeastern GA	292	147(50.3)	114(39.0)	31(10.6)		Blumberg et al., 1964
Boston, MA	407	234(57.5)	146(35.9)	27(6.6)		Murray and Robinson, 1968
Tecumseh, MI	7,658	3,910(51.06)	3,139(40.99)	609(7.95)		Schreffler et al., 1971
California	4,488	(50.2)	(41.0)	(8.5)	2	Grunbaum et al., 1978
Detroit, MI	503	242(48.11)	213(42.35)	45(8.95)	3,16	Stolorow et al., 1979
Miami, FL	365	168(46)	160(44)	37(10)	16	Stuver, 1979
Los Angeles, CA	109	59(54.1)	47(43.1)	3(2.8)	16	Siglar, 1979
Southeastern PA	110	59(53.6)	40(36.4)	11(10)	4	Kueppers and Harpel, 1979
California	1,050	537(51.1)	429(40.9)	74(7)	5	Grunbaum et al., 1980
NEGRO						
New York, NY	144	115(79.86)	28(19.4)	1(0.69)	1	Cleve and Bearn, 1961a
New York, NY	120	98(81.67)	19(15.83)	3(2.50)		Cleve and Bearn, 1961b
Southeastern GA	231	192(83.1)	38(16.5)	1(0.4)		Blumberg et al., 1964
California	832	(74.5)	(21)	(2)	6	Grunbaum et al., 1978
Detroit, MI	504	366(72.62)	112(22.22)	4(0.79)	7,16	Stolorow et al., 1979
Miami, FL	339	263(77.6)	64(18.9)	6(1.8)	8,16	Stuver, 1979
Los Angeles, CA	43	34(79.1)	7(16.3)	2(4.7)	16	Siglar, 1979
Southeastern GA	219	185(84.5)	26(11.8)	4(1.8)		
Philadelphia, PA	273	198(72.5)	61(22.3)	3(1.1)	9	Kueppers and Harpel, 1979
Combined	492	383(77.8)	87(17.7)	7(1.42)		
California	867	638(73.6)	183(21.1)	18(2.1)	10	Grunbaum et al., 1980
HISPANIC						
California	1,417	(59.1)	(35.3)	(5.2)	11	Grunbaum et al., 1978
Miami, FL	360	207(57.5)	126(35)	24(6.7)	12,16	Stuver, 1979
Los Angeles, CA	102	83(81.4)	17(16.7)	2 (1.9)	16	Siglar, 1979
California	1,908	1,160(60.8)	655(34.3)	80(4.2)	13	Grunbaum et al., 1980
OTHER						
New York, NY (Chinese)	117	69(59)	42(35.9)	6(5.1)	1	Cleve and Bearn, 1961a
California and Hawaii (Asians)	3,043	(50.4)	(39.2)	(6.4)	14	Grunbaum et al., 1978
California and Hawaii (Chinese and Japanese)	1,566	780(49.8)	590(37.7)	118(7.5)	15	Grunbaum et al., 1980

1. Calculated from gene frequencies
2. 0.4% were "rare"
3. One was Gc 1-Y
4. Subtyping: 3(2.7) 1F, 19(17.3) 1F-1S, 37(33.6)1S, 8(7.3)2-1F and 32(29)2-1S
5. 10 were "rare"
6. 2.4% were "rare"
7. 18(3.57) were Gc 1-Y, 3(0.6) were Gc 2-Y and 1 was "rare"
8. 6(1.8) were Gc 1-Y
9. Subtyping:
   GA — 141(64.4) 1F, 40(18.3) 1F-1S, 4(1.8) 1S, 21(9.6) 2-1F, and 5(2.3) 2-1S;
   PA — 130(47.6) 1F, 58(21.2) 1F-1S, 10(3.7) 2S, 51(18.7) 2-1F, and 10(3.7) 2-1S;
   Combined — 271(55.1) 1F, 98(19.9) 1F-1S, 14(2.8) 1S, 72(14.6) 2-1F and 15(3.0) 2-1S;
   in PA, there were 6(2.2) Ab-1F, 3(1.1) Ab-1F and 2(0.7) Ab-2;
   in GA, there were 3(1.4) Ab-1F and 1(0.5) Ab-2.
10. 28 were "rare"
11. Population was "Chicano/Amerindian; 0.5% were "rare"
12. 2(0.6) were Gc 1-Y and 1(0.3) was Gc 2-Y
13. Population was "Mexican" and many samples were from Mexico City; 13 were "rare"
14. 4% were "rare"
15. 78 were "rare"
16. And see Shaler, 1978

SECTION 42. TRANSFERRIN (SIDEROPHILIN)

42.1 Introduction

Most of the acid-soluble iron in blood plasma is reversibly associated with a special iron transporting protein, which shuttles Fe^{3+} to marrow, and to organs such as the liver. The presence of such a protein was suspected for decades. Starkstein and Harvalik (1933) localized it as a globulin protein, and Vahlquist (1941) found that it had the electrophoretic mobility of a β-globulin. Holmberg and Laurell (1945) and Schade and Caroline (1946) independently established that a specific protein in plasma was responsible for the iron-binding properties. They also established the physiological role of the protein, and recognized its clinical importance. Schade *et al.* (1949) called the protein "siderophilin", and showed that it took up two Fe^{3+} per mole, the uptake of one anion (HCO_3^-) for each iron being necessary for the binding to take place. Ehrenberg and Laurell (1955) carried out magnetic measurements on Fe-transferrin which indicated that the iron was ferric, and ionically bound. Holmberg and Laurell (1947) established that the protein bound copper ions as well, but that the binding was much weaker than with ferric ions which would readily displace copper. They suggested that the iron binding protein be called "transferrin".

42.2 Genetic Variation

In 1956, Smithies and Poulik introduced two-dimensional electrophoresis, the initial run being carried out on a filter paper strip, which was then inserted into a starch gel for electrophoresis in the second direction, at right angles to the first. Further studies (Poulik and Smithies, 1958) indicated several components in the β-globulin region, the most heavily staining one being third in its migration rate. This band was designated "C" for that reason. In 1957, Smithies reported that a β-globulin had been found in the sera of two Black women from New York (out of 49 tested) and in the sera of 5 Australian aborigines (out of 23) which had not previously been seen. Two-dimensional electrophoresis was necessary to see these differences. In most sera, β-globulins called A, B, C, and E were observed, but in the sera just mentioned, a "D" component was found as well. This D had not been observed in several hundred sera from Caucasians. Horsfall and Smithies (1958) extended these studies to a larger sample of aboriginal sera, including samples from members of families. The original sera had exhibited approximately equal amounts of components C and D, but a few were seen in the later sample that had only D. It was suggested that these β-globulins were under the simple Mendelian genetic control of a pair of alleles, called β^C and β^D. The "C" and "D" type sera represented the homozygotes, and the "CD" sera were from heterozygotes. In five out of 420 Canadian Caucasian sera, Smithies (1958) found a slightly faster protein called "B". Family studies indicated that B was conditioned by a third allele, β^B. It was soon established that the β-globulin protein exhibiting this genetically controlled variation was equivalent to transferrin (Smithies and Hiller, 1959). Independent lines of evidence based on independent experiments by Pert, Poulik, Allison, and by Sutton and Bishop (communicated to Smithies and Hiller, 1959) all pointed to the β-globulin-transferrin identity. The locus was, therefore, named *Tf*, and the known alleles Tf^C, Tf^D and Tf^B. Harris *et al.* (1958b) found two more variants, in addition to those seen by Smithies and coworkers. The faster one was called "D_2" (Smithies' "D" becoming "D_1"), and the slower one was called "B_2" (Smithies' "B" becoming "B_1"). The subscripts were designed to classify the faster and slower (than C) bands according to electrophoretic mobility. Giblett *et al.* (1959) then found three new transferrins (as heterozygotes of C), CD_0, CD_3 and B_0C. In 1960, Harris *et al.* found a CD_4 in two European families. By this time, it had become apparent that the nomenclature scheme which had been started was not going to be able to absorb the flow of new variants. Parker and Bearn (1961b) found a Tf in Navajo Indians that migrated between B_0 and B_1. This one was named "B_{0-1}", and was observed as a CB_{0-1}. Two further variants were found in New York Oriental populations by Parker and Bearn (1961a). One was a B_3D in a Japanese. The other, in a Chinese, ran very much like D_1, but rather than try to continue the numbering system, it was called "D_{Chi}". Some of the variants found subsequently were still given numerical subscripts, while others took on the names of the places or populations in which they were seen. The basic symbols "C" for the usual Tf, "D" for the slower, and "B" for the faster variants, were kept. Giblett (1969) suggested dropping numerical subscripts and sticking to geographical designations. Ultimately, as the chemical structures of the variants are worked out, a nomenclature descriptive of the chemical change can be adopted (Putnam, 1975d), as was done for the hemoglobins (section 38).

References to some further variants are: B_{1-2}, seen in a Venezuelan family as CB_{1-2} (Arends *et al.*, 1962); B_{Lae}, seen as CB_{Lae} and homozygous in New Guinea (Lai, 1963); $D_{Adelaide}$, seen in Australia and perhaps identical to D_0 (Cooper *et al.*, 1964); $B_{Atalanti}$, seen in Greece, and apparently not clearly distinguished from B_1 (Murray *et al.*, 1964); D_{Wigan}, seen in England (Glen-Bott *et al.*, 1964); $D_{Finland}$, seen in Finns, and perhaps the same as D_2 (Seppälä, 1965; Seppälä *et al.*, 1967); $B_{Lambert}$, perhaps the same as B_{1-2} (Barnett and Bowman, 1968); D_{Madiga}, $D_{Mudiraj}$, $B_{Goldsmith}$ and B_{Madiga}, seen in India (Rao *et al.*,

1979). There are several other variants as well, and further characterization will be required in many cases to demonstrate which of them are actually identical. Some of them are very rare, and serum is not available for the comparisons. Variants are discussed by Kirk (1968b), Bowman (1968), Giblett (1969) and Putnam (1975d). Relative electrophoretic mobilities of the Tf variants are indicated diagramatically in Figure 42.1.

The variants can be arranged on a scale of relative electrophoretic mobility, as has been done by Sutton and Jamieson (1972). They classified many of the Tf variants according to their relative mobilities on polyacrylamide disc gel electrophoresis, setting the mobility of C = 0. This kind of scheme provides a basis for comparing newly discovered variants with older ones, even if one does not have access to panels of sera containing variants, assuming the exact same electrophoretic procedures are followed. A representation of this scheme is shown in Figure 42.2. In 1975, Rittner and Rittner suggested a variation of this approach. They compared a number of variants by high voltage electrophoresis on agarose gels, and in longer runs could resolve most of them. They wanted to use Tf B_2 as the "reference variant", setting its relative electrophoretic mobility equal to +0.7 for practical reasons. The variants could then be designated according to their mobilities, e.g. B 0.78, D 1.06, etc. This nomenclature is analogous to that adopted for C3 variants (section 45). This nomenclature scheme is also shown in Figure 42.2.

The many population and family studies which have been carried out are fully consistent with the variant forms being representatives of alleles at the *Tf* locus (e.g. Kirk *et al.*, 1964; Kurz and Ritter, 1972). At least two families have been studied in which two non-C Tf genes occurred in heterozygous combination in some members. Beckman (1962) found a B_2D_1 combination, the first in which "fast" and "slow" variant types occurred together. Robinson *et al.* (1963) described an individual of $B_{1\text{-}2}B_2$ phenotype.

There are several reports of families with Tf deficiency (Heilmeyer *et al.*, 1961; Goya *et al.*, 1972). Other reports have been discussed by Gitlin and Gitlin (1975). These families suggest the possibility of a rare silent allele at *Tf*, although the data are somewhat complicated, and other genetic explanations are possible.

42.3 Further Genetic Heterogeneity in Tf—Tf C Subtypes

In 1978, Kühnl and Spielmann (1978a) in Germany, and, somewhat independently, Thymann in Denmark demonstrated that the common transferrin, Tf C, was resolved into three patterns by isoelectric focusing on polyacrylamide gels (PAGIF). Family studies revealed that these patterns represented three phenotypes, called Tf C1, C2-1 and C2, due to a pair of codominant alleles Tf^{C1} and Tf^{C2}. Thymann (1978) found the same polymorphism in Denmark. Apparently, Kühnl and Spielmann had first reported at a conference (the 7th International Congress of Forensic Haemogenetics in Hamburg) that this was some kind of an Hp polymorphism. She called the alleles Tf^1 and Tf^2. Using Kühnl and Spielmann's nomenclature, the German sample showed $Tf^{C1} = 0.8195$ and $Tf^{C2} = 0.1720$, while the Danish one showed $Tf^{C1} = 0.81$ and $Tf^{C2} = 0.19$. The Tf^{C1} of Kühnl and Spielmann is the Tf^2 of Thymann. Kühnl and Spielmann (1978a) showed that this was not a sialic acid effect, for neuraminidase did not obliterate the three phenotype behavior of the samples. The new Tf C subtypes were quickly confirmed by Stibler *et al.* (1979), who had earlier noticed heterogeneity of Tf C upon isoelectric focusing (Stibler *et al.*, 1978), and by Hoste (1979). In 1979, Kühnl and Spielmann (1979a) found a third allele, which was called Tf^{C3}. Phenotypes conditioned by the third allele were detected by a modified PAGIF procedure using a shallower pH gradient. The gene frequencies in the extended system were $Tf^{C1} = 0.795$, $Tf^{C2} = 0.155$ and $Tf^{C3} = 0.042$. There are, thus, six subphenotypes: Tf C1, C2, C3, C2-1, C3-1 and C3-2. Family studies indicated the accuracy of this genetic model. In 1979, Kühnl *et al.* looked at a series of Tf B and Tf D heterozygotes on PAGIF. In all CB or CD samples, only C1 or C2 was seen, but never both, suggesting that the new series of Tf^C alleles are true alleles of those conditioning the B and D variants. If all these *Tf* genes are indeed allelic, then any of the variant alleles discussed in section 42.2 could be paired with Tf^{C1}, Tf^{C2} or Tf^{C3}, increasing the number of possible Tf phenotypes by three-fold. Transferrin genetic variant nomenclature is due for some standardization in view of the new findings.

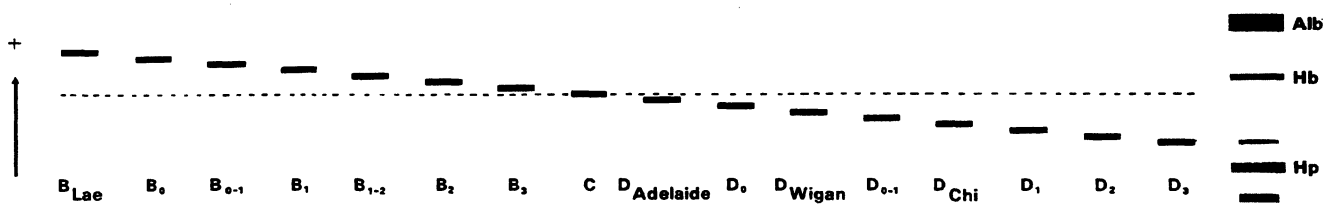


Figure 42.1 Relative Electrophoretic Mobility of Some Tf Variants at Alkaline pH.
Dotted line indicates position of the Common Tf C.

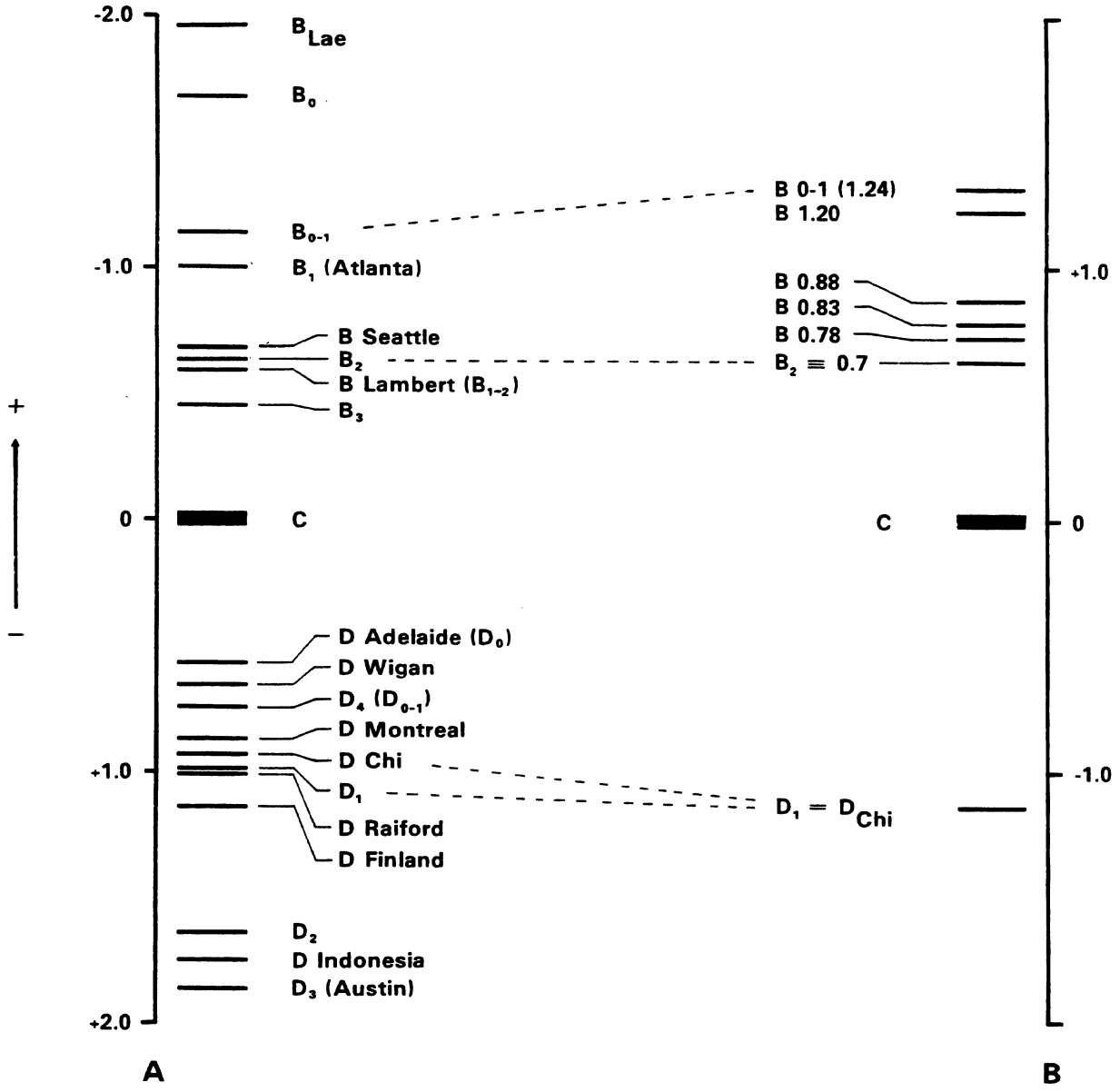


Figure 42.2 Arrangements of Transferrin Variants according to Relative Electrophoretic Mobility on (A) Polyacrylamide Gels (Sutton and Jamieson, 1972) and (B) Agarose Gels after High Voltage Runs (Ritner and Ritner, 1975).

42.4 Biochemical Studies on Transferrin

42.4.1 Structure of transferrin

There have been many studies on the amino acid composition of human Tf, which are reviewed by Putnam (1975d). There is strong evidence that the Tf molecule is a single polypeptide chain (Greene and Feeney, 1968; Mann *et al.*, 1970; MacGillivray and Brew, 1975) of MW about 76,000–77,000 (about 630 amino acid residues). There are 15 or 16 intrachain disulfide bridges, and apparently no free -SH groups. The molecule can be cleaved with cyanogen bromide to yield 9 fragments (see in MacGillivray and Brew, 1975, and Putnam, 1975d), and a number of these have been sequenced. Brune *et al.* (1978) reported the sequence of the N-terminal peptide fragment (50 residues), which they said completed about a third of the total sequence. The N-terminal peptide fragment contained two intrachain disulfide bridges, between Cys9 and Cys48, and between Cys19 and Cys39. There is considerable similarity in the composition of human and animal transferrins (Palmour and Sutton, 1971; Graham and Williams, 1975). There is also quite a bit of structural homology in different segments of human Tf C (MacGillivray and Brew, 1975), and between Tf and lactoferrin (Metz-Boutique *et al.*, 1978).

Transferrin is a glycoprotein, and its carbohydrate structure has been worked out completely (Jamieson *et al.*, 1971). There are two identical carbohydrate chains per Tf molecule, attached through an internal GlcNAc residue to Asn in the peptide chain. Each carbohydrate chain has two N-acetyl neuraminic acid "ends", so that there are four in the complete molecule. The structure is shown in Putnam (1975d) as well as in the original paper. Graham and Williams (1975) compared the carbohydrate structure of human Tf with that of the transferrin from several animal species. The presence of terminal sialic acid residues in human Tf was recognized early (Parker and Bearn, 1961c and 1962). Action of sialidase alters the mobility of Tf C in stepwise fashion, yielding five different bands which appear to differ from one another by a single unit of negative charge (Parker and Bearn, 1961c). This result shows that all four sialic acid residues are accessible to the sialidase. Sialidase treatment of Tf variants yielded the same kind of behavior observed with Tf C (Parker and Bearn, 1962). Similarly, neuraminidase treatment alters the pI of the Tf C subtypes (Kühnl and Spielmann, 1978a). However, neither the sialic acid nor the complete carbohydrate moiety appears to have anything directly to do with the polymorphism, which almost certainly reflects peptide chain compositional differences. There is evidence that the carbohydrate moiety has a biological role in Tf function (see in Putnam, 1975d).

42.4.2 Structural differences among Tf variants

Much work remains to be done in this field, and it is made more difficult by the size and internal complexity of the Tf molecule in terms of amino acid composition and by the rarity of many variants. Most of the information is based on differences in peptide fingerprints of a few of the variants. Wang and Sutton (1965) indicated that D_1 differed from C by an Asp→Gly substitution, which was detected in chymotryptic digests. This same substitution is found in Tf D_1 samples from American Blacks or from Australian aborigines (Wang *et al.*, 1967). Wang *et al.* (1966) found that on the basis of tryptic digest patterns, Tf B_2 differed from Tf C by a single Gly→Glu substitution. A simple substitution of His→Arg was reported for D_{Chi} by Wang *et al.* (1967). It now appears (Sutton *et al.*, 1975) that Tf D_1 may have two substitutions, Asp→Gly and Asn→Gly, while D_{Chi} has only the one. Immunological differences between Tf C, D_1, D_{Chi} and D_3 were not detected by Wang *et al.* (1968) using a radioimmune inhibition of precipitation technique.

Most authorities are inclined to think that the variants derive from one-step mutations resulting in single amino acid changes. There are some serious difficulties with such a view (Putnam, 1975d). It is hard to explain the large number of Tf variants, which appear to differ by at least a single unit of charge, by single amino acid substitutions in a molecule that appears to have no subunit structure. It is possible that there are size differences, which are reflected in the electrophoretic mobility, but there is no evidence for significant size differences. Perhaps more extensive structural differences do exist, although if these are different single amino acid substitutions, double or multiple mutations might have to be invoked as an explanation. Deletions or additions are possible; another possibility is that of repeating sequences within Tf.

42.4.3 Metal binding properties

This subject is complex, and reviewed in more detail by Putnam (1975d). Each Tf molecule binds two iron atoms (Schade *et al.*, 1949), and these are Fe^{3+} (Ehrenberg and Laurell, 1955). Bonding is ionic and bicarbonate anion is taken up for each ferric ion bound. Further, the binding is pH dependent. Tf can combine loosely with other metals, such as Cu (Holmberg and Laurell, 1947; Aasa *et al.*, 1963). Earlier work tended to suggest that the binding sites in Tf were independent and identical (e.g. Aisen *et al.*, 1966). More recent evidence indicates, however, that they are not identical (Harris, 1977; Aisen *et al.*, 1978). The sites are designated "A" and "B" (or "a" and "b"). Makey and Seal (1976) showed that four transferrins, representing iron free Tf (apo-Tf), Tf with Fe bound to site A, Tf with Fe bound to site B, and Fe_2-Tf, could be separated by electrophoresis in polyacrylamide gels containing 6M urea in Tris-EDTA-borate buffers at pH 8.4. It was not clear just exactly why the forms could be separated in this way. These results may help to explain the immunological differences seen by Jager and Gubler (1952) and Kourilsky and Burtin (1968). Leibman and Aisen (1979) took advantage of the ability to separate the forms in order to estimate their relative amounts in normal sera. Binding was not random, nor was it determined by the relative binding strengths of the A and B sites. The more acid-labile and weakly-binding B site of Tf was predominantly occupied.

42.5 Medicolegal Applications

The Tf system is used in some laboratories in disputed parentage cases (Mauff *et al.*, 1975; Dykes and Polesky, 1978). The exclusion probability is low in Europeans and in American Caucasians (about 1%), and about 5% in Black Americans. Chakraborty *et al.* (1974) quoted slightly higher values. The finding that Tf C can be subtyped by electrofocusing techniques, however, and that the alleles are reasonably well distributed (at least in Europeans), greatly enhances the value of the system. The exclusion probability increases to about 13–15% in Europeans if Tf C is subtyped (Thymann, 1978; Kühnl and Spielmann, 1979a; Hoste, 1979). Use of the Tf C subtypes is made more economical by the fact that the *Gc*[1] subtypes (section 41.3) and the Tf^{C} subtypes can be determined in the same isoelectric focusing gel (Thymann, 1978; Hoste, 1979).

Turowska (1969) noted that Tf could be typed in fresh bloodstains. There are a few laboratories in which Tf typing is done in bloodstains at the present time. It is possible to type Tf in some stains which are a matter of months old. The commoner variants can be detected on agarose gels, followed by immunofixation detection, and Tf typing is normally combined with Gc typing in the same gel.

Tf can be detected in a variety of human tissues (Mason and Taylor, 1978). Transferrin can also be detected in seminal fluid but not on sperm cells (Blake, 1976; Blake and Sensabaugh, 1976). The minimum quantity of semen required for typing is about 50 $\mu\ell$ compared with about 5 $\mu\ell$ whole blood, since the concentration of Tf is 10 times lower in semen (Blake and Sensabaugh, 1978). Tf typing might, therefore, be possible in seminal stains under favorable conditions.

42.6 The Distribution of Tf Phenotypes in U.S. Populations

The data for American populations which have been studied are given in Table 42.1.

Table 42.1 Distribution of Tf Phenotypes in U.S. Population

Population	Total	Frequency — Number (Percent) C	B_1 C	B_2C	CD_1	Note	Reference
CAUCASIAN							
Southeastern GA	107	103(96.3)		1(0.9)	2(1.9)	1	Cooper et al., 1963
Southeastern US	2,221	2,194(98.8)	2(0.09)	15(0.68)	10(0.45)		Roop et al., 1968
Chicago, IL	101	101(100)					Shih and Hsia, 1969
Tecumseh, MI	7,654	7,560(98.8)		83(1.1)	11(0.14)		Schreffler et al., 1971
Greater Philadelphia, PA	203	202(99.5)			1(0.5)	2	Pakstis et al., 1978
	196	193(98.5)		2(1.0)	1(0.5)		
California	801	786(98.1)				3	Grunbaum et al., 1980
NEGRO							
New York, NY	99	89(89.9)			9(9.1)	4	Parker and Bearn, 1961a
Southeastern GA	133	120(90.2)			13(9.8)		Cooper et al., 1963
Southeastern US	418	399(95.5)			19(4.5)		Roop et al., 1968
Chicago, IL	101	93(92.1)			8(7.9)		Shih and Hsia, 1969
Greater Philadelphia, PA	164	151(92.1)			13(7.9)	2	Pakstis et al., 1978
	169	158(93.5)			10(5.9)		
California	502	467(93)				3	Grunbaum et al., 1980
OTHER							
New York, NY Chinese	116	109(94)				5	Parker and Bearn, 1961a
California and Mexico City Mexican	765	742(97)				3	Grunbaum et al., 1980
California and Hawaii Asians	1,295	1,259(97.2)					

1. One was B_{1-2} B_2
2. The sampling area included sections of PA and southern NJ. The two populations listed are separate sums of two members of twin pairs. Among the 169 members of the Black co-twin 2 population, one was D_1.
3. The following numbers (percents) listed as "rare": Caucasian 15(1.87); Negro 35(6.97); Mexican 23(3.01); Asian 36 (2.78).
4. One was D_1
5. 7 were CD_{Chi}.

SECTION 43. α_1–ANTITRYPSIN (Pi)

43.1 Introduction

The principal protease inhibitors in human plasma are α_1-antitrypsin (α_1AT), α_2-macroglobulin (α_2M), inter-α-trypsin inhibitor (IαI), antichymotrypsin (Achy; α_1X), antithrombin III (AT III) and C1 activator (c1-Ina) (Laurell and Jeppsson, 1975). The most important of these are α_1AT and α_2M, and α_1AT is the subject of this section because of the extensive genetic polymorphism it exhibits.

Proteolytic enzyme inhibiting activity in blood was recognized many years ago (Hahn, 1897; Camus and Gley, 1897). The complexity of the proteinase inhibitor system of plasma became more clear with the more recent studies of Grob (1943), Shulman (1952) and Jacobsson (1955). Shulman (1952) was able to differentiate plasmin inhibition activity from the inhibition of trypsin or chymotrypsin. Jakobsson (1955) recognized two trypsin inhibiting activities in plasma, one associated with α_2 globulins and the other with α_1 globulins. The latter was much more plentiful. The α_1 globulin associated trypsin inhibitory activity was isolated by Schultze *et al.* in 1955, but it was not recognized as such by this group until later (Schultze *et al.*, 1962c), and was called "3.5S α_1 glycoprotein". It has also been studied by Poulik and Smithies (1958), who called it α_1-globulin, Norman (1958) who called it α_1-antiplasmin, Bundy and Mehl (1959) who called it α_1-trypsin inhibitor, and Burtin (1964) who called it α_{1_A}-globulin. Schultze *et al.* (1962c) named the protein "α_1-anti-trypsin", and it is this designation that has persisted in the literature. The biochemical journals tend to call it α_1-protease inhibitor. Nomenclature is still something of a problem with this system, despite efforts to standardize it (see further below).

43.2 Genetic Variation

43.2.1 Multiple alleles controlling α_1-antitrypsin

In 1963, Laurell and Eriksson noted that the sera of several patients suffering from pulmonary degenerative diseases exhibited markedly reduced concentrations of α_1-antitrypsin, and suggested that there was a correlation between the two things. The possibility of a genetically controlled 'inborn error of metabolism' was raised. Eriksson (1964) showed that the pathological condition was hereditary, behaving like a recessive, and that homozygotes for the trait seemed to be most likely to have the emphysema. Family members presumed to be heterozygous had roughly half the normal α_1–AT levels in their serum. These observations were confirmed independently by Kueppers *et al.* (1964) and others. Eriksson and Laurell (1963) found an electrophoretically slow α_1–AT band, accompanied by the usual band, in the serum of a patient. The serum concentration was normal, and each band accounted for about half of it. The α_1-antitrypsins represented by the two bands were immunologically indistinguishable. The patient's three children had normal α_1–AT patterns. When a similar case was found (Axelsson and Laurell, 1965), genetic and family studies indicated not only that the slow α_1–AT variant was genetically determined, but that the gene for it was allelic to that for the previously observed α_1–AT deficiency.

In 1965, Fagerhol and Braend discovered a system of protein zones in the "prealbumin" region, which exhibited observable variation in low pH (4.95) starch gels. Five phenotypes were seen in the sera of 390 healthy Norwegian blood donors, and family studies showed that the system was inherited, and under the control of three alleles. The name "Pr proteins" was suggested for the system. The locus was designated *Pr*, and the alleles Pr^F, Pr^M and Pr^S (for "fast", "medium" and "slow"). The phenotypes were called MM, MS, SS, FM and FS. It was soon realized that the "Pr system" proteins were not "prealbumins", but that they corresponded to the α_1–AT proteins (Fagerhol and Braend, 1966; Kueppers and Bearn, 1966b), and Fagerhol and Laurell (1967) suggested that, since these proteins represented the major plasma proteinase inhibitors, the locus controlling their variation should be called *Pi* (for *P*rotease *i*nhibitor). The system exhibited a remarkable degree of genetic polymorphism. By 1968, Fagerhol and Tenfjord could list seven alleles at *Pi*. Arranged in order of decreasing electrophoretic mobility of their products on acid starch gels, these are Pi^F, Pi^I, Pi^M, Pi^S, Pi^V, Pi^X and Pi^Z. A full description was given by Fagerhol (1968). An additional phenotype, MW, accounted for by a new allele Pi^W, was found in two Spaniards by Fagerhol and Tenfjord (1968). The slow moving variant originally described by Eriksson and Laurell (1963) and Axelsson and Laurell (1965) corresponds to the product of Pi^X, while the gene for α_1–AT deficiency (homozygous serum having about 10–15% of the normal α_1–AT concentration) is Pi^Z. Descriptions of the alleles Pi^I, Pi^F, Pi^S and Pi^V may be found in Fagerhol (1967) and Fagerhol and Gedde-Dahl (1969). The latter study also included family studies confirming the codominant mode of inheritance of the various *Pi* alleles. Similar family studies were done by Kellerman and Walter (1970).

A number of additional phenotypes and variants have been described. Some are better characterized than others, and family data are apparently not available for all of them. The ninth allele, Pi^P, detected as MP, and of low frequency, was described by Fagerhol and Hauge (1968). Pi^E and Pi^G were described by Fagerhol at an international meeting in 1971 (discussed by Cox and Celhoffer, 1974). Pi MW^2 was described by Gedde-Dahl *et al.* (1972), MY^2 and ZY^2 by Porter *et al.* (1972), Pi^B seen as BM by Martin *et al.* (1973), and Pi^L in a Bantu population by Vandeville *et al.* (1974).

Cox and Celhoffer (1974) described Pi^N, seen as MN, and they reviewed the variants known up to that time.

43.2.2 Quantitative variation and complete deficiency

Several of the *Pi* alleles appear to be associated with reduced α_1-AT synthesis, in addition to coding for proteins that are structural variants. These are Pi^Z, Pi^P and Pi^S. Association of deficient allele phenotypes with diseases is discussed further below. In 1969, Kueppers found that the Pi^Z allele yielded a product electrophoretically distinguishable from that of the common Pi^M, and that MZ and ZZ people could be distinguished in this way. The frequency of this allele was about 7% in 100 random blood donors from California. Talamo *et al.* (1973) found a 24 year old man with advanced pulmonary emphysema whose serum had no detectable α_1-AT by any technique. Members of his family had half the normal activity. The total deficiency phenotype was called "Pi-", and the man was considered a homozygote for a Pi^- allele. The half activity family members were thought to be Pi^MPi^-, and their phenotypes were written "M/-" or "M-".

43.2.3 Refinements of Pi typing methods, isoelectric focusing, Pi M subtypes and other variants

A number of the Pi phenotypes can be detected by acid starch gel electrophoresis. Unequivocal characterization of some of the variants requires two dimensional crossed immunoelectrophoresis. Electrophoresis in the first direction is carried out in acid starch gels, and that in the second direction is done in antibody-containing gels at 90° to the first direction. Up to eight zones can be detected by the crossed immunoelectrophoresis technique, and the patterns differ according to phenotype. Agarose gel electrophoresis can be used for Pi typing as well, with protein staining or immunofixation for detection (Laurell and Persson, 1973; Jeppsson *et al.*, 1979). A standard must be run alongside unknown samples if simple protein staining is used to detect Pi zones (Laurell and Persson, 1973).

Application of isoelectrofocusing to Pi typing has shown that it provides a somewhat easier method for phenotyping some of the variants than two-dimensional immunoelectrophoresis, and it has further revealed some additional genetic variations in the Pi system. Procedures for Pi typing by isoelectric focusing on polyacrylamide gels have been described by Allen *et al.* (1974), Arnaud *et al.* (1975 and 1977) and Kueppers (1976b). Detection has been carried out by protein staining, which may be accompanied by densitometric tracing, and by immunofixation techniques (Arnaud *et al.*, 1977). Immunofixation can be done in the gel itself, or by "printing" onto an overlaid cellulose acetate membrane strip. Hercz and Barton (1978) described a Schiff's reagent staining technique for α_1-AT in polyacrylamide gels following isoelectric focusing, which had the advantages of showing only the isoproteins of α_1-AT, and of giving some information about the carbohydrate content of the components. Some of the phenotypic patterns are improved by prior treatment of the samples with cysteine, and isoelectric focusing of the mixed disulfides (Pierce *et al.*, 1976).

Isoelectric focusing has revealed that the common phenotype on starch gels, Pi M, can be resolved into at least six subtypes, accounted for by three different Pi^M alleles which are comparatively well distributed in Europeans. The nomenclature of the new variants can be quite confusing to the nonspecialist, in spite of the efforts of an international group to standardize it (see below).

In 1976, Frants and Eriksson found three phenotypes within Pi M, which they called M_1, M_1M_2, and M_2. The alleles responsible were Pi^{M1} and Pi^{M2}. Another allele, Pi^{M3}, which was difficult to detect by conventional isoelectric focusing, was reported by Frants and Eriksson in 1978. The product of the Pi^{M3} allele could not be detected very well by conventional PAGIF when it was paired with another Pi^M allele, but could be distinguished when paired with a different *Pi* allele. However, the introduction of so-called "separators" into the gels along with the ampholines, a technique called "separation isoelectric focusing" or "SIEF", enabled the differentiation of the products of all three Pi^M alleles. The separators cause "plateaus" in the pH gradients, which can improve resolution in certain systems (Frants *et al.*, 1978). According to Frants and Eriksson (1978), the previously described Pi M_1 resolves to M_1, M_1M_2 and M_2, Pi M_1M_2 becomes M_1M_3, and Pi M_2 resolves to M_2M_3 and M_3 in the new three allele system. Kueppers (1976a) found one of these alleles, and called it Pi^{M1} where Pi^M was the usual allele. Van den Brock *et al.* (1976) found the same allele, and termed it Pi^{MN}. In 1978, Kueppers and Christopherson described the other allele. Klasen *et al.* (1977) also described the third common allele of Pi^M. Genz *et al.* (1977) found all six phenotypes, and they called the alleles Pi^{Ma}, Pi^{Mb} and Pi^{Mc}. The phenotypes were Pi Ma, Mb, Mc, Mab, Mac and Mbc. The equivalencies between these different designations, framed in terms of the Pi Committee recommendations, are: The Pi M_1, M_1M_2 and M_2 of Frants and Eriksson (1976) are equivalent to M, MM_1 and M_1 (Kueppers, 1976a), Ma, Mac and Mc (Genz *et al.*, 1977), and M, MM_N and M_N (van den Brock *et al.*, 1976). The committee recommended that these be called, respectively, M_1, M_1M_2 and M_2. The Pi^{M3} of Kueppers and Christopherson (1978) is apparently the same as that of Klasen *et al.* (1977) and of Frants and Eriksson (1978). The Pi^{M2} is also equivalent to the Pi^N of Constans and Viau (1975), but not to the "Pi^N" of Cox and Celhoffer (1974). The M_1, M_1M_2 and M_2 phenotypes have been studied in the Japanese population by Harada *et al.* (1977).

Some additional phenotyes reported in the Pi system are M_{Lamb} and $M_{Baldwin}$ (Johnson, 1976), Pi W3 Constantine (Khitri *et al.*, 1977), Pi Nhampton (or Nham) (Arnaud *et al.*, 1978a), Pi B Alhambra (Yoshida *et al.*, 1979), Pi T (Kühnl and Spielmann, 1979b and 1979c), Pi <L (Kühnl and Spielmann, 1979b) and Pi Gam (Welch *et al.*, 1980). There are quite a few others as well, some better characterized than others, and they are discussed in Cox *et al.* (1980).

It may be noted here that *Pi* has been shown to be linked to *Gm* (Gedde-Dahl *et al.*, 1972). Further discussion and additional data are given by Fagerhol (1976). Recombination frequency was dependent in certain ways upon the Pi alleles

present, and the phenomenon is apparently not yet fully understood.

43.2.4 Standardization of nomenclature and techniques

Pi phenotype identification is apparently not as easy to master nor as straightforward as that for many other systems, especially when variant phenotypes are involved. Some variants are more difficult to diagnose than others. Special isoelectrofocusing techniques are required to be certain about the identity of the Pi M_3 phenotypes. In 1978, an international committee met to consider the complexities of the Pi system, and their work is reported by Cox (1978) and Cox *et al.* (1980). The committee recommended that the protein be designated α_1-antitrypsin (α_1AT), and its genetic locus as *Pi*. Several reference laboratories have been established, and they try to maintain panels of variants against which "new" ones can be compared by various different techniques. In our part of the world, one reference laboratory is maintained at the Hospital for Sick Children in Toronto (Dr. Diane Wilson Cox), and another is at the University of North Carolina Medical Center in Chapel Hill (Dr. A. Myron Johnson). Details are published in Cox *et al.* (1980). The committee established rigorous standards for confirmation of variants as "new". They noted that several different techniques must be used to compare a "new" variant with known ones. In addition, they recommended some standardization of the nomenclature. A laboratory manual has been prepared under NIH sponsorship in this country (Talamo *et al.*, 1978), and it contains the detailed standard procedures. The papers should be read for the details. A few highlights are: The M1, M2 and M3 designations for the common subtypes of Pi M were retained, their alleles being Pi^{M1}, Pi^{M2} and Pi^{M3}; upper case letters will continue to be used to indicate relative position in isoelectric focusing gradients, and descriptive names can be appended as needed; lengthy descriptive names can be abbreviated by using the first few letters of the name, e.g. Pi $E_{Cincinnati}$ = Pi E_{Cin}, Pi $M_{chapelhill}$ = Pi M_{cha}, and so forth; the complete deficiency allele is called Pi^{Null}. A number of additional variants are discussed in Cox *et al.* (1980). It is also clear from the report that unequivocal diagnosis of some variant Pi types is not always an easy matter, even for experienced laboratories. They recommended against Pi typing in ordinary clinical laboratories, suggesting instead that a few reference laboratories be set up for the purpose, each serving a geographical area containing 5 to 10 million people. These larger labs would have the resources and volume of work to maintain complete sets of reference sera, and so forth.

Not everyone agrees completely with the committee's recommendations. Yoshida (1979) took exception to several of them. He noted that the name "alpha-antitrypsin" had become widely used by clinical workers, but that the biochemical journals still preferred "α_1 protease inhibitor". He preferred the latter, since the ". . . . .protein is not an antibody against trypsin", and because that name is compatible with the locus name, *Pi*. He said further that the guidelines for the identification and publication of new variants imposed unreasonable restrictions on investigators, and should not be adopted. Restricting the criteria to electrophoretic or electrofocusing mobilities was a mistake, he said, because variants not differing in charge would be missed. Biochemical properties, including kinetic properties, amino acid analyses, etc., should be included too, he said. It would also be helpful if standard electrophoretic and isoelectrofocusing procedures were recommended, and all established variants run in the standard systems, and the results published.

43.3 Relationship of α_1–Antitrypsin Deficiency and Disease

There is a well established connection between certain kinds of degenerative lung diseases and the Pi^Z allele. Homozygotes for the gene, Pi ZZ people, have a high risk of developing chronic obstructive pulmonary disease (COPD), which leads to emphysema. Similarly, Pi ZZ infants are at high risk for developing neonatal hepatitis and cirrhosis of the liver. This material is discussed by Kueppers and Bearn (1966a), Kueppers and Black (1974), Fagerhol (1976) and has been reviewed by Morse (1978). In homozygous deficient subjects (ZZ), and less so in Z-heterozygotes, some of the liver cells accumulate α_1–AT (see, for example, Lieberman *et al.*, 1972), and this leads to liver cell necrosis. Most investigators think that Pi SZ people are at higher risk for COPD as well, but there is some controversy about the risk of MZ or MS individuals. The pathogenesis of the lung disease almost certainly has nothing to do with trypsin, which rarely gets into circulation. α_1-Antitrypsin can inhibit other proteases, notably leucocyte collagenase and elastase. These enzymes have been implicated in the production of emphysema lesions, and their excess activity in α_1–AT deficient subjects may provide a basis for understanding the relationship. There is much more to it, and more work will be required before an understanding is achieved. For one thing, there is quite a bit of individual variation among ZZ people with respect to clinical manifestations in lung or liver. Another thing is that α_1–AT deficiency is implicated in only a small percentage of degenerative lung disease patients. There is some evidence of the association of α_1–AT deficient phenotypes with other diseases. Although the frequency of Pi^Z is significant in many populations, and 2 to 5% of some European and American populations may be considered "at risk" on the basis of the preliminary data, there is still a question as to whether mass screening programs are appropriate. These matters are discussed by Fagerhol (1976) and Morse (1978). Chapuis-Cellier and Arnaud (1979) recently reported that the Pi^Z allele is preferentially transmitted to offspring when it is present in the father suggesting that Pi^Z-carrying sperm enjoy a selective advantage of some sort.

43.4 Biochemical Studies

Some biochemical properties of α_1–AT were mentioned at the beginning of the section, in connection with the earlier studies on its isolation and characterization. More recent purification and isolation studies are reviewed by Laurell

and Jeppsson (1975). Schultze *et al.* (1963) described the purification procedures used at that time. α_1-Antitrypsin is an inhibitor of a number of proteolytic enzymes besides trypsin, including plasmin, chymotrypsin, thrombin (see Rimon *et al.*, 1966), elastases and collagenases. The esterase activity of these enzymes is inhibited as well. The mechanism of inhibition involves some kind of complex formation, but its exact nature has not been completely elucidated. α_1-AT is unstable at low pH (less than 5) and loses its inhibitory capacity. The activity can be recovered, however, by titrating back up (Glaser *et al.*, 1977). Aggregates form at about pH 4 which retain immunological identity, but which do not inhibit proteases. There is microheterogeneity associated with α_1-AT regardless of the phenotype, and its biochemical basis is not very clear. Musiani *et al.* (1976) isolated 8 fractions from Pi M sera on a preparative scale, and the fractions showed various distributions in a pH 4–6 isoelectric focusing gradient. These fractionation results will now have to be interpreted in terms of the now recognized Pi M subtypes. Hodges *et al.* (1979) isolated and characterized the oligosaccharide chains of the α_1-AT molecule (from Pi M people). There are two types of chains, a large one with a complicated structure, and a smaller one. It appeared that each protein contains one large and three small chains. Both kinds are attached to the peptide through GlcNAc—Asn linkages. The large chain can have up to three terminal N-acetyl-neuraminic acid residues, and the small chain has two. Therefore, there could be up to 9 terminal NANA residues in the intact molecule, if these relative amounts are correct.

There are a few biochemical studies on the protease inhibitor from specific different phenotype sera. Only in a few cases have the amino acid differences between variant and M molecules been worked out. The difference between Pi M and Pi S resides in a single amino acid (Owen *et al.*, 1976; Yoshida *et al.*, 1977). Pi S has Val where Pi M has Glu, and this was clear in two completely different examples of the protein. Jeppsson *et al.* (1978) purified Pi M, S and Z molecules, and said that they had detected a single amino acid difference in one cyanogen bromide peptide between M and Z. M had Glu where Z has Lys. Yoshida *et al.* (1979) characterized their Pi B Alhambra mutant fully, and found two amino acid substitutions, Lys→Asp and Glu→Asp. Hercz and Barton (1977) have purified Z protein, and found that it contained more glycine residues than the M protein, but that it did not differ in carbohydrate content. They also discussed some of the apparently conflicting results of various workers. Bauer *et al.* (1978) have isolated and studied Z protein material from the liver of a Pi ZZ individual. It was homogeneous, and antigenically intact, but had no proteinase inhibitor activity. There was no galactose or sialic acid in the carbohydrate moiety and GlcNAc was reduced. Mannose was present in excess, however.

Arnaud *et al.* (1978b) studied a number of individuals who had the Pi^{i} allele. It was found that this allele is in the class of "deficient" alleles, in that people who had it showed reduced protease inhibitor levels. Further, two subtypes of Pi^{i}, called I_1 and I_2, were detected by electrofocusing. Kueppers *et al.* (1977) described a woman with a Pi M phenotype, but who was deficient in α_1-AT. She had emphysema, and her father had COPD. He had normal levels of α_1-AT, but her mother and two sibs had about 50% normal levels. This family may have a variant "M", which is indistinguishable from the usual M, and the possibility of Pi^{Null} cannot be ruled out in the family. Kahn *et al.* (1977) conducted studies on a number of normal, partially deficient and fully deficient individuals, and the results indicated that α_1-AT contributes >90% of the total antitrypsin activity of normal plasma.

Reviews of the Pi system may be found in Fagerhol (1968 and 1976), Giblett (1969), Fagerhol and Laurell (1970) and Laurell and Jeppsson (1975).

43.5 Medicolegal Applications

No direct references to the application of the Pi system to disputed parentage cases were found in the literature, although a number of authors mention its obvious applicability. The system is much more powerful if Pi M subtyping is performed, and specialized laboratories will probably be employing the system in this way.

There has been little reported work on Pi phenotyping in bloodstains. Frants and Eriksson (1979) indicated that they had obtained promising preliminary results on Pi phenotyping in dried blood by isoelectrofocusing. The system would be most useful if Pi M subtypes could be determined. As was noted above, difficulties can be encountered with many of the variant phenotypes. Stored sera tend to develop "storage" bands, which make the already complex patterns more difficult to interpret, but sulfhydryl reagents alleviate this problem appreciably. A study of the ability to determine Pi phenotypes in postmortem bloods was done by Conrad *et al.* (1979). Sera stored frozen (−20°) retained their phenotypic characteristics quite well if the pH was 7.0–7.5. 86% of the sera stored for 2–2½ years, but only 30% of those stored for more than 4 years, could be typed. There was some decrease in the typability of sera that were not collected immediately after death.

α_1-AT is found in genital secretions as well, where it is suspected of having some role in fertilization (Hirschhäuser *et al.*, 1972), and it is possible that the protein might provide a genetic marker in these fluids. The cervical mucus shows cycle-dependent variations in α_1-AT content, which are affected by oral contraceptive therapy (Schumacher, 1970). α_1-AT is found in semen at mean levels of 68–87 $\mu g/m\ell$, but not in sperm cells (Schumacher, 1970; Blake, 1976; Blake and Sensabaugh, 1976 and 1978). Blake and Sensabaugh (1978) estimated that about 100 $\mu\ell$ semen would be needed to detect α_1-AT, compared with about 5 $\mu\ell$ whole blood. They did not regard it as a very good prospect for typing in forensic samples containing semen. It is not completely clear whether the Pi phenotypes observed in serum are expressed in semen in the same way.

43.6 Distribution of Pi Phenotypes

There have been very few studies in U.S. populations, and in some cases only the gene frequencies were reported. Leaving aside Pi M subtyping for a moment, it is found that Pi M is the common phenotype in most populations examined. In Europeans, the Pi^S frequency is polymorphic, and is higher in Spain, Portugal and the south of France than elsewhere. The frequency of FM varies from about 0.2 to about 2%, and that of MZ is between about 0.2 and 5%. Pi^Z is more frequent in Caucasians, and relatively few non-M phenotypes seem to occur in Africans, Asians, Finns, Lapps and Greenland Eskimos.

In St. Louis, MO, Dew *et al.* (1973) reported $Pi^M = 0.9480$, $Pi^F = 0.0027$, $Pi^I = 0.0012$, $Pi^S = 0.0344$ and $Pi^Z = 0.0127$ in 2,047 Caucasians. In another St. Louis study, 1,933 Caucasians were 90% M, 6.7% MS and 2% MZ, while 204 Negroes were 96.1% M, 2% MS and 1% MZ (Pierce *et al.*, 1975). The gene frequencies observed in 188 Caucasians in San Francisco were very similar to those in St. Louis (Kueppers, 1971). Kueppers and Christopherson (1978) reported the frequencies of Pi phenotypes, including subtypes, in 240 Whites (Rochester, MN and southeastern PA), and in 304 Blacks (southeastern GA and Philadelphia, PA). Among the Caucasians were: 98 (41%) M_1, 14 (5.8%) M_2, 2 (0.8%) M_3, 51 (21.3%) M_1M_2, 39 (16%) M_1M_3, 7 (2.9%) M_2M_3, 1 (0.4%) FM_1, 2 (0.8%) IM_1, 12 (5%) M_1S, 6 (2.5%) M_2S, 2 (0.8%) M_3S and 6 (2.5%) M_1Z. Among the Negroes were: 248 (81.6%) M_1, 1 (0.3%) M_3, 15 (4.9%) M_1M_2, 29 (9.5%) M_1M_3, 2 (0.7%) M_2M_3, 1 (0.3%) IM_1, 3 (1%) FM_1, 3 (1%) M_1S and 2 (0.7%) M_1Z. The Pi^{M1}, Pi^{M2} and Pi^{M3} allele frequencies were, respectively, 0.64, 0.19 and 0.11 in Whites, and 0.903, 0.028 and 0.054 in Blacks.

SECTION 44. GENETIC MARKERS OF THE IMMUNOGLOBULINS—Gm, Km, Am AND Hv

44.1 Introduction

The genetically determined antigenic markers which comprise the Gm and Km (and the Am and Hv) systems are located on the immunoglobulins, the family of serum proteins which possess antibody activity. The classes and structure of the immunoglobulins were discussed in section 1.3.3.2. There are five classes of these molecules, each possessing a different type of heavy chain. Immunoglobulins have the general structure H_2L_2, where H stands for a heavy chain and L for a light chain. There are two types of light chains, κ and λ, both of which occur in individuals, but not in the same molecule. IgM is a polymer of the basic H_2L_2 structure.

Gm and Km are among the most complicated of the genetic marker systems, and they are very useful in forensic problems. Among the many excellent reviews of these systems are Prokop and Bundschuh (1963), Natvig and Kunkel (1968), Franklin and Fudenberg (1969), Giblett (1969), Görtz (1969), Grubb (1970), Mage *et al.* (1973), Natvig and Kunkel (1973), Ropartz (1974) and Giblett (1977).

44.2 Genetic Variation in the γ Chains of IgG—The Gm System

Some terminology arises in discussions of Gm that is not commonly used in most of the other systems, and it will be introduced briefly here. Three degrees of specificity related to structural variation have been differentiated, based on the antigenic properties of the immunoglobulin molecules within a given species (Giblett, 1969). *Isotypic* specificities are common to all members of a species, but differentiate immunoglobulin molecules into classes and subclasses. *Allotypic* specificities differ within a species, and reflect inherited variation of molecular structure. The polymorphic genetic marker systems (Gm, Km) represent allotypic specificity differences, and one can and does refer to the "Gm allotypes". *Idiotypic* specificities are characteristic of the products of single cells (or their clones). Myeloma proteins are a good example. The term *haptotype* is not used too much in discussions of Gm genetics, but it could be. It is a very common term in HLA genetics. When a chromosome segment carries closely linked genes that are part of the same system, and which tend to be inherited together as a unit, the gene makeup of one or the other homologous chromosome segment is called a *haplotype*. In the Fisher-Race conception of Rh genetics, therefore, one could speak of Rh haplotypes. Similarly, one could use the term in MNSs and in Gm genetics, though it seems to be encountered most often in discussions of HLA.

44.2.1 The Gm factors

In 1956, Grubb found that Rh+ cells sensitized with some examples of incomplete anti-D were agglutinated by the sera of some patients who had rheumatoid arthritis. Further, this agglutination could be inhibited by the sera of some 60% of normal random donors. Similar observations were made by Milgrom *et al.* (1956) and by Waller and Vaughn (1956). The inhibiting property of normal serum was quickly shown by Grubb and Laurell (1956) to be inherited as a simple Mendelian dominant. The responsible factor was found in the gamma globulin fraction (now known as IgG), and it was called Gm(a). The active rheumatic sera contained an "anti-Gm(a)" which agglutinated anti-D sensitized Rh+ cells, provided the anti-D IgG possessed the Gm(a) determinant. Normal sera having Gm(a) on the IgG molecules reacted with the anti-Gm(a) in the active rheumatic sera, thus inhibiting its ability to agglutinate the sensitized Rh+ cells. Soon afterward, two other genetically determined, serologically detectable differences in the IgG were found using different reagent pairs (rheumatoid serum agglutinator and sensitizing anti-D), and they were called Gm(c) (Harboe and Lundevall, 1959) and Gm(b) (Harboe, 1959).

By 1965, approximately 14 distinct Gm specificities had been identified, and nomenclature had become something of a problem. A great deal of new information had also become available about the structure and properties of immunoglobulins around this time. In 1964, the nomenclature of human immunoglobulins was standardized (W.H.O., 1964). As a logical outgrowth of that work, a standardized nomenclature for the Gm and Km allotypes was recommended in the following year (W.H.O., 1965; Ceppellini and many others, 1966). Numbers were assigned to the Gm and Km specificities, and their use was recommended instead of the letters that had been used. In the more recent nomenclature revision, however, letter designations and their numerical equivalents are apparently equally acceptable (W.H.O., 1976). More will be said about the recent recommendations in appropriate sections below.

Almost 30 different Gm factors have been described. Some have been found to be the same as others, and in some cases, the specificities can no longer be tested because there are no longer any reagents available. In a few cases, the relationships of particular factors to the rest of the system are not very clear. The list of factors, and some of their equivalent names, is shown in Table 44.1.

The original anti-Gm antibody, now called anti-Gm(a), was found in the serum of a rheumatoid arthiritis patient, and many of the subsequent examples of antibodies defining

Table 44.1 Genetic Markers of the Immunoglobulins

Chain Location	Recommended Designation: Alphameric	Recommended Designation: Numeric	Other or Previous Designations: Alphameric	Other or Previous Designations: Numeric	Reference(s)
Allotypic Markers of the γ Chains					
IgG1:	G1m(a)	G1m(1)	Gm(a)		Grubb, 1956
	G1m(x)	G1m(2)	Gm(x)		Harboe and Lundevall, 1959
	G1m(f)	G1m(3)	Gm(b^w), (b2), (f)	Gm(3),(4)	Steinberg and Wilson, 1963; Gold et al., 1965; Steinberg, 1965
	G1m(z)	G1m(17)	Gm(z)	Gm(17)	Litwin and Kunkel, 1966a and 1966b
IgG2:	G2m(n)	G2m(23)	Gm(n)	Gm(23)	Kunkel et al., 1966; Natvig and Kunkel, 1967
IgG3:	G3m(b0)	G3m(11)	Gm(b^β), (b0)	Gm(11)	Ropartz et al., 1963; van Loghem and Martensson, 1967
	G3m(b1)	G3m(5)	Gm(b), (b1), (Jb^γ)	Gm(5),(12)	Harboe, 1959; Ropartz et al., 1963; van Loghem and Martensson, 1967
	G3m(b3)	G3m(13)	Gm(b3), (Bet)	Gm(13),(25)	Steinberg and Goldblum, 1965
	G3m(b4)	G3m(14)	Gm(b4)	Gm(14)	Steinberg and Goldblum, 1965
	G3m(b5)	G3m(10)	Gm(b^a), (b5)	Gm(10)	Ropartz et al., 1963; van Loghem and Martensson, 1967
	G3m(c3)	G3m(6)	Gm-like, (c), (c3)	Gm(6)	Steinberg et al., 1960
	G3m(c5)	G3m(24)	Gm-like, (c), (c5)	Gm(24)	van Loghem and Martensson, 1967
	G3m(g)	G3m(21)	Gm(g)	Gm(21)	Natvig, 1968
	G3m(s)	G3m(15)	Gm(s)	Gm(15)	Martensson et al., 1966
	G3m(t)	G3m(16)	Gm(t)	Gm(16)	Martensson et al., 1966
	G3m(u)	G3m(26)	Gm(Pa)		van Loghem and Grobbelaar, 1971; Steinberg, 1977
	G3m(v)	G3m(27)	Gm(Ray)		
		G3m(28)		Gm(28)	Rivat et al., 1978
Markers whose status is unclear and/or for which reagents are no longer available					
–			Gm(r)	Gm(7)	Brandtzaeg et al., 1961
–			Gm(e)	Gm(8)	Ropartz et al., 1962
IgG1			Gm(p)	Gm(9)	Waller et al., 1963
IgG1			RO2, Rouen 2	Gm(18)	Ropartz et al., 1967
–			RO3, Rouen 3	Gm(19)	Ropartz et al., 1966
–			San Francisco 2	Gm(20)	Klemperer et al., 1966; Ropartz et al., 1966
–			Gm(y)	Gm(22)★	Litwin and Kunkel, 1967
Light Chain Markers					
κ	–	Km(1)	InV, Inv(1)(l)	Inv(1)	Ropartz et al., 1962
	–	Km(2)	Inv(a)	Inv(2)	Ropartz et al. 1961a and 1961b
	–	Km(3)	Inv(b)	Inv(3)	Steinberg et al., 1962
Isoallotypic Markers (the so-called "non-markers")					
	nG1m(a)	nG1m(1)	non-a		Natvig et al., 1969
	nG1m(z)	nG1m(17)	nG1m(z)		
	nG3m(b0)	nG3m(11)	non-b0		Gaardner and Natvig, 1972
	nG3m(b1)	nG3m(5)	non-b1		Gaardner and Natvig, 1972
	nG3m(g)	nG3m(21)	non-g		Natvig et al., 1969
	nG4m(a)		4a		Kunkel et al., 1970
	nG4m(b)		4b		Kunkel et al., 1970
		nA2m(2)		nA2m(2)	
Other Markers					
IgA2:	A2m(1)			$Am(1), Am_2$	Vyas and Funderberg, 1969
	A2m(2)			$A_2M(2)$	van Loghem et al., 1973
IgG1				Isf(1)	Ropartz et al., 1966 and 1968
Heavy chains (variable region) of IgG, IgM and IgA				Hv(1)	Wang et al., 1978

Recommended nomenclature after W.H.O. (1976)

★ Equivalent to nG1m(a) = nG1m(1) (Ropartz, 1974)

Gm specificities have been found in such sera. These are called "Ragg" antibodies (for *R*heumatoid *Agg*lutinating). Examples of various anti-Gm of various specificities are also found in non-rheumatoid sera (Ropartz *et al.*, 1960), especially in multiply transfused patients, women who have been pregnant, and in children. These antibodies are called "SNagg" (for *S*erum *N*ormal *Agg*lutinating). The former are usually quite potent, but may have autoantibody activity, multiple specificity and they often show prozones. The latter are usually of lower titer, but they are often monospecific and do not show prozones. They are the preferred reagents for Gm typing.

Gm(1), or Gm(a), was the first specificity to be described (Grubb, 1956) as noted above. Antisera to Gm(1) have been found in several hundred people. Hemagglutination inhibition remains the best typing method, and the anti-D must come from a Gm(1) person, preferably someone containing as few other Gm factors as possible. It has occasionally been reported that Gm(1) can be detected by one reagent system but not another, and there are reports of reagent systems that appear to differ in the detection of Gm(1) in different racial groups. Gm(2), or Gm(x) was described by Harboe and Lundevall (1959), and many additional examples of anti-Gm(2) from rheumatoid and normal sera have been found. Gm(2) is rare in Black populations. Gm(3) was defined by the serum of a 4 year old Black boy (Steinberg and Wilson, 1963), and was called Gm(b^w). It has also been called Gm(b^2) (Steinberg and Goldblum, 1965). Anti-Gm(3) has not been found in anyone else (Grubb, 1970). Gm(4) was described by Gold *et al.* (1965) who called it Gm(f). Examples of anti-Gm(4) have been found in more normal people than rheumatoid arthritis patients. Discrepant results have been reported in Gm(4) detecting systems, depending upon the reagents used. There is considerable evidence to suggest that Gm(4) is not different from Gm(3) (Steinberg, 1965), and they are regarded as identical. Gm(5) was described by Harboe (1959) who called it Gm(b). It has been called Gm(b^1) (Steinberg and Goldblum, 1965), and Gm(b^γ). Anti-Gm(5) antibodies have been found on a number of occasions. Those which readily distinguish Gm(5) from Gm(−5) in Caucasians can give markedly different reactions when used in other populations. This discovery has led to the delineation of a number of additional Gm factors. Gm(6) was first called "Gm-like" by Steinberg *et al.* (1960), and later Gm(c) (Steinberg and Wilson, 1963). It has also been called Gm(c5). A number of examples of anti-Gm(6) have been found. Most of the antibodies have come from Whites, many with rheumatoid arthritis, although the factor is mostly found in Blacks. Gm(7) was described by Brandtzaeg *et al.* (1961), and called Gm(r). Another anti-Gm(7) has been found, but apparently most of the reagents have now been exhausted. Gm(8) was first described by Ropartz *et al.* (1962), and then named Gm(e). The original anti-Gm(8) is the only one that had been found up to 1970, and it came from a rheumatoid arthritis patient. Gm(9) was originally called Gm(p) (Waller *et al.*, 1963), and two examples of anti-Gm(9), both from patients with rheumatoid arthritis, were described. Further examples had not been found by 1970. Gm(10) was described by Ropartz *et al.* (1963) as Gm(b^α). The anti-Gm(10) came from a healthy Caucasian, and no further examples had been seen by 1970. Grubb (1970) said that further studies would be needed to show that Gm(10) was different from Gm(13). Indeed, Johnson *et al.* (1977) regarded Gm(10) and Gm(13) as identical. Gm(11) and Gm(12) were also found by Ropartz *et al.* (1963), and called Gm(b^δ) and Gm(b^γ), respectively. Gm(11) has been called Gm(b^0) (van Loghem and Martensson, 1967). Other examples of anti-Gm(11) have been found. Gm(11) and Gm(5) are usually found together in Caucasians. Gm(12) is not clearly distinguishable from Gm(5), and they are considered identical. Gm(13) was the Gm(b^3) of Steinberg and Goldblum (1965). It has also been called Gm(Bet). Further anti-Gm(13) sera have been found. As noted above, Grubb (1970) said that Gm(13) and Gm(10) need to be more clearly distinguished. Gm(14) is the Gm(b^4) of Steinberg and Goldblum (1965). Anti-Gm(14) sera are apparently rare. Gm(15) and Gm(16) were described as Gm(s) and Gm(t) by Martensson *et al.* (1966). Some further examples of antisera to both have been found. Gm(17) is the Gm(z) of Litwin and Kunkel (1966a and 1966b). It was detected by a rabbit anti-Gm(17) prepared by immunization with the Fab fragment of IgG1 myeloma protein, followed by absorption with Gm(−17) immunoglobulin. Gm(18) is the Gm (Ro2), or 'Rouen-2' of Ropartz *et al.* (1967). Antisera are apparently very rare. Gm(19) is Gm(Ro3) or 'Rouen-3' of Ropartz *et al.* (1966). The antiserum was difficult to work with. Gm(20) was described by Klemperer *et al.* (1966), defined by an antibody from a rheumatoid arthritis patient. No further examples have been found. Gm(20) was only found in Gm(1) people. Gm(21) is the Gm(g) of Natvig (1966). The first antisera were human Raggs, but anti-Gm(21) has been produced in animals. Gm(21) is almost always reciprocally related to Gm(5) and Gm(11). Gm(22) is the Gm(y) of Litwin and Kunkel (1967), and was defined by a rabbit anti-IgG1 (Gm−1,4) myeloma protein. Anti-Gm(22) are apparently very scarce, if there is still any available at all. Gm(23) is Gm(n) (Kunkel *et al.*, 1966), and was detected by an antiserum prepared in primates against IgG2 myeloma protein. Gm(23) is the only allotypic marker known on IgG2. Gm(23) can be detected by precipitation in gels, the first Gm specificity to be detected in this way. The technique requires too much precious antiserum, however. No anti-D with Gm(23) has ever been found, so a different technique was used (Natvig and Kunkel, 1967). IgG2 myeloma protein with Gm(23) can be coated onto red cells using bis-diazotized benzidine, thus providing the "coat" for the test system. Tanned red cells did not work very well (see sections 1.3.4.1 and 16.3). Gm(24) is apparently the Gm(c^5) of van Loghem and Martensson (1967). Gm(25) appears to be identical with Gm(13) and no longer treated separately. Thus, as noted above in connection with Gm(10), Johnson *et al.* (1977) regard Gm(10) = Gm(13) = Gm(25). Gm(26) was described by van Loghem and Grobbelaar (1971) on the basis

of immune sera prepared in baboons. Steinberg (1977) described further studies on it. Gm(26) is also called Gm(u), and has been called Gm(Pa). Gm(27) is known as Gm(v) and Gm(Ray). Gm(28) was recently described by Rivat *et al.* (1978). A Gm factor called "L1" was described by Blanc *et al.* (1976) in two families, one French and the other Algerian. It was transmitted with Gm(1) and Gm(17), was always present in Gm(21) sera, and always absent in Gm(5) sera. It is unclear whether it has yet been assigned a number. Ropartz (1974) said that Gm(22) or Gm(y) had been found to be equivalent to Gm(non-a), which according to the nomenclature recommendations should be designated nGlm(1) or nGlm(a) (see further below).

44.2.2 Assignment of Gm factors to IgG subclasses

Existence of isotypic subclasses of IgG has been recognized for some time. In certain neoplastic diseases, especially multiple myeloma, immunoglobulins are synthesized in excess. Urine may contain an unusual protein in these conditions, now known to represent immunoglobulin light chains, and called Bence-Jones protein. The IgG synthesized in multiple myeloma is unique in its high degree of homogeneity, as compared with the normal complex mixture of these molecules. The homogeneity of the myeloma protein is attributable to their synthesis by a highly selected population of cells, rather than by the sum total of all immuno-globulin-synthesizing cells. The neoplastic cells producing myeloma proteins are regarded as being derived from a single clone. Because of the homogeneity of myeloma proteins, and the fact that they can be obtained in large amounts, they are very important tools for immunochemists and immunologists. In 1964, Grey and Kunkel made rabbit antisera to various myeloma proteins, and absorbed them with other myeloma proteins. They were able by subsequent immunodiffusion tests to distinguish four subclasses of IgG, which they called We, Ne, Vi and Ge. Other investigators obtained similar results independently, but used different names for the subclasses. We now call the subclasses IgG1, IgG2, IgG3 and IgG4, according to the W.H.O. recommendations. The equivalent older names are IgG1 = We, γ2b, C; IgG2 = Ne, γ2a; IgG3 = Vi, γ2c, Z; and IgG4 = Ge, γ2d. All four subclasses are present in normal sera, though not in equal concentrations. The antigenic determinants which characterize them are, therefore, isotypic. There is considerable structural similarity in the peptides derived from the four subclasses (Grey and Kunkel, 1967).

Through studies of IgG myeloma proteins for both subclass and Gm specificity, the Gm antigens have been assigned to the various subclasses of IgG on which they reside. These assignments are now reflected in the formal nomenclature (W.H.O., 1976). Those antigens residing on IgG1 are denoted "Glm(1)", "Glm(2)", etc. Those on IgG3 are called "G3m(11)", "G3m(5)", etc. The letter designations may be used as well, as indicated in Table 44.1. Gm(23) or Gm(n) is formally designated G2m(23) or G2m(n), and is the only known Gm specificity residing on IgG2. This nomenclature is extended to cover genetic markers on other immunoglobulin heavy chains as well (see below).

Each subclass of IgG is thought to be under the control of a separate genetic locus. Thus, if genes determining two or more Gm factors are present on a single chromosome, they may be expressed together, separately, or not at all, on a given IgG molecule. It will depend on the subclass of that molecule. It may also be noted that myeloma proteins do not possess the same phenotype as serum from the same person. In any given plasma cell, only one of the paired genes for immunoglobulin synthesis on homologous chromosomes is active. Immunoglobulins provide the only known example of autosomal allele exclusion in humans, i.e., only one allele is active in a given cell. This phenomenon apparently also occurs with the entire X chromosome in females (see sections 1.2.4.4 and 33.1.4). In a Gm(1,3,5) Caucasian, for example, IgG myeloma proteins contain only one, or none, of these determinants. The reason is that Gm(1) and Gm(3) are at different locations in IgG1, and the genes are on opposite homologous chromosomes, while that for Gm(5) is in IgG3.

44.2.3 Isoallotypic markers of immunoglobulins—The nonmarkers

Because myeloma proteins have been extensively used in the study of immunoglobulin markers, it has been possible to define a new class of genetic marker. Myeloma proteins represent only one subclass of IgG, and as noted above, correlation studies on these proteins of subclass and Gm antigens have permitted assignment of Gm factors to particular subclasses. Antigens have been discovered which are shared by different subclasses, but which exhibit genetic variation in only one of them. The immunoglobulin system thus shows two classes of markers. In one, a genetic event is related to a subclass-specific region, and two regular allelic genes control a pair of markers, like Glm(3) and Glm(17). In the other, a genetic event is shared by other subclasses, reflecting the structural homologies preserved in subclass genes. Here, one may have a regular Gm marker behaving as an allele in one subclass, like Glm(1), but where the antithetical marker is shared by other subclasses. These latter are referred to as "nonmarkers" by Natvig and Kunkel (1973), and are called "isoallotypic markers" by the W.H.O. Nomenclature Committee. The human nonmarkers were detected by immunization of animals with myeloma proteins reflecting a particular subclass. The first was "non-a" (Frangione *et al.*, 1966; Natvig *et al.*, 1969). Both the peptide and the antigenic marker were present in all Glm(−1) IgG1 proteins, and in all IgG2 and IgG3 regardless of type, but absent in IgG4. It is recommended by the W.H.O. Nomenclature Committee that "non-a" be designated "nGlm(1)" or "nGlm(a)", as indicated in Table 44.1. There are several other nonmarkers as well. "Non-g" was found in G3m(g) [G3m(21)]-negative IgG3 proteins,

and in all IgG2 (Natvig *et al.*, 1969). Antigens "non-b0" and "non-b1" [nG3m(b0) and nG3m(b1)] are present on IgG3 molecules which lack G3m(b0) and G3m(b1), respectively, and also on all IgG1 and IgG2 (Gaardner and Natvig, 1972). The IgG4 class shows no regular genetic markers. One antigen, however, called "4a" is present on some IgG4, whereas "4b" is present on all 4a-negative IgG4 (Kunkel *et al.*, 1970). No IgG4 with both was found, but 4a is present on all IgG1 and IgG3, and 4b is present on all IgG2. Since normal sera are positive for these "nonmarkers", they do not serve as genetic markers in the ordinary sense (hence, the name "nonmarkers"), but they have been important as markers in isolated subclasses of IgG. For example, Michaelson and Natvig (1971) devised a procedure for separating IgG1 and IgG3 Fc fragments from normal serum, and could use non-a and non-g as markers. Abel (1972) has demonstrated the amino acid differences in IgG4 accounting for 4a and 4b. The IgG 4a protein has a Leu residue at position 309 which the IgG 4b protein lacks.

44.2.4 Gm genetics

No other marker system, with the exception, perhaps, of HLA, shows such a high degree of variability in different racial and ethnic groups. The formal genetics of the Gm system is complicated because of the structures of the genes. Each subclass of IgG has its own cistron in the genome, and these are very closely linked to one another. Some of the marker and nonmarker genes are truly allelic, probably reflecting base changes at the same position of the structural gene. Natvig and Kunkel (1973) call these pairs "homoalleles". Examples are Glm(f)–Glm(z) [Glm(3)–Glm(17)], Glm(a)–Glm(non-a) [Glm(1)–nGlm(1)] on IgGl, Glm(g)–Glm(non-g) [Glm(21)–nGlm(21)] on IgG3, and G3m(b0)–nG3m(b0) and G3m(b1)–nG3m(b1) on IgG3. Markers in different positions within allelic genes of the same cistron are called "heteroalleles" by contrast. Because of the close linkage, they may be used as "allelic" markers in populations, though. The formal genetics of Gm is discussed by Grubb (1970), van Loghem (1971), Natvig and Kunkel (1973) and Stedman and Wainwright (1979).

As noted above, the W.H.O. Committee recommended nomenclature for the presence or absence of individual Gm factors. Glm(1) indicates that the antigen is present; and Glm(−1) indicates that it is absent. If a series of allotypic markers is expressed, the designation is written in subclass order: e.g. Glm(1,2,3)G2m(23)G3m(5,13,14). A haplotype is also expressed in subclass order, e.g. $Gm^{1,2,3;23;5,13,14}$. If G2m(23) were absent, the haplotype would be $Gm^{1,2,3;-23;5,13,14}$, or $Gm^{1,2,3;-;5,13,14}$. Phenotypes are written in subclass and haplotype order, e.g. Gm(1,2,17,3;23), where the Glm subclass haplotypes are $Gm^{1,2,17}$ and Gm^{3}, and G3m antigens were not tested. Partial phenotypes may be written which indicate only the presence or absence of antigens tested, e.g. Gm(1,2,3;-14). Stedman and Wainwright (1979) cover this material, and the other aspects of Gm in a brief but clear and understandable way. Genotypes may be written as the haplotypes, separated by a slash, e.g. $Gm^{1,17;-;21}/Gm^{3;23;5}$.

As mentioned above, various haplotypes tend to be characteristic of certain racial or ethnic groups, and some factors are very seldom present in particular population groups. One of the difficulties in population work is the rarity of some of the testing reagents, with the result that a population may be tested only for certain allotypes for which antisera are available to the investigators. Studies using antisera for many specificities are rare. Stedman and Wainwright (1979) summarized the factors and haplotypes that are relatively common in various groups: In Caucasians, $Gm^{1,17;21}$; $Gm^{1,2,17;21}$; $Gm^{3;5,10,11,13,14}$; and $Gm^{3;23;5,10,11,13,14}$; In Negroes, $Gm^{1,17;5,6,11,24}$; $Gm^{1,17;5,6,10,11,14}$; $Gm^{1,17;5,10,11,14}$; $Gm^{1,17;5,10,11,13,14}$; and $Gm^{1,17;10,11,13,15}$; In Mongoloids, $Gm^{1,17;21}$; $Gm^{1,2,17;21}$; $Gm^{1,3;23;5,10,11,13,14}$; and $Gm^{1,17;10,11,13,15,16}$. Further information may be found in Johnson *et al.* (1977).

A number of years ago, when Gm population studies were first being carried out, and the extensive biochemical data from studies of myeloma proteins was not available, there were several ways of looking at the genetics. Giblett (1969) discussed this point. Steinberg, for example, entertained at least for some time a conception of Gm genetics that was analogous to Wiener's notion of Rh genetics (see section 22.6.1) (e.g. Steinberg, 1965). Such a model postulates multiple alleles at a single locus, particular alleles being able to code for proteins that contain one or several antigenic determinants. The alternative way of looking at it imagines the series of closely linked genes in the various subclass cistrons, as outlined in the first paragraph of this section.

Extensive studies of myeloma proteins of the various subclasses, and fragments obtained from them, have resulted in the assignment of many of the markers to specific regions of the IgG chains—and thus, the genes determining them to specific regions of the cistrons (see, e.g. Turner, 1976). In some cases, amino acid sequence studies have been carried out on the chains, revealing the chemical basis for the antigenic differences. Assignment of the antigens to specific regions of the chains is discussed by Natvig and Kunkel (1973), and well summarized by Stedman and Wainwright (1979). The information is summarized in Figure 44.1. Because of the close linkage of the IgG subclass genes, different genetic events can take place because of unequal homologous crossovers. Examples are deletions, hybridizations and duplications. Families have been studied in which apparent deletions are segregating, and Natvig and Kunkel (1973) thought that this was a better explanation than silent Gm alleles. Lefranc *et al.* (1976) reported on a deletion chromosome affecting IgG3. Kunkel *et al.* (1969) have studied an example of a hybrid IgG chain, which is analogous to the "Lepore" hemoglobins (section 38.2.3.5). There is also evidence for duplication of the IgG1 genes on one chromosome (Natvig *et al.*, 1971a; Natvig and Kunkel, 1973). The arrangement of structural genes on the chromosome is thought to be in the order IgG4–IgG2–IgG3–IgG1.

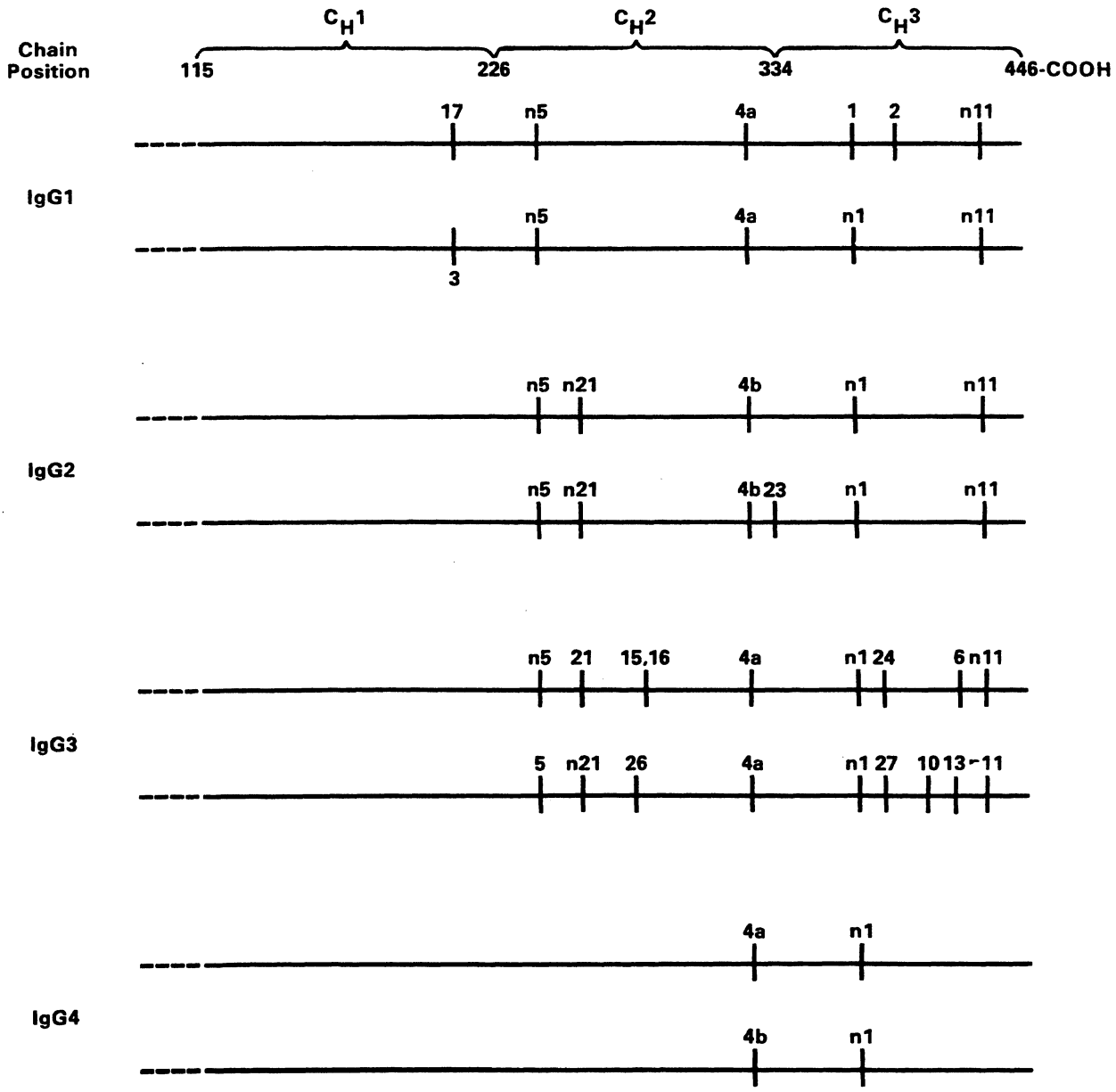


Figure 44.1 Distribution of the Genetic Markers on the IgG Subclasses
CH1 through CH3 refer to the constant homology regions.
The "nonmarkers" are denoted n1, n5, etc.

44.3 Light Chain Markers—The Km System

Ropartz *et al.* (1961a and 1961b) found an inheritable property of serum which was detected in the same way as Gm antigens, but was independent. They called it "InV", and later "Inv(a)". An additional Inv factor, called "Inv(1)" was described by Ropartz *et al.* (1962). The "(1)" in "Inv(1)" was apparently a lower case "L", but this cannot be distinguished from a number one ("1") character in printing or typing. Steinberg *et al.* (1962) described another factor called Inv(b), and it behaved as though it were determined by a gene allelic to the Inv(a) gene. Terry *et al.* (1965) showed that the Inv factors were located on the κ light chains of immunoglobulins, but not on the λ chains. In the interests of consistency, the W.H.O. Nomenclature Committee recommended that the system name be changed to "Km". Thus, as shown in Table 44.1, Inv(1) (lower case L) or Inv(1) (number one) became Km(1); Inv(a) or Inv(2) became Km(2); and Inv(b) or Inv(3) became Km(3).

The antigens of the system are inherited by way of the codominant alleles Km^1, $Km^{1,2}$ and Km^3 (Grubb, 1970). A number of populations have been tested with anti-Km(1), but the other antisera are apparently quite rare. The $Km^{1,2}$ allele is far more common than is Km^1. In Caucasians, almost all Km(1) are Km(1,2) if anti-Km(2) serum is used, and the figure is only slightly less for other populations. Similarly, over 90% of Caucasians and over 80% of Negroes who are Km(1) are Km(1,3) if tested with anti-Km(3). Km(–1,–2,–3) people are known, and the phenotype implies the existence of a Km^- gene, an inhibitor of Km expression, or of another allele to which an antiserum has not yet been found.

The amino acid substitutions in the κ chains associated with the Km allotypes have been worked out by Milstein *et al.* (1974). The residues which vary are 153 and 191. Km(1,2,–3) has 153Ala, 191Leu, Km(–1,–2,3) has 153Ala, 191Val, and Km(1,–2,–3) has 153Val, 191Leu. Additional information on Km may be found in Grubb (1970), Johnson *et al.* (1977) and Stedman and Wainwright (1979).

44.4 Other Immunoglobulin Markers

The immunoglobulin marker called "Isf(1)" (Ropartz *et al.*, 1966) received its designation because the antiserum defining it came from healthy donors from San Francisco. It is apparently independent of Gm and Km, and resides on Ig-Gl. It is clearly and simply inherited. Ropartz *et al.* (1968) found that its expression was age-dependent in Caucasians but not in Negroes.

The first genetic marker on a heavy chain from a non-IgG immunoglobulin was independently found in IgA by Vyas and Fudenberg (1969) and by Kunkel *et al.* (1969). Like IgG, IgA molecules may be subclassified (into isotypes) according to antigenic determinants on their heavy (α) chains. The genetic marker is associated only with IgA2 molecules, and was first called Am(1) by Vyas and Fudenberg (1969) and Am_2 by Kunkel *et al.* (1969). It is now called A2m(1) to be consistent with the Gm nomenclature. A2m(1) occurred in most Whites, roughly half the Blacks tested, and in 73% of Japanese and 56% of Chinese. The numbers of people tested were small. Soon afterward, a second marker was found by van Loghem *et al.* (1973), which they called "A_2m(2)", and it behaved as if its gene were allelic to that for A2m(1). There are, thus, three A2m types: (1,–2), (1,2) and (–1,2). The frequency of the allele for A2m(1) is high in Caucasians, and much lower in Black and Oriental populations. Van Loghem (1974) has discussed the properties and inheritance of the A2m system. The complete amino acid sequence of an $\alpha 2$ heavy chain from immunoglobulin molecules of the A2m(2) allotype has recently been determined (Toraño and Putnam, 1978).

All the IgG markers described above reside on the constant homology regions of the heavy chains. A marker was recently described on the variable regions of the heavy chains of IgG, IgM and IgA (Wang *et al.*, 1978). It was called "Hv(1)".

44.5 Determination of Immunoglobulin Types

There are essentially only two ways of determining immunoglobulin types. The first is serological, and is probably the most widely used. The second is immunoprecipitation using precipitating antibodies raised in animals. Precipitating anti-Gm antibodies can be prepared to some antigens in rabbits, and in primates such as the rhesus monkey or the baboon (Hess and Bütler, 1962; Alepa and Steinberg, 1964; Litwin and Kunkel, 1966c; Yount *et al.*, 1967; Natvig *et al.*, 1968; Van Loghem and Grobbelaar, 1971).

The serological method is based on antibody neutralization (inhibition test). The presence of an antigen in a sample is indicated by its ability to prevent agglutination of red cells coated with specific IgG molecules which have the antigen after incubation with the antiserum. The serological test, therefore, requires two reagents: the anti-Gm reagent (often called the "agglutinator"), and an IgG incomplete antibody, containing the Gm factor, which will sensitize the red cells without agglutinating them (often called the "antibody coat" or simply the "coat"). Anti-D is customarily used as a coat, in part because of its availability, and in part because of its sufficiently higher titer. There can be variability in the behavior of both reagents of their pair, even though they may appear to have the same specificity. The differences between Ragg and SNagg agglutinators have been noted. The latter are preferable if available. There can be variability in the antibody coat as well, and these reagents must be carefully selected and standardized. Giblett (1969) noted that Rh+ cells with as many D sites as possible should be employed (section 22.7.1). Some Gm specialists suggest that R_1R_1 cells are preferable to R_2R_2 cells. The sensitized cells should be strongly Coombs positive. Giblett (1969) discussed both tube and tile techniques. Borel *et al.* (1967) described a microprocedure on microtiter plates. These reagents are often very precious, and microtechnique is preferable wherever possible. A special technique was described

in section 44.2.1 above for artificially coating red cells with appropriate myeloma proteins to detect a specificity for which a proper anti-D coat could not be found. A clearly presented and very useful methods monograph has recently been published by Kipps (1979).

44.6 Medicolegal Applications

44.6.1 Disputed parentage

The Gm system has been employed in disputed parentage cases in specialized laboratories for quite some time. This application is discussed in detail by Planques *et al.* (1961), Harboe and Lundevall (1961), Prokop and Hunger (1961), Prokop and Bundschuh (1963), Ellis *et al.* (1973), van Loghem and Nijenhuis (1979) and Sebring *et al.* (1979), among others. The value of Gm typing in parentage cases depends on the number of specificities which can be tested, and this varies to some extent from one laboratory to another. In this country, the use of Gm in paternity testing seems to be more common in the AABB Reference Laboratories (Polesky and Krause, 1977). In the older papers, fewer specificities were employed for the testing than in the more recent ones.

There is general agreement that the system is very useful, and improves the overall exclusion percentages provided that the typing is carried out skillfully and that all the intricacies of Gm and Km genetics are appreciated. The article by Sebring *et al.* (1979) provides excellent coverage of the subject. They presented results on 925 cases in which Gm typing was carried out using anti-Glm(1), (2), (3), (17), G3m(5), (10), (6), (24), (21), (15), (16) and Km(1). G2m(23) typing was employed to sort out a case involving an uncommon haplotype. They said that the exclusion probability was about 24% for Caucasians and 17% for Negroes in this country, using anti-Glm(1), (2), (3) and anti-G3m(b1). The figure would increase to about 33% for Black Americans if Glm(17), G3m(21), (10), (6), (24) and (15) were added.

44.6.2 Gm and Km typing in bloodstains

Efforts to group Gm antigens in bloodstains began about 20 years ago, and it is well established that reliable Gm typing is possible with bloodstains. In practice, it appears that Gm typing is limited to specialized laboratories that have developed experience with the system. Km antigens can likewise be determined in bloodstains.

Among the most extensive earlier studies on Gm stain typing are those of the group in Toulouse. Background for the serum group systems was presented by Ruffie (1961). In 1961, Planques *et al.* presented their studies on the determination of Glm(1), Glm(2) and G3m(5) in bloodstains. Stains were deposited on a number of substrata, both absorbent and nonabsorbent, and both Ragg and SNagg reagents were tested. Dried blood on nonabsorbent substrata were easier to work with. Nonspecific absorption of the anti-Gm was not observed, but they said that the SNagg antisera gave more consistently complete inhibitions with stains containing the corresponding Gm antigen than did Raggs. There was no doubt that all the antigens tested could be grouped specifically and reliably in stains. In some cases, stains that had been stored for years could be typed, attesting to the stability of the Gm antigens. Further studies on the typing of Glm(1), Glm(2) and G3m(5) were carried out by Ducos *et al.* (1962 and 1963a). In Germany, similar studies were done by Fünfhausen and Sagan (1961), Fünfhausen *et al.* (1962) and Sagan and Fünfhausen (1965). They studied Glm(1) and Glm(2) determination in bloodstains on a variety of substrata. Even stains exposed to 100° conditions for an hour (7 days old stains) could still be typed. In 1962, Prokop *et al.* devised a rapid microprocedure for Gm typing, which was applicable to bloodstain typing. Ducos *et al.* (1963b) briefly reiterated the value of Gm typing. Kobiela (1963) confirmed that Glm(1) was reliably determinable. Nielsen and Henningsen (1962) also confirmed that Gm factors could be determined in bloodstains, as did Lenoir and Muller (1966) and Turowska (1969). The factors were not detectable in older stains kept at higher humidity conditions, but the stains kept dry seemed to retain activity well. In 1964, Brocteur and Moureau reported that they had been able to sort out a car accident case, involving a determination who had been driving one of the cars, based in part on Gm typing of a bloodstain in the car, and comparison of the result with the Gm types of the occupants. A lengthy and extensive study of Gm stain grouping was carried out by Görtz in 1969. The conditions of the inhition test were studied in detail, and he tested more than 950 bloodstains. Under favorable conditions, Glm(1) and Glm(2) could be typed using 0.1 mg of stain material, somewhat more being required for G3m(5). Stains which were still comparatively soluble were more readily groupable than those which had become insoluble upon aging. Like other investigators, he emphasized the careful selection and evaluation of reagents for the tests. The studies extended to Glm(3), G3m(6) and G3m(10) as well, indicating that all could be reliably determined. Görtz said that negative inhibition results had to be interpreted with great caution in older, insoluble stains, since these might fail to inhibit the agglutinator even though the corresponding factor was present. A full paper on Glm(3) appeared in 1969, emphasizing that information about the racial origin of stains could be obtained by combining Glm(3) and G3m(5) typing (Ducos *et al.*, 1969). The Gm(-3,5) type was characteristic primarily of Black people (cf. above in section 44.2.4). Stains as old as 15 years could be typed. In 1971, Blanc and Görtz reported on the typing of Gm(Bet) [G3m(13)] in bloodstains. In this paper, they showed that typing results in sera and the corresponding bloodstains were concordant for Glm(1), (2), (3), G3m(5), (6), (10), (13) and for Km(1) and (2). Blanc *et al.* (1971) discussed the value of Gm phenotyping in bloodstains in terms of determining racial origin. Certain haplotypes and phenotypes occur in very much higher frequency in certain populations than others, as noted in 44.2.4, and careful selection of the factors to be tested can be informative. They also noted that only the positive identification of an antigen should be used in the interpretation. Görtz *et al.* (1970) discussed the Gm typing technique and possible

sources of error. The agglutinator should be SNagg, and have a titer of 1:16 to 1:32. They seemed to think, too, that the coat reagents should be prepared in specialized laboratories. Most of the same types of problems encountered with any kind of stain grouping are encountered in Gm typing as well.

Km antigens can be determined in bloodstains. There are a number of reports on Km(1) (Merli and Rouchi, 1967; Blanc and Görtz, 1971; Blanc *et al.*, 1973; Khalap *et al.*, 1976), and on Km(2) (Blanc and Görtz, 1971; Blanc *et al.*, 1973).

Khalap *et al.* (1976) described a procedure for the typing of Glm(1), Glm(2) and Km(1) in stains. This procedure appears in the MPFSL (1978) manual as well. They noted that woolen substrata can give "cloth reactions," i.e., nonspecific inhibition of the agglutinator. Khalap and Divall (1979) reported on the typing of G3m(5) in bloodstains. The antigen was reliably determinable, and they noted that the addition of G3m(5) to their routine testing protocol had additional value in the interpretation of negative results with Glm(1) and Glm(2). A substantial number of the samples tested in London (from British Caucasians) were Gm(-1,-2;5). If only Glm(1) and (2) were included in a test on a stain from such a blood, the negative results would probably be uninterpretable. However, if G3m(5) is found, the likelihood is much stronger than the Gm(-1,-2) result actually represents the type and is not a reflection of deterioration of the antigens. They noted that G3m(10) could be used in a similar way. This matter is clearly discussed by Shaler (1982) as well, using casework illustrations. A negative result for a factor can be interpreted as such if a positive result is obtained in the same sample for another factor residing on the same IgG subclass. A very detailed discussion of Gm and Km typing procedures in forensic samples was given by Kipps (1979). Davie (1979) described a procedure for Gm and Km typing on microtiter plates.

The immunoglobulin markers may represent the only genetic marker system used in forensic serology which by their nature allow some control over the interpretation of negative results. In general, failure to detect an antigen in a bloodstain cannot normally be taken to mean that it was never present. In the case of some Gm antigens, however, the finding of one specificity can be used as a positive control for the presence of the immunoglobulins in the stain, as Khalap and Divall (1979) have noted. In this way, it may be possible to interpret negative reactions with more confidence. Such positive marker control antigens are even better if they reside on the same subclass of IgG molecules. One wonders if the so-called nonmarkers, or antisera defining particular subclasses (isotypic), could not be put to good use as indicators of the presence of the IgG molecules containing the Gm factors being sought.

Perhaps the most appealing property of the Gm and Km system antigens for bloodstain work is their extraordinary stability. No other genetic marker known, except for ABO, shows anything even approaching the stability of the Gm antigens in dried blood. Various Gm and Km factors have been typed in bloodstains many years old (Görtz, 1969; Ducos *et al.*, 1969; Blanc *et al.*, 1973). Budyakov (1967) detected Glm(1) in 5 year old stains regardless of exposure to often adverse environmental conditions. Similarly, Turowska (1969) found that Glm(1) and Glm(2) could be typed in 5 year old stains. Hoste *et al.* (1978) recently detected the Glm(1), G3m(10) and Km(1) types in 33 year old bloodstains.

The greatest difficulty with the Gm system is the availability of reagents. SNagg reagents and appropriate anti-D (or even other) coats for many of the specificities are not very common. Antisera can be developed in animals, but unless there is more commercial interest in such reagents, they will probably continue to be rather hard to obtain for some time. Since the reagents are ordinarily precious, it is useful to develop systematic approaches to Gm typing, taking into consideration the phenotypic distributions in the population of interest, so as to be able to obtain the maximal discrimination using the fewest reagents. Such a flowsheet for the English population is given by Stedman and Wainwright (1979).

44.6.3 Gm and Km typing in body fluids and tissues

Any tissue or fluid that contains immunoglobulins of the appropriate classes will contain the associated genetic markers, but not necessarily in concentrations adequate for typing, particularly in stains. Nielsen and Henningsen (1963) could not detect Gm factors in 23 seminal specimens. Klose and Schraven (1962) had detected Glm(1) in some specimens from secretor patients, but reported to Nielsen and Henningsen (1963) that they could not always detect it in semen from healthy donors. Krämer (1963) found Glm(1) in 9 of 30 seminal samples. Davie and Kipps (1976) showed that seminal Km(1) types match the type in serum from the same donor (31 samples). Special techniques were needed for grouping liquid semen (or saliva) samples because of their ability to cause nonspecific aggregation of the test cells. A freeze-thaw step, followed by centrifugation, prevented this effect in most cases. Many typing reagents perfectly suitable for serum typing were not very satisfactory for semen and saliva, because of the low immunoglobulin concentrations in these fluids. Jorch and Oepen (1977) reported that a number of Gm factors and Km(1) could be detected in semen if the quantity were sufficient, but that contamination by other body fluids which contained immunoglobulins was a danger in medicolegal samples. It was considered more likely that markers on IgG1 would be detectable than those on IgG3 in semen. Both nasal and vaginal mucus were said to be possible sources of problems if they were found in mixtures with semen. Blake and Sensabaugh (1978) noted that there is about 50 times more IgG in blood than in seminal plasma, and that one would expect to require at least the equivalent of 0.1 mℓ of semen to detect an IgG marker. Davie and Kipps (1976) reported some limited success with seminal stains, but said that more sensitive techniques would be needed.

In saliva, immunoglobulin levels are about 1000 times less

than in blood (Waissbluth and Langman, 1971). Krämer (1963) did not detect Glm(1) in normal saliva. Davie and Kipps (1976) found that Km(1) types correlated in paired saliva and semen samples, but the saliva had to be treated by freezing, thawing and centrifugation to remove its red cell aggregating property. Only selected examples of anti-Km(1) were found to be suitable for this typing. Some success was reported with saliva stains, but increased sensitivity was needed in the test because of the relatively low immunoglobulin levels. Km(1) was selected because it is not restricted to IgG, and might be expected to be present in somewhat higher concentratons than Gm factors. Jorch and Oepen (1977) found that Km(1) could be detected in saliva and saliva stains, but they had no success with Gm factors.

Gm factors appear to be detectable in urine only if it contains a pathologically high protein concentration (Nielsen *et al.*, 1963). Sweat does not have levels of immunoglobulins sufficient to permit Gm or Km antigen detection, but nasal and vaginal mucus do have (Jorch and Oepen, 1977). Caution must therefore be exercised if mixtures are suspected, and typing is to be attempted. Human inner ear fluid can be typed for Glm(1) and Km(1) (Turowska and Trela, 1977). Tausch *et al.* (1977) studied the possibility of detecting Glm(1), (2), (3), G3m(10) and Km(1) in decaying blood and organ materials. Serum remained typable the longest as a rule. Organ exudates were typable for varying lengths of time, from 1 to 8 weeks. Henke and Bauer (1980) recently reported that a number of Gm factors and Km(1) can be detected in the pulp material of human teeth.

44.7 Distribution of Gm and Km Phenotypes in Populations

There are a number of studies on various U.S. populations, but they differ in the number of antigens determined. An older study by Blumberg *et al.* (1964a) tested Glm(1), (2), G3m(5), (6), Km(2) and Km(3) in both White and Black populations of southeastern Georgia. The best overall reference is probably Johnson *et al.* (1977).

SECTION 45. OTHER SERUM PROTEIN GENETIC MARKERS

45.1 Serum Lipoproteins—The Ag, Lp and Ld Systems

45.1.1 Introduction

The lipoproteins are a heterogeneous and complex group of serum proteins. They are macromolecular complexes having reproducible lipid-protein ratios and stability in aqueous solution. There are several ways of classifying lipoproteins, each based on a different separation criterion. The nomenclature is imprecise and sometimes ambiguous, and there is no universal agreement on it as yet. In part, this situation is due to the complexity of the systems, and in part it is due to the rapid expansion of knowledge in the field. An *apolipoprotein* is a homogeneous protein, containing no detectable noncovalently bound lipid. A *lipoprotein* is an apolipoprotein-lipid complex held together by noncovalent forces. The lipid molecules are arranged such that there are no clearly defined surface areas where polar groups shield intramolecular hydrophobic regions from solvent. A *lipoprotein particle* is an apolipoprotein-lipid complex, again held together by noncovalent bonds, except that here there are extensive regions where polar groups of lipids form a surface to shield intramolecular hydrophobic regions from solvent. These useful definitions were given by Osborne and Brewer (1977) in their extensive review of plasma lipoproteins.

Lipoproteins have been classified on the basis of (1) electrophoretic mobility; (2) hydrated density; and (3) apolipoprotein composition. According to electrophoretic migration criteria, lipoproteins may be defined as those at the origin (nonmigrating) and those migrating with pre-beta (α_2), β and α (or α_1) mobilities on paper or agarose. The migration may be a little different on starch blocks (Scanu *et al.*, 1975). Hydrated density is the most widely employed classification criterion. The relatively low density of these proteins, compared with other serum proteins, is an advantage in separating them. They were originally classified as: chylomicrons ($\rho < 1.006$), very low density lipoproteins (VLDL; $\rho < 1.006$), low density lipoproteins (LDL; $\rho = 1.006$–1.063), and high density lipoproteins (HDL; $\rho = 1.063$–1.21). Refinements of technique have resulted in the division of LDL into two classes and of HDL into three. So-called very high density lipoproteins (VHDL; $\rho = 1.21$–1.25) have also been recognized. Apolipoprotein composition studies of the varous density fractions has led to a classification of lipoproteins into families, based on the apolipoprotein composition. The families are called Lp A through Lp E, and each contains characteristic proteins or peptides, some of which have been better characterized than others. The specific peptide or protein constituents of Lp A are called A-I and A-II; those of Lp C are C-I, C-II, etc. This material has been thoroughly reviewed by Scanu *et al.* (1975) and by Osborne and Brewer (1977).

The polymorphic serum lipoprotein antigens that are discussed in the remainder of section 45.1 are associated with the low density lipoproteins (LDL).

45.1.2 The Ag system

The first antigen of the Ag system was observed by Allison and Blumberg in 1961. There had been previous observations in animals, and even in human beings, of the formation of precipitating antibodies by one individual against the serum proteins of another in response to transfusion. No thorough or systematic studies had ever been done on these "isoprecipitins", however. The formation of such antibodies indicates serological variations in the serum proteins within the same species. Allison and Blumberg (1961) described a precipitating antibody in the serum of a patient at N.I.H. (Mr. C.deB.) who had received many transfusions. The antibody reacted with some but not all examples of human sera from other people. The antigen in human serum that was reacting with C.deB. serum was called "Ag" (which just stood for "antigen"). The Ag factor was inherited in a simple Mendelian way, and Blumberg *et al.* (1962a) said that the gene determining the antigen should be called Ag^A, and its allele Ag. Ag^AAg^A and Ag^AAg people would thus be Ag(a+), while $AgAg$ ones would be Ag(a−). Ag(a+) was shown to be associated with the low density (β) lipoproteins of serum (Blumberg *et al.*, 1962b). In 1963, Blumberg and Riddell described another precipitating antibody in a patient's serum (the 'New York' serum) which defined an apparently different Ag factor, and this was called Ag(b). It was thought to be conditioned by another gene, Ag^B, and Blumberg (1963) suggested that this gene was allelic to Ag^A, at least in some populations. Blumberg and his collaborators continued to screen the sera of multiply transfused patients for precipitating antibodies, and, by 1964, they said that the antibodies all taken together defined from 5 to 7 separate antigenic specificities (Blumberg *et al.*, 1964b). Information about the various antisera, and about the frequencies of their corresponding antigens, and their relationships and inheritance, was not very detailed, however. In addition, it was apparent by then that some of the antisera were polyspecific. The very early studies with Allison were discussed by Blumberg (1977) in his Nobel address. The study of antibodies in polytransfused patient sera led Blumberg and his collaborators off in a different direction once the "Australia" antigen had been discovered (section 50.2.3), and these studies culminated in the discovery of the infectious agent responsible for hepatitis B. Blumberg shared the Nobel Prize for Physiology or Medicine in 1976 for the hepatitis work.

Hirschfeld and Blombäck (1964) described another anti-Ag serum (the "L.L." serum) which reacted with about 40% of Swedish sera, and had a peculiar relationship to C.deB. serum. All C.deB.-negative samples were also L.L.-negative, but C.deB.-positive sera could be either L.L.-positive or L.L.-negative. The relationship was similar to that existing between Gm(a) and Gm(x) (see in section 44), and the specificity in the L.L. serum was thus named Ag(x). C.deB. serum was found to contain not one, but three different Ag antibody specificities. One of these, anti-Ag(x), it shared with L.L. serum. The other two were named anti-Ag(a_1) and anti-Ag(z) (Hirschfeld *et al.*, 1964). These findings tended to simplify somewhat the conception of the Ag system (Hirschfeld, 1968b). Some of the antisera shared antibody specificities, and were polyspecific, and some of the earlier complexities could be understood in these terms, rather than in terms of relationships between the different antigens. The original so-called "Ag(a)". and its gene "Ag^A", disappeared in the revised interpretation.

A number of different isoprecipitin sera have been described, and sorting out the different specificities and their immunological and genetic relationships has not always been an easy task. Until around 1964, there were really only three fairly well characterized antisera, the C.deB., the New York, and the L.L. ones. C.deB. was an anti-Ag(a_1,x,z). The L.L. serum was considered to be anti-Ag(x), and possibly the only monospecific reagent of the three, since the New York serum was suspected of being more than just anti-Ag(b) (Blumberg *et al.*, 1964b). In 1966, Geserick *et al.* found an "anti-Ag(b)" antibody in a patient in Berlin who had been transfused over 200 times. It reacted with about 93% of German sera. Hirschfeld *et al.* (1966) described another serum ("B.N.") which appeared to define a new specificity called Ag(t). It seemed that B.N. serum was a monospecific anti-Ag(t). Then, in 1967, Hirschfeld and Bundschuh compared the Berlin example of "anti-Ab(b)" (the "New York" serum was no longer available) and the "B.N." [anti-Ag(t)] serum, and said that they were identical. Another serum, called "C.P.", was described by Hirschfeld *et al.* (1967). It reacted with an antigen in about 90% of Italian and Swedish sera, and was thought to be defining a new antigen, Ag(y), whose determining gene, Ag^y, appeared to be allelic to Ag^x. Two other sera sent in by other workers for evaluation appeared to be anti-Ag(y) as well. Data in a number of populations were consistent with the assumption that Ag^x and Ag^y were allelic (Hirschfeld and Okochi, 1967), and examples of anti-Ag(x) and anti-Ag(y) were found in the Japanese population (Okochi, 1967). Bütler *et al.* (1967a) examined eight serums with anti-Ag activity. Six had anti-Ag(x), and in two, an additional antibody defining a new specificity called Ag(r) was found. One serum appeared to have a monospecific anti-Ag(r), and another, a monospecific anti-Ag(y) as well. In 1966, Bütler and Brunner described a passive hemagglutination technique for typing Ag specificities. It was sensitive, and offered a number of advantages compared with the double diffusion technique which had been employed exclusively up to that time. Red cells were "coated" with LDL of known Ag specificity by a diazotization procedure. Corresponding anti-Ag sera would then give agglutination of the cells. This kind of "coating" technique was introduced by Coombs *et al.* (1952) and discussed in section 16.3. It was further discussed in section 44.2.1 in connection with the determination of G2m(23) [G2m(n)] for which a suitable anti-D "coat" was never available. Bütler *et al.* (1967b) described an anti-Ag activity which was not precipitating, and could be detected only by the passive hemagglutination technique. This antibody defined an apparently new Ag specificity called Ag(c). Further studies on Ag(c) and another new specificity, Ag(e), were reported by Bütler and Brunner (1969). In 1968, Contu found a serum ("R.M.") which appeared to define another Ag antigen, which he called Ag(m). He found that all "L.L. serum"-positive sera were Ag(m+) while all "L.L. serum"-negative ones were Ag(m−). Although "L.L." serum was regarded as an anti-Ag(x), Contu thought that it was probably anti-Ag(x,m). Bütler *et al.* (1970a) described polyspecific antisera which contained, in addition to anti-Ag(a_1) and anti-Ag(x), a new anti-Ag(g). Bütler *et al.* (1970b) described two sera containing antibodies to yet another specificity, Ag(d). Boman (1971) described an antiserum whose activity was detected by passive hemagglutination technique. This was not an anti-Ag(x), but its relationship to the other factors is not clear.

The complexity of the Ag system, and the difficulty in understanding the relationships between the antigens, can be accounted for in several ways. Antibodies are apparently found only in the sera of people who have received many transfusions. Many of the antibody-containing sera have several antibodies in them. In addition, anti-Ag sera are comparatively rare. Geserick and Dufková (1967) found an anti-Ag(b) [anti-Ag(t)] in the serum of a patient who had received 205 transfusions. After 12 additional transfusions, an anti-Ag(x) developed in the individuals's serum. Vierucci *et al.* (1966) reported screening 80 sera from multiply transfused patients, and finding anti-Ag activity in 6 of them. Hirschfeld (1968b) said that about 60 reagents had been described by the time that review was written. There are technical problems and variations associated with Ag typing as well. It is difficult to "standardize" the test procedure because of variations in the strengths of antisera, and perhaps because of differences in the reactivity of the same lipoprotein antigen in different sera. Hirschfeld (1968b) conducted extensive studies on 28 different isoprecipitin sera with a panel of 462 sera from unrelated individuals. Particular antisera do not always give the same results with the same panel serum in repeated tests over the course of time, although the great majority of repeated determinations were concordant. The "L.L." serum, for example, tested with 108 panel sera in 536 determinations gave deviant results in 15 instances. Some series of immunodiffusion plates give better results than others, and nonspecific precipitin zones, formed around the antigen wells, can sometimes cause problems of interpretation. Incubation time is another variable that is difficult to optimize because it is dependent on which

reagents are used, and the amounts of each, the distance between the wells, and the temperature. Polyspecific reagents can contain antibodies with different optimal incubation times. There can be differences in the reactivity of reagents at different times during storage as well. Sera kept at −20° sometimes showed negative results after having reacted with the same antiserum when they were fresh. In a few other cases, sera previously determined to be negative with a particular antiserum reacted with it after being stored frozen for 2 years. These technical factors obviously have an effect on the results obtained with particular reagents, and the subsequent conclusions that are drawn about the Ag system.

Much of the evidence (Morganti *et al.*, 1970; Bütler *et al.*, 1971; Hirschfeld, 1971) tends to support a genetic model for the Ag system that postulates four closely linked loci controlling a total of eight antigens. $Ag^{x,y}$ can designate the locus at which Ag^x or Ag^y alleles may be found. The other three loci are $Ag^{a_1,d}$, $Ag^{c,g}$ and $Ag^{t,z}$. This model permits 16 different chromosomal arrangements, which can theoretically give rise to 136 genotypes and 81 phenotypes. Not all of them have been found. In addition, Ag^z is never present in the absence of Ag^{a_1} (Hirschfeld, 1971). The four closely linked loci model is not the only possible one. A single locus for the Ag system with multiple alleles is conceivable, if each allele produces a product with four different antigenic determinants, or factors. These two models are closely analogous to the Fisher-Race and Wiener conceptions, respectively, of Rh (section 22.6.1). Hirschfeld (1971) has noted that, in either case, antibodies are regarded as being monospecific (simple) while each Ag chromosome produces four different antigens which are inherited as genetically nonsegregating units ("complex antigens"). He therefore calls these models "simple-complex". One can imagine other kinds of models in which the chromosome produces only one antigen with a single determinant, and where antibodies are complex, i.e., they can react with the product of more than one Ag chromosome. If both antigens and antibodies are allowed to be complex, even more involved models can be constructed (Hirschfeld, 1971). While it is convenient to think of Ag system genetics according to a model having four closely linked loci, there are undeniable problems with it. Apart from those mentioned above, it is not clear where Ag(m) fits into the picture. Bütler *et al.* (1971) said that Ag^m might be an allele of Ag^x and Ag^y. Bütler *et al.* (1974) have reported an additional specificity called Ag(h), thought to be conditioned by an allele Ag^h. This allele was said to occupy a fifth closely linked locus, and its hypothetical partner was designated $Ag^{[i]}$. Giblett (1977) brought up the interesting idea that the five Ag loci, assuming the model is correct, might have a correlation with the five classes of LDL, in a manner somewhat analogous to the relationship between the Gm antigens and the IgG subclasses (section 44).

The Ag system was reviewed by Bütler (1967a), Bütler *et al.* (1971) and Hirschfeld (1968b and 1971). Allison (1963) noted that the Ag system was not a particularly promising prospect for forensic blood identification because the antisera were so rare. For this reason, and perhaps others, the system has been used very little in medicolegal work. The Ag(x)-Ag(y) pair, which is believed to be conditioned by a pair of alleles at a single locus, has been employed as a marker in paternity cases in the United Kingdom (Bradbrook *et al.*, 1971). According to Lee (1975), the PE for a falsely accused White father would be about 14% using Ag(x,y) testing.

45.1.3 The Lp system

In 1963, Berg reported that he had detected a new, genetically controlled serum lipoprotein antigen in humans. Serum produced in rabbits by immunization with human LDL, and then absorbed with various human sera to remove the "anti-LDL" activity, retained a precipitating activity against about 35% of sera from unrelated donors. The antigen being detected was called "Lp(a)", where "Lp" stood for "lipoprotein". Reactors were designated Lp(a+), and nonreactors were Lp(a−). The gene postulated as being responsible was called Lp^a, and it behaved as a mendelian dominant. Further genetic studies (Berg and Mohr, 1963) indicated that the genetic model was probably correct. The hypothetical allele of Lp^a was designated Lp^a. In one family, however, an Lp(a−) × Lp(a−) mating had produced an Lp(a+) child. While there was no evidence of illegitimacy, it could not be ruled out either. Lp(a) was different from any of the antigens of the Ag system (Berg, 1964a). Problems could be encountered in typing Lp(a), and Berg (1964b) discussed an albumin-β-lipoprotein precipitation which he said could be a source of misinterpretation in reading immundiffusion gels. Lp(a) was not very stable to storage, or to freezing and thawing, and Berg (1964c) noted that it should be typed in fresh serum. Protocols for preparing rabbit anti-Lp(a) were given by Berg (1965a), and he also found (1965b) that the antibody the rabbits made was a 7S γ-globulin. Additional family and population studies (Berg, 1966) were consistent with the proposed genetic model. Wassenich and Walter (1968) reported on another family, however, in which an Lp(a−) × Lp(a−) mating had produced three Lp(a+) children (out of a total of ten). The *Lp* locus is apparently not closely linked to *Ag* (Berg, 1967).

In 1964, Bundschuh found that "anti-Lp(a)" produced in horses behaved differently from the rabbit reagent. Two precipitin lines were produced against Lp(a+) sera, one showing immunologic identity with Lp(a) and one showing nonidentity. Prokop and Uhlenbruck (1969) discussed these studies in more detail. The horse antiserum was thought to be detecting a different but related antigen, called Lp(x). All Lp(x+) sera were Lp(a+), but not conversely. The relationship between Lp(a) and Lp(x) is not very clear. Berg (1968) tested the horse serum as well, and confirmed the results of the German workers.

Simons *et al.* (1970) partially purified the Lp(a) lipoprotein. They said it differed from LDL and HDL in amino acid composition, but was similar to the former in lipid composition. They further said that the protein had a characteristic mobility upon disc electrophoresis, and that this

technique might be used for Lp typing. Utermann and Wiegandt (1970) found that they could not characterize Lp(a+) sera by gel electrophoresis. Rittner (1971) reported electrophoretic variation in the lipoproteins using disc electrophoresis. This appeared to be genetically controlled, but its relationship to Lp is not very clear. Seegers *et al.* (1965) also described an inherited electrophoretic variation in the β-lipoprotein pattern, and this too may be related in some way to Lp.

In 1970, Harvie and Schultz found that Lp(a−) sera contained measurable quantitites of Lp(a) when concentrated. They regard the Lp polymorphism, therefore, as being a quantitative genetic trait. Utermann and Wiegandt (1970) interpreted their electrophoretic results as being consistent with Lp(a) as a quantitative trait. Enholm *et al.* (1971) found that serum Lp(a) levels varied over a very wide range (about 30-fold), and this finding would be consistent with quantitative variation. Albers *et al.* (1974) measured Lp(a) concentrations by radial immunodiffusion, and did not detect bimodality in the concentration distribution. They concluded that the variation was consistent with the polygenic inheritance. Sing *et al.* (1974) and Schultz *et al.* (1974) carried out quantitative studies on Lp(a) in serum using a sensitive radioimmune inhibition technique. They found that all individuals had Lp(a), but that the concentrations were distributed bimodally. They imagine that Lp^a, the dominant allele, determines one distribution of Lp(a) concentrations, while the allele determines another distribution with a lower mean value. Fisher *et al.* (1975) have found that human LDL seem to exist in several different MW classes, and that the classes possessed by a given individual are under genetic control, apparently at a single locus. The relationship of this finding to the Lp system is not yet clear, but it may be related to the concept of Lp(a) as a quantitative genetic variant. The studies of Sing *et al.* (1974) and of Schultz *et al.* (1974) indicated that there is a slight overlap in the Lp(a−) concentration range and that of Lp(a+) people. In this view, the Lp(a+) and Lp(a−) phenotypes are not unambiguously related to genotype. This model would help to explain some peculiarities of the system, the most troublesome of which is the occasional observation of Lp(a+) children from Lp(a−) × Lp(a−) matings.

The Lp(a) protein has been isolated in a number of different ways, and characterized to some extent (Enholm *et al.*, 1971; Utermann and Wiegandt, 1969; Dubarry and Moullec, 1976; Desreumaux *et al.*, 1977a and 1977b). The Lp(a) lipoprotein is closely related to LDL in some respects, but is apparently not identical with them. Upon storage or detergent treatment, the Lp(a) preparation may dissociate into LDL, albumin and Lp(a) apoprotein.

The Lp system has been reviewed by Bütler (1967), Berg (1968 and 1971a) and Cooper (1978). There have been almost no reports on applications of Lp to forensic or medicolegal problems. In view of the complexities of Lp(a), many of which are not yet completely understood, it is probably not a very suitable system for forensic work. Haferland (1965) did studies on the detection of Lp(a+) in 24 hour old dried blood on filter paper. He optimized the pH and buffer concentration of the buffer used to make the immunodiffusion gels, and then tested a number of samples. The antigen was detected in 20 of 21 Lp(a+) samples, and the instability of Lp(a) was noted.

45.1.4 The Ld system

Berg (1965c) found an isoprecipitin in the serum of a multiply transfused boy which reacted with about 42% of the sera of unrelated people. The antigen was associated with LDL, and was called "Ld(a)". It was not related to the Lp system, and reacted best at lower temperatures. Its controlling allele was called Ld^a, which behaved as a Mendelian dominant in family investigations. Berg and Reinskou (1967) found that the anti-Ld(a) was an IgG antibody. Berg (1971a) said that the original serum had later been found to contain both anti-Ag(x) and anti-Ld(a), but that monospecific antisera for Ld(a) had been found. Another antiserum had also been found which appeared to react with the product of the allele of Ld^a.

45.2 Complement Components

45.2.1. Introduction

The complement system was discussed briefly in section 1.3.5. Genetic variation has been found in a number of the components of the complement system, and both quantitative and structural variants have been reported. Complement component variants are typed by electrophoresis or by isoelectrofocusing, and may be detected by protein staining, immunofixation, or by functional detection overlays, depending upon the component. A recent review of this material may be found in Hauptmann (1979), and other reviews are Cooper (1978), Hobart and Lachmann (1976) and Gitlin and Gitlin (1975). The complement component deficiency states, which are under genetic control, were reviewed by Agnello (1978).

45.2.2 C2 Component

C2 is a single chain protein of approximate MW 102,000. Genetic variation in this component was detected by Hobart and Lachmann (1976), and independently by Alper (1976). There are two or three prominent bands and several additional minor bands in the C2 patterns of most individuals. C2 patterns are usually determined by polyacrylamide gel isoelectrofocusing (PAGIF), and detected by functional techniques, using an agarose overlay containing sensitized red cells and a C2-deficient serum. Alper (1976) called the common pattern "C2–C", and it accounted for about 96% of the samples. The rarer variants are of two kinds, acidic and basic, and were designated "C2–A" and "C2–B". These were accounted for by three alleles, $C2^C$, $C2^A$ and $C2^B$. Meo *et al.* (1977) studied this polymorphism further. They used the designation "C 2–1" for the common type, and "C 2–2" designated the rare basic type. The rare acidic type would be "C 2–3". The responsible alleles are $C2^1$ (= $C2^C$), $C2^2$ (= $C2^B$) and $C2^3$ (= $C2^A$). Olaisen *et al.*

(1978) confirmed these findings, and they used the designations of Meo *et al.* (1977). $C2^2$ was found to be rare in the Norwegian population as well. C2 deficiency is the most common deficiency of a classical complement component. The deficiency is inherited and quite rare. In view of the finding of structural variation at the *C2* locus, the deficiency states are now usually attributed to homozygosity for a rare silent allele *C2°* (Klemperer, 1974). The *C2* locus is linked to the *HLA* locus (Fu *et al.*, 1974; Alper, 1976; Meo *et al.*, 1977; Olaisen *et al.*, 1978).

45.2.3 C3 Component

C3 is a pivotal protein in the complement system. It occurs in relatively high concentrations in human serum, and it can be detected comparatively easily by simple staining for protein after high voltage electrophoresis. For these reasons, it is perhaps the best studied of the complement component polymorphisms.

The genetic variation in C3 was detected by Wieme and Demeulenaere (1967) using agarose gel electrophoresis. The polymorphism was described by Azen and Smithies (1968) and independently by Alper and Propp (1968). Azen and Smithies (1968) used high voltage starch gel electrophoresis to separate the proteins, and found 6 phenotypes, which could be attributed to four alleles. Only three phenotypes, 1, 2-1 and 2, accounted for by $C3^1$ and $C3^2$, were fairly common. Alper and Propp (1968) used prolonged agarose gel electrophoresis to detect the variations. They found 8 phenotypes, and could account for them on the basis of three certain and 2 probable additional alleles. The common variants were designated C3 F, F-S, and S, where "F" and "S" stood for "fast" and "slow". The fastest variant was called C3 F_1 and the slowest, C3 S_1. The other variants were designated according to their relative mobility taking C3 F_1 and C3 S_1 as unity. They were C3 $F_{0.8}$, $F_{0.5}$ and $S_{0.6}$. Technical refinements for C3 typing have been described (Azen *et al.*, 1969; Teisberg, 1970a). Family and population studies by a number of workers have amply confirmed the codominant inheritance pattern, and many additional variants have been described (Azen *et al.*, 1969; Teisberg, 1970b, 1971a, 1971b and 1971c; Brönnestam, 1971; Brönnestam *et al.*, 1971; Dissing and Sørensen, 1971; Segers *et al.*, 1974; Seger and Salmon, 1977; Schlesinger *et al.*, 1979). C3 S is more common in most Caucasian populations ($C3^S$ frequency about 0.8) than C3 F. The $C3^S$ allele has a very high frequency in the Lapps (Brönnestam *et al.*, 1971; Teisberg, 1971b). A possible association of $C3^F$ and rheumatoid arthritis has been suggested (Brönnestam, 1973), but no association of the C3 polymorphism with Graves disease could be detected (Pepper and Farid, 1979).

The nomenclature and recommended typing methodology for C3 is based upon agreements reached by an international panel of specialists convened at Bonn in June of 1972 (Rittner, 1973). The common C3 variants are designated C3 F and C3 S, and the responsible alleles $C3^F$ and $C3^S$, respectively. Variant types are designated according to their electrophoretic mobility on agarose gels under a specified set of conditions, with C3 S and C3 F_1 defined as having unit mobility. Thus, fast phenotypes have designations like $F_{0.8}$, $F_{1.1}$, etc., and the slow ones are similarly called $S_{0.4}$, $S_{0.65}$, etc. A heterozygote having one $C3^F$ and one $C3^{S0.4}$ gene would have the phenotype C3 FS0.4. Phenotypes representing the expression of at least 22 rarer alleles had been found by the time the Bonn conference was held. Dr. Rittner's laboratory was designated the C3 Reference Laboratory at the meeting, and an updated report of new variants was published for 1973-1974 (Rittner and Rittner, 1974). The nomenclature and other standards adopted at the June, 1972, meeting are sometimes referred to in the literature as the "Bonn conventions". C3 mobility is very sensitive to the concentration of Ca^{++} in the buffers. This cation has the effect of decreasing C3 mobility and, hence, of making the C3 bands easier to visualize by simple protein staining. The Bonn convention recommended that C3 typing be done on agarose gels in buffers containing 1.8 mM Ca lactate. Other variables in the typing procedure were discussed by Kühnl and Strobel (1974).

In 1969, Rose and Geserick observed genetic variation in a serum protein which they designated "Pt" (for "post-transferrin"). There were three phenotypes, accounted for by a pair of alleles, and the variation was seen only in aged serum samples. Before too long, it became apparent that the "Pt" system was intimately related to C3 (Geserick and Rose, 1973). Pt A is related to C3 F, Pt AB to C3 FS, and Pt B to C3 S. C3 is converted to C3a and C3b during complement activation *in vivo*, and C3b can be further converted to C3c and C3d by serum factors. It appears that C3c is a major storage fragment of C3 in serum. The so-called "Pt" system variants are apparently equivalent to the common C3 variants, which are preserved in the C3c fragments (Alper, 1973; Mauff *et al.*, 1974; Hauptmann, 1979). The conversion of C3→C3c is inhibited by 45.6 mM Na_2EDTA (Patzelt *et al.*, 1977). The common C3 variants are evident in the C3c fragment (the "Pt" system), but rarer variants cannot be readily typed using the Pt typing system. If antigen-antibody crossed electrophoretic techniques are used, C3 variants can be detected more readily in C3c fragments as well as in native C3 (Schwamborn *et al.*, 1976). C3 apparently does not cross the placental barrier, and fetal C3 corresponds to the type of the fetus and not to that of the mother (Propp and Alper, 1968; Azen *et al.*, 1969). Reviews of the C3 polymorphism have been given by Alper (1973), Seth and Seth (1976), Hobart and Lachmann (1976) and Hauptmann (1979).

The C3 polymorphism has been applied to disputed parentage problems in a number of countries (Teisberg, 1972; Farhud and Walter, 1973; Spielmann, 1973; Hoste *et al.*, 1977; Geserick *et al.*, 1980). The probability of excluding a falsely accused western European Caucasian father with C3 is about 13%. No references to C3 typing in bloodstains were found.

45.2.4 C4 Component

C4 is a glycoprotein of Mw about 206,000, consisting of

three polypeptide chains. Normal serum contains a C4 binding protein which can form stable complexes with C4. This complex formation is prevented by EDTA or heparin, however, and EDTA must be incorporated into buffers used for C4 polymorphism studies if EDTA or heparin plasma is not available (Hauptmann, 1979).

Genetic variation in C4 was described by Rosenfeld *et al.* (1969) and studied further by Bach *et al.* (1971). They studied EDTA-plasma by antigen-antibody crossed electrophoresis employing an anti-C4 serum for immunofixation detection. Ten different patterns could be distinguished, but the underlying genetics was not very clear. Teisberg *et al.* (1976) investigated C4 patterns using high voltage agarose gel electrophoresis, and immunofixation detection. They detected three common phenotypic patterns and one rarer one, and family studies indicated the involvement of three codominant alleles at an autosomal locus, which were closely linked to the *HLA* locus. The common phenotypes were C4 F, S and FS, accounted for by $C4^F$ and $C4^S$. Another phenotype, FM, was attributed to heterozygosity of $C4^F$ with a rarer allele $C4^M$. These studies were extended (Teisberg *et al.*, 1977), and an additional allele $C4^{F1}$ was found. Mauff *et al.* (1978a) confirmed these findings in a German population, and gave a modified electrophoretic procedure enabling easier detection of the F1 types. In addition to the common F, FS and S types, F1F, F1S, F1, MF, MS, MF1 and M phenotypes were observed.

Recent studies by O'Neill *et al.* (1978a, 1978b and 1978c) have resulted in a different picture of C4 genetics. It is clear that *C4* is closely linked to HLA, especially to *HLA-B* and *Bf*, but there is no clear association with *GLO* (Teisberg *et al.*, 1977; Mauff *et al.*, 1978a). The *C4* locus is thus apparently within the HLA complex. O'Neill *et al.* suggest that C4 phenotypes are controlled not by codominant alleles, but by two separate but closely linked loci. In addition, they have shown that the "red cell" antigens Chido (Ch^a) and Rodgers (Rg^a) are in fact distinct antigenic components of C4. C4 F is said to be controlled by one locus, with alleles *F* and f^0 determining the presence or absence, respectively, of the fast bands. Similarly, C4S is controlled by a second locus having alleles *S* and s^0. Ch^a is believed to be a component of C4 S, and Rg^a of C4 F, because C4 F people (FFs^0s^0 or $Ff^0s^0s^0$) are always Ch(a−), while C4 S people (f^0f^0SS or $f^0f^0Ss^0$) are always Rg(a−). The C4 antigenic determinants are associated with the C4d part of the molecule (Tilley *et al.*, 1978). Hauptmann (1979) has noted that known variations in C4 FS patterns would be consistent with the prediction of the four different genotypes for phenotype C4 FS in the two locus model. There are characteristic associations of certain HLA-B and Bf types in individuals of C4 F and C4 S type. The levels of C4 show variation according to phenotype as well, C4 F and C4 S individuals having significantly lower levels than C4 FS ones (O'Neill and Dupont, 1979). Awdeh *et al.* (1979) have recently shown that antigen-antibody crossed electrophoresis shows different and distinguishable patterns in FS, f^0S, Fs^0, FSs^0 and Ff^0S types, thus allowing their discrimination, and lending support to the two locus hypothesis. Hauptmann (1979) said that O'Neill and collaborators had recently reported finding Ch and Rg antigens on the red cells of a homozygous C4 deficient individual, but absent in the serum. This finding appears to indicate the Ch^a and Rg^a may be controlled by a locus independent of *C4*. The relationship between Ch/Rg and C4 will require further study. Hereditary C4 deficiency is quite rare, but most workers now attribute it to a rare silent allele $C4^0$ in the one locus model, or to the genotype $f^0f^0s^0s^0$ in the two locus model.

Olaisen *et al.* (1979) have recently found that the *C4* locus is duplicated, at least in some people. They suggested a change in the nomenclature for the C4 polymorphism to take this fact into account. In addition, they do not appear to regard the model proposed by O'Neill and coworkers, in which *C4* is duplicated on all chromosomes but has a high frequency of silent alleles at both loci, as proven.

45.2.5 C6 Component

Polymorphism of C6 was detected by Hobart *et al.* (1974) using isoelectric focusing and functional detection with a specific C6 hemolytic assay system. Three common patterns, called A, AB and B, could be accounted for by a pair of codominant alleles $C6^A$ and $C6^B$. Some rarer patterns were thought to be due to the expression of a less common allele, called $C6^R$. They said that $C6^R$ might not be a single allele. The common types of C6 are fairly well distributed in western Europeans, and the inheritance pattern has been confirmed (Hobart and Lachmann, 1976; Rittner *et al.*, 1979). *C6* is closely linked to *C7*, the locus controlling the seventh complement component, but it is not linked to *HLA* (Hobart and Lachmann, 1976; Kagan *et al.*, 1979). Inherited deficiency of C6 has been described, and is attributed to homozygosity for a silent allele $C6^0$ (Glass *et al.*, 1978). Ritter *et al.* (1979) and Mauff *et al.* (1979) have recommended incorporation of C6 testing into paternity investigations. The probability of excluding a falsely accused man is about 18% in western Europeans.

45.2.6 C7 Component

Hobart *et al.* (1978) found three C7 phenotypes by isoelectric focusing. There was one homozygous type, C7 1, and two apparently heterozygous types, C7 2-1 and C7 3-1. Three codominant alleles, $C7^1$, $C7^2$ and $C7^3$, were postulated to explain the types. In one family, a 2-1 × 3-1 mating had produced a 3-2 child. They noted that *C7* is closely linked to *C6*.

45.2.7 C8 Component

Raum *et al.* (1979) found genetic polymorphism in C8 using PAGIF. Two common alleles, $C8^A$ and $C8^B$, give rise to three common phenotypes. $C8^A$ is more common, having frequencies of about 0.65 in Blacks and Whites, and about 0.7 in Orientals. A rarer allele, $C8^{A1}$, is most frequent in Blacks (about 0.05), very rare in Caucasians, and it was not seen at all in Orientals. C8 deficiency is attributed to a silent ($C8^0$) allele. *C8* is not linked to *HLA*.

45.2.8 Properdin factor B (Bf)

Properdin factor B was discovered (and named) by Pillemer *et al.* (1954). Properdin is a name that has been given to several factors which can activate complement by an alternative pathway to the classical one (section 1.3.5). Thus, properdin factor B is a C3 proactivator. The protein was isolated by Haupt and Heide (1965) and called "β_2-glycoprotein II". It was also isolated by Boenisch and Alper (1970) and called "glycine-rich-β-glycoprotein" or "GBG". There was a suggestion in the work of Boenisch and Alper (1970) that the protein might exist in multiple molecular forms. Alper *et al.* (1971 and 1972) described the polymorphism of "GBG", and suggested that the protein had some relationship to the complement system. The "F", "FS" and "S" (for "fast" and "slow") types were common in all populations tested. The locus controlling the protein was named *Gb*, and the alleles Gb^F and Gb^S. A phenotype determined by a rarer allele Gb^{F1} was seen in Blacks, and one determined by another rarer allele Gb^{S1} was seen in Whites. It was then realized that GBG was identical to factor B of the properdin system (Alper *et al.*, 1973) as well as to a protein isolated by Götze and Müller-Eberhard (1971) that had been called C3 proactivator. The locus has been re-named *Bf*, and the common alleles are Bf^F and Bf^S. The less common alleles described by Alper *et al.* (1972), Gb^{F1} and Gb^{S1} are now called Bf^{F1} and $Bf^{S0.7}$. By 1978, there were seven more alleles. An internationally agreed upon nomenclature for Bf types was adopted (Mauff *et al.*, 1978b), and a Reference Laboratory headed by Prof. Dr. Mauff was set up at Cologne. Variants other than F, S and F1 are now designated "F" or "S" (depending upon whether they are "fast" or "slow") with a relative mobility value listed after the upper case letter. The reference distance in this case is S→F1. Kühnl and Spielmann (1978b) have done population studies on Bf in the German population. Weidinger *et al.* (1979) presented evidence for a silent allele, Bf^0, in one of the families in their material.

The *Bf* locus is closely linked to *HLA* (Allen, 1974; Rittner *et al.*, 1975), and it probably lies closest to *HLA-B*. There are striking linkage disequilibria between HLA-B and Bf alleles. As noted above, the *C2* and *C4* structural loci are closely associated with *Bf* in the HLA complex.

45.3 α_2–Macroglobulin (α_2M)—Xm, AL–M and α_2M

α_2-Macroglobulin is one of the major protease inhibitors of human serum (Laurell and Jeppsson, 1975), as noted in section 43.1. The molecule was purified and characterized by Hall and Roberts (1978). It had a MW of 718,000, and probably consists of 4 polypeptide chains of MW about 185,000. These smaller MW units are cleaved to yield 85,000 MW fragments upon interaction with a variety of proteases. The serum concentration of α_2M is dependent upon age and sex, mean values being of the order of 175 mg/100mℓ for men and 206 mg/100 mℓ for women. The value is 2–3 times higher in small children.

In connection with studies on Lp(a) (section 45.1.3), Berg produced an antiserum in rabbits which, after absorption, reacted with some but not all human sera. The precipitates stained for protein, but not for lipid, and the antigen was, therefore, not a lipoprotein. The antiserum had been produced by immunization with the serum of a single Lp(a–) female donor. Reactions of the antiserum strongly indicated that the antigen might be controlled by an X-linked gene. The antigen could also be localized to the macroglobulins, and the system was named "Xm" to indicate the X-linked macroglobulin protein antigen. The antigen was called Xm(a) and the gene controlling its presence Xm^a (Berg and Bearn, 1966a and 1966b). The antigen was shown to be localized in α_2M, and extensive linkage studies were carried out with other known X markers (Berg and Bearn, 1968). *Xm* mapped fairly near "deutan" and a little farther from *Gd*. Cooper (1978) said that the limited supply of anti-Xm(a) reagent had been exhausted, and that no further examples had been found.

In 1969, Kasukawa *et al.* found an isoprecipitin in a chronic hepatitis patient who had never been transfused. It reacted with about 30% of random sera in Japan, and the antigen, which was said to be an α_1-globulin, seemed to be inherited in a simple Mendelian way. Another example of the antiserum was then found by Leikola *et al.* (1972), and they showed that the antigen resided in the α_2M fraction. The antigen was named "AL-M", and it was inherited as an autosomal dominant.

In 1974, Gallango and Castillo reported another polymorphism in α_2M. This one was detected by immunoelectrophoresis in the sera of a Venezuelan Mestizo population using anti-α_2M sera. The types were distinguished by electrophoretic mobility upon immunoelectrophoresis. There were apparently three homozygous types, A, B and C, and three heterozygous types AB, BC and AC. Genetic data indicated that the types could be accounted for by three codominant alleles at an autosomal locus, α_2M^A, α_2M^B and α_2M^C. The autosomal inheritance pattern indicated nonidentity with the Xm system. Identity with AL-M seemed to be excluded by the fact that no "negatives" had been observed. Mroueh and Adham (1970) assayed semen for α_2M levels, and found measurable quantities in 11 out of 34 samples. Concentrations in the 11 ranged from 30 to 130 mg/100mℓ with a mean of 70.4. There was no correlation between α_2M level and oligo- or azoospermia.

45.4 α_1–Acid Glycoprotein (Orosomucoid)

α_1-Acid glycoprotein is a plasma globulin characterized by a high carbohydrate content, a large number of sialyl residues and a very acidic pI. The protein occurs in the so-called "seromucoid" fraction of plasma, and is the fastest moving serum protein component on starch gel electrophoresis using borate buffers at pH 8.6. One of the first purified preparations of the protein was described by Weimer *et al.* (1950). At that time, it was referred to as the "MP-1 component". Schmid (1953) obtained a crystalline preparation

of α_1-acid glycoprotein. The purified protein had a MW of about 40,000, and about 45% of its weight is attributable to the carbohydrate content. The primary structure of α_1-acid glycoprotein has been completely solved (Schmid *et al.*, 1973), and upon close analysis, revealed an extraordinary number of amino acid substitutions in different examples of the protein. 21 different residues, out of 181 in the complete molecule, have been found to be substituted. The sequence also showed structural homology with the immunoglobulins.

In 1964, Schmid *et al.* found that α_1-acid glycoprotein exhibited electrophoretic heterogeneity, and suggested that this behavior might have a genetic basis. Further studies were carried out on desialyzed material, and indicated that the three different patterns which could be observed were the result of a pair of alleles (Schmid *et al.*, 1965). Because of its molecular properties, the protein shows considerable microheterogeneity upon electrophoresis, in addition to the macro polymorphic patterns. This microheterogeneity is even more apparent if isoelectric focusing is used as the separation technique (Gordon and Dykes, 1972).

The α_1-acid glycoprotein phenotypes were studied by immunofixation electrophoresis on agarose gels by Johnson *et al.* (1969). Further family and population studies, extending the work of Schmid *et al.* (1965), were conducted to establish the genetic basis for the polymorphism. The phenotypes were called SS, SF and FF (where "S" and "F" stand for "slow" and "fast"). These are accounted for by a pair of autosomal codominant alleles called Or^S and Or^F (the protein is also called "orosomucoid"). The Or^S frequency in U.S. Caucasians (n = 220) was 0.36, and varied from about 0.33 to 0.57 in other populations. The polymorphism is thus fairly well distributed, and may well become another valuable serum group system marker.

Sayce and Rees (1977) reported a series of experiments on seminal orosomucoid. There appeared to be two different and distinguishable immunological reactions using anti-orosomucoid serum. These experiments weere discussed briefly in section 10.13.2. The relationship between these immunologically distinguishable forms and the serum polymorphic types, if there is any relationship, is not clear.

The properties of α_1-acid glycoprotein have been discussed in detail by Schmid (1975). The biological role of the protein is not yet fully clear.

45.5 Ceruloplasmin (Cp)

Copper is an essential trace element in the nutrition of many animals, including human beings. Most of the copper in human serum is bound to an α_2 globulin protein (Holmberg and Laurell, 1947). Cp has a MW of about 150,000 and can bind 8 atoms of Cu per mole. The isolated protein has a strikingly beautiful sky blue color, which is often mentioned in discussions of it. The name "ceruloplasmin" was given to the protein by Holmberg and Laurell (1948). Cp has been extensively purified, and a crystalline preparation was obtained by Deutsch (1960). The detailed properties of the protein are described in the review of Poulik and Weiss (1975). The detailed structure is apparently still not completely clear. Subunit structure has been proposed for Cp (see in Poulik, 1968), but there is also evidence to suggest that the molecule consists of a single polypeptide chain (Poulik and Weiss, 1975). Kingston *et al.* (1979) determined the sequence of a lengthy segment of the molecule (159 amino acid residues), and appeared to favor a single chain structure for the intact protein. This segment did not contain any of the carbohydrate.

The exact function of Cp is still not settled altogether. The protein exhibits oxidase activity with a number of artificial substrates. Cp is usually detected by its oxidase activity after electrophoretic separation. Uriel (1958) devised such a detection procedure with p-phenylenediamine as substrate. The protein is also an effective "ferroxidase", catalyzing the conversion of Fe^{2+} to Fe^{3+}. It has been assigned a name and number by the Enzyme Commission on this basis: ceruloplasmin = ferroxidase; iron(II):oxygen oxidoreductase; E.C. 1.16.3.1. The ferroxidase activity is one of the three functions of Cp that have been assigned physiological significance, the others being copper transport and detoxication, and the maintenance of copper homeostasis in tissues. The functions are not necessarily mutually exclusive. At the present time, a number of investigators think that the ferroxidase activity of Cp is its most important function. Ferrous ions are oxidized by Cp to ferric ions, which are then incorporated into transferrin (section 42). Goldstein *et al.* (1979) have shown that Cp can also act as a scavenger of superoxide radicals, thus mimicking the action of SOD (section 37.4) in this respect.

Molecular heterogeneity was observed in Cp by a number of the earlier workers, such as Uriel (1958), Deutsch (1960), Morell and Scheinberg (1960) and Hirschman *et al.* (1961). A genetic basis for the variation was considered, but Poulik and Bearn (1962) indicated that it would be difficult to interpret the genetic significance of the heterogeneity. Other explanations, such as polymerization and denaturation caused by methods of preparation or storage, were considered more likely.

Qualitative genetic variation in Cp is now known, however. A possible genetic variant was described by Martin *et al.* (1961) and more fully by McAlister *et al.* (1961). The variant form was detected by vertical starch gel electrophoresis at pH 8.6. It was faster than the usual Cp (called "Cp 1") and was called Cp 1-F. It was easier to detect, they said, in pH 9.1 buffers. The variant Cp was transmitted through four generations of a family as a dominant trait. In spite of large scale Cp typing in a number of laboratories, however, this variant was not encountered again (Poulik and Weiss, 1975).

Schreffler *et al.* (1971) described three genetically determined forms of ceruloplasmin, called Cp "A" (the fastest), "B" (intermediate) and "C" (the slowest), which could occur alone, or in combinations. Five phenotypes were seen: A, AB, B, AC and BC. These phenotypes were attributable to three codominant alleles at an autosomal locus, Cp^A, Cp^B and Cp^C. Cp B is the common phenotype. Cp^A oc-

curred in Caucasians but was more frequent in Negroes, while Cp^C was not seen in Caucasians. These phenotypes were detected by starch gel electrophoresis in borate buffers, pH 9. Shokeir and Schreffler (1970) described two additional variants in the sera of American Blacks, Cp New Haven (Cp NH) and Cp Bridgeport (Cp Bpt). Cp NH migrated between Cp B and Cp C, and was due to an allele of Cp^A and Cp^B called Cp^{NH}. Cp Bpt was seen in a Black family, and migrated between Cp A and Cp B. Its genetic relationship to the other forms was not as clear. Cp^{NH} had a higher frequency in American Blacks than did Cp^C. Shokeir (1971) compared the biochemical and immunological properties of Cp A, AB, AC, ANH, BNH and BptB. All the forms were of similar molecular size, and charge differences were not attributable to differences in sialic acid content. No immunological differences were detected with an anti-human Cp, but there were some differences in the inhibition of oxidase activity by cyanide or azide. McCombs *et al.* (1970) have described another variant, Cp Galveston, detected by slab polyacrylamide gel electrophoresis in pH 9.1 buffers. The variant protein was seen in serum and in ascites fluid, and although an additional allele was suspected, family studies could not be carried out.

One, and possibly two, new variants were described by Buettner-Janusch *et al.* (1973) in the Malagasy population of Madagascar. They found that Cp B was the common phenotype, as in other populations. Examples of Cp NH, Bpt and Galveston containing phenotypes were observed among the 405 sera. A previously undescribed phenotype, called Cp 'Tananarive' (or Cp 'Tan') was observed as A/Tan and Tan/Tan in this population. No B/Tan heterozygotes were seen. The failure to find B/Tan types might mean that the allele determining 'Tan' was not codominant with Cp^B, but it could also mean that the sample of the population typed simply did not include a person of this type. Sera were observed that typed as 'Cp O' by normal testing procedures. If these were subjected to prolonged activity staining, however, a weak band, which was named 'Cp X', eventually appeared. The band appeared to represent Cp and not some other plasma protein, but its relationship to the other Cp variants was not completely clear.

Genetically controlled quantitative variation in Cp is known, the most noted manifestation of it being Wilson's disease. The first complete description of this comparatively rare disorder was given by Wilson in 1912. The disease has been called hepatolenticular degeneration, since it is characterized in adults by deterioration of the cerebral lenticular nuclei and by progressive cirrhosis of the liver. It is also characterized by greatly decreased levels of serum Cp (hypoceruloplasminemia). The major biochemical feature in Wilson's disease is the disruption of normal copper metabolism. The defect is inherited as an autosomal recessive. Wilson's disease patients have been studied quite extensively, but a full understanding of the molecular pathology does not appear to have emerged as yet. Gitlin and Gitlin (1975) and Poulik and Weiss (1975) discussed the subject in detail. There has been an inclination to regard the primary biochemical lesion as the inability to synthesize Cp, but evidence is conflicting. Studies on "Wilsonian" Cp as compared to that from normal individuals (e.g. Neifakh *et al.*, 1972) have not yielded a clear set of differences. Some have suggested that the disease may result from homozygosity for different genes in different populations, or perhaps from different modifying genes.

45.6 Transcobalamin II (Tc II)

Vitamin B_{12} binding and transport in serum are mediated by two types of transcobalamins, called I and II. Tc I is about three times larger in MW than Tc II (Hall, 1969). Tc II has been purified from human serum and characterized to some extent. Allen and Majerus (1972) obtained an affinity purified Tc II that had a MW of 53,900 by ultracentrifugation, and bound one Vitamin B_{12} per mole of protein. It dissociated in SDS to yield two peptides, and did not appear to contain carbohydrate. Lindemans *et al.* (1979) also purified Tc II by affinity chromatography on cyanocobalamin-Sepharose. The MW of the native molecule was about 37,000, but a faint band of MW about 29,000 could also be seen on SDS-polyacrylamide gel electrophoresis. The preparation was heterogeneous by isoelectric focusing. Haus *et al.* (1979) have found that human Tc II seems to be distinctly different immunologically from the protein in other species.

In 1975, Daiger *et al.* found polymorphism in Tc II by polyacrylamide gel electrophoresis and autoradiographic detection using radiolabelled Vitamin B_{12} (Daiger *et al.*, 1975b). There were some 7 different patterns, with 2–4 bands each. They were stable in a given person and inherited. Four alleles at an autosomal locus were postulated to explain the types. Tc^3 and Tc^4 were quite common, while Tc^1 and Tc^2 were rare. Tc^1 bands were the fastest migrating, and Tc^4 bands, the slowest. Heparinized plasma gave very poor results, perhaps because heparin binds these proteins. Similar observations on the polymorphism have been made by Fràter-Schröder and Hitzig (1977) and Fràter-Schröder *et al.* (1979a and 1979b). They too used polyacrylamide gel electrophoresis and radiolabelled vitamin B_{12} autoradiography for typing, and could distinguish eleven phenotypes, three of which were fairly common. These were called Tc II 3-3, 1-3 and 1-1. They were accounted for by two common alleles $TcII^1$ and $TcII^3$ at an autosomal locus. Three additional alleles, $TcII^2$, and $TcII^4$ and $TcII^5$ had lower frequencies. Family studies were consistent with the five codominant allele explanation. Occasional families with an inherited Tc II deficiency have been described (Hitzig *et al.*, 1974), and deficient children usually suffer from megaloblastic anemia. Two unusual Tc II patterns in patients with anemias have been described as well (Fràter-Schröder *et al.*, 1979a)

45.7 Thyroxine Binding α–Globulin (TBG)

Throxine in human plasma may be bound to three different proteins: thyroxine-binding prealbumin (TBPA),

thyroxine-binding globulin (TBG), and albumin. TBPA was first isolated by Schultze *et al.* (1962d) and designated "tryptophan-rich prealbumin" because its electrophoretic mobility was faster than that of albumin at pH 8.6. The protein has since been more thoroughly characterized. The amino acid sequence has been worked out (Kanda *et al.*, 1974), and there are only two Try residues per polypeptide chain of 127 amino acids. The x-ray structure of TBPA at a resolution of 2.5Å has also been published (Blake *et al.*, 1974). TBPA can bind the retinol-binding protein (RBP), the specific vitamin A carrier in plasma, on a one for one mole basis. The TBPA exists in a tetrameric structure for the binding of RBP or thyroxine, and complexation with TBP apparently has no effect on thyroxine binding (Putnam, 1975b; Cooper, 1978). Characteristics of retinol-binding protein are further discussed by Putnam (1975b). The majority of protein-bound thyroxine appears to be associated with TBG, the thyroxine-binding capacity of TBPA apparently being secondary to its RBP-binding function (Cooper, 1978). Thyroxine binding to albumin is nonspecific, and apparently not of much physiologic significance (Putnam, 1975b).

TBG has not been characterized as well as TBPA, in part because the former is present in serum at significantly lower concentrations (1–2 mg/100mℓ) than that of the latter (10–40 mg/100 mℓ) (Putnam, 1975b). Giorgio and Tabachnik (1968) developed a purification scheme for TBG from human plasma, resulting in a preparation that was homogeneous by starch gel and polyacylamide gel electrophoresis as well as by ultracentrifugation. The purified glycoprotein had a MW of 58,000.

Familial quantitative variation in TBG has been reported. Nicoloff *et al.* (1964) first noticed the near absence of the protein in six members of a family whose members had no evidence of Thyroid disease. Nikolai and Seal (1966 and 1967) observed TBG deficiency in a second family, and could show that the characteristic was X-linked. Other pedigrees showing this same behavior have been noted, and in one family the TBG deficiency was linked to the Xg^a blood group (Grant *et al.*, 1974). Shane *et al.* (1971) found an inherited elevated level of TBG in four generations of a family. The characteristic was X-linked, and other families are apparently known which show it as well.

Daiger (1976) described an electrophoretic variant of TBG in Black and Oriental populations. The variant gene occured in about 15% of American Blacks. The variant locus is apparently X-linked as well, but it is not clear whether it is identical to the locus controlling the quantitative variations.

45.8 Albumin

Serum albumin is the most familiar and most plentiful of the serum proteins. Serum normally contains 42 ± 3.5 g/ℓ of albumin (with a range of 35–50. Albumin represents about 60% of the total serum protein. It has been assigned numerous functions, including maintenance of the osmotic pressure of blood, fatty acid transport and sequestration, and transport of bilirubin. Albumin was extensively reviewed by Peters (1975).

Serum albumin has been extensively purified and characterized. It is a simple protein, containing one polypeptide chain and no carbohydrate. Some fatty acid is apparently associated with even the purest examples of the protein. Human albumin has a MW of 66,248 (calculated from the amino acid composition). Its sequence has been completely determined (Brown, 1975), as has that of the bovine protein (Behrens *et al.*, 1975). Both sequences may also be found in Peters (1975). There are 17 intrachain disulfide bridges, creating a structure with 9 loops. Albumin is well known for its ability to associate with a great number and variety of small molecules, including cations, anions (especially fatty acid, but including hormone and amino acid), drugs and organic dyes. Albumin is a good antigen, and anti-albumin is surely a major constituent of the "anti-human serum" prepared in animals (section 16.1). Atassi *et al.* (1979) have recently reported that the bovine protein has five antigenic sites.

Strictly speaking, albumin is not polymorphic in most human populations. It does exhibit a large number of structural variants, most of which are exceedingly rare. A few variants reach significant frequencies in isolated populations. Albumin variants have been reviewed at length by Gitlin and Gitlin (1975). Structural variants are of two types. One of these is represented by the group that is said to give rise to "paralbuminemia", "bisalbuminemia", or "alloalbuminemia". The terms are essentially equivalent in most of the literature, and mean simply that more than one albumin band (usually two) is seen upon electrophoretic separation. The other type is represented by albumins which tend to dimerize. Quantitative variation is known, and is represented by markedly low albumin levels, usually termed "analbuminemia".

Genetic variation was first noted by Nennstiel and Becht (1957) and by Knedel (1958). They observed two albumins in serum by electrophoresis, undoubtedly the reflection of heterozygosity for a variant structural gene. A large number of variants have since been described, especially by Weitkamp and collaborators. Normal albumin (the usual kind) is often called "A". Many others are named after places. Until the variants can be studied structurally, it will be difficult to know exactly which ones are really different. Weitkamp *et al.* (1969) described eight distinguishable "types" of albumin, but they said that each "type" might be a collection of a number of different variants. In 1973, Weitkamp *et al.* carried out extensive studies on 23 variants. The most frequent variant among Europeans is called "B", but even it is quite uncommon. Weitkamp *et al.* (1973) said that the frequency of albumin variants may be as low as 1 in 2,000 in Europe. Several electrophoretic typing systems must be used to distinguish all the different variants. Fine *et al.* (1976) examined 24 cases of albumin variants in France, and 16 of them were type B. Efremov and Braend (1964) saw only one variant in 1,015 Norwegians, and no variants were found by

Persson *et al.* (1971) in Greenland Eskimos. Some albumin variants have been found only in isolated populations (Lie-Injo *et al.*, 1971; Frohlich *et al.*, 1978). People with familial analbuminemia have been described (Bennhold and Kallee, 1959), but are very rare. Apart from the family studies by Bennhold and Kallee (1959), Gitlin and Gitlin (1975) said that only 10 additional cases had been seen. It is not clear what the exact nature of the defect is in these cases, since "analbuminemics" do make a small amount of albumin. In addition, the known cases may not all have the same genetic basis. The albumin locus is known to be linked to *Gc* (Weitkamp *et al.*, 1966 and 1970).

Since albumin is not significantly polymorphic in most populations, it does not provide a useful genetic marker system for medicolegal problems. Nevertheless, Reinskou (1968b) reported that one of two putative fathers in a paternity case had an albumin variant which had been inherited by the child. The man had also passed a Gc^2 gene to the child. The case illustrates the value of rarities when they do occur in a case, and are detected.

45.9 β_2–Glycoprotein I (Bg)

This protein was isolated from human plasma by Schultze *et al.* (1961). It may be recalled in passing that this group of investigators isolated a "β_2-glycoprotein II" (Haupt and Heide, 1965), which was later recognized as being identical with factor B of the properdin system (section 45.2.8). Heimberger *et al.* (1964) described a number of the characteristics of the molecule. Liu and Putnam (1975) have reported similar studies more recently. The MW is about 40,000, and the molecule appears to be a single polypeptide chain about 300 amino acids long with 8 or 9 disulfide bridges. It occurs in normal plasma at concentrations of 15–30 mg/100 mℓ. It has no known function.

In 1968, Haupt *et al.* found a family in which two sibs had no β_2-glycoprotein I and whose parents had about half the normal amount. Cleve (1968) then carried out a population study using radial immunodiffusion technique. 94% of people had concentrations ranging from 16–30 mg/100 mℓ with a mean of 21, while the remaining 6% had concentrations of 6–14 mg/100 mℓ with a mean of 10 in their serum. The distribution was sharply bimodal in these initial data, and it was proposed that β_2-glycoprotein I concentration was controlled by a pair of codominant alleles called Bg^N and Bg^D at an autosomal locus. Bg^NBg^N people have the higher concentration range, while Bg^NBg^D people have the lower one. "N" and "D" stood for "normal" and "deficient". The two sibs of Haupt *et al.* (1968) with no detectable protein were presumably uncommon Bg^DBg^D homozygotes. Bg^D is thus probably a silent allele. Various nongenetic factors like sex, age, pregnancy and disease affect the concentration, however. One family in the material studied by Cleve (1968) did not fit the model. Cleve and Rittner (1969) studied 88 families with 213 children for this characteristic. In 9 families, one parent and about half the children appeared to be Bg^NBg^D. But a few families did not fit the model. Parents with normal serum concentrations can have children who have intermediate levels. Koppe *et al.* (1970) studied an additional 49 families, and again, a few exceptions were found. Atkin and Rundle (1974) looked at 381 people in the British population, and found almost the same Bg^N frequency as in the German population (about 0.94). They also found one deficient person. It is assumed by most investigators that the basic genetic hypothesis is correct, and that most of the variation is controlled by a single *Bg* locus. The situation resembles that of Lp^a in some ways (section 45.1.3), except that the codominant nature of the *Bg* alleles, in contrast to the dominance of Lp^a, makes the variation somewhat easier to interpret (Cooper, 1978).

SECTION 46. THE HLA SYSTEM AND THE HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX

46.1 Introduction

In his Nobel address in 1930, Landsteiner drew a parallel between hemolytic reactions caused by the transfusion of blood-group incompatible blood and skin graft rejections. The two phenomena, he said, ". . . .are basically related and rest on chemical differences of a similar kind".

The immunological system is fairly similar in all vertebrates, and vertebrates reject foreign skin transplants as well as react against foreign particles or fluids by the formation of specific antibodies. Rejection of "foreign" skin grafts may be acute (within weeks), or chronic (within a few weeks or months). In species able to reject acutely, it has been possible to identify a small region on a single chromosome in which numerous genes controlling immune reactions are located at closely associated loci. This genetic region is called the "major histocompatibility region", or "system", or "complex" (MHR, MHS, MHC). Mammals are included among those vertebrates having a MHC. In animals capable only of chronic rejection, the responsible genes are distributed throughout the genome. Even in acutely rejecting species, which have a MHC, however, there are additional genetic loci whose products are associated with chronic rejection, and these are distributed throughout the genome.

The pioneering work in the recognition of mammalian major histocompatibility regions was done by Gorer and Snell and their collaborators beginning in the 1930's. Their work was with mice, and resulted in the definition of the H-2, or major histocompatibility system, in the mouse. Major histocompatibility complexes are now recognized in the mouse, rat, chicken, guinea pig, dog, rhesus monkey, chimpanzee and in human beings (Albert and Scholz, 1978; Götze, 1977). The H-2 system in the mouse has been an important model in many respects for the studies responsible for unravelling the MHC in humans.

In 1952, Dausset and Nenna found antibodies in a multiply transfused patient which agglutinated the leucocytes of some, but not all other people. The presence of leucoagglutinins in multiply transfused people was found to be a more general occurrence (Dausset, 1954). In 1958, Dausset described the first antigen in what was to become the HLA system. It was called "Mac" (today, HLA-A2). A second leucocyte polymorphism was found by van Rood (1962). He called the responsible locus "4" (or "FOUR"), and the two supposed alleles "4a" and "4b". Recognition of these specificities was achieved primarily by statistical genetic analysis of many antibody-containing sera which had been tested against panels of leucocytes from selected, unrelated donors. Using similar methods, Payne *et al.* (1964) described another polymorphism independent of FOUR. It was called "LA" (where "L" stood for "leukocyte" and "A" was supposed to indicate the first locus). From these beginnings, an understanding of the HLA system has emerged over the past 15 or 20 years. The evolution of developments in this field has been characterized by an impressive and very productive degree of international cooperation and agreement. In 1980, Dausset and Snell shared the Nobel Prize for Physiology or Medicine with Benacerraf, for their work on the "genetically determined structures of the cell surface that regulate immunological reactions" (Marx, 1980). Gorer, who died in 1961, would probably have been included in the group, had he lived.

The HLA and genetically associated systems constitute the most intricate and complex array of polymorphisms yet described. The impressive degree of international cooperation and agreement that has characterized the developments in HLA serology has surely simplified the nomenclature problems, and provided order in what could otherwise have been a chaotic nightmare of complicated data. Even given the relatively organized history of the system, the complexities of trivial nomenclature, workshop nomenclature and official nomenclature, along with the recognition of such a large number of different specificities, can be quite bewildering to the nonspecialist. Many of the antisera contain antibodies to multiple specificities, others contain cross reacting antibodies, and it has taken time to recognize and understand all these facts. Not all the specificities are detected by the same methods either. Several different methods have been used to recognize and type the lymphocyte-defined antigens especially, and the different methods do not always give exactly concordant results. The sheer extent of the HLA system, however, makes this system the best genetic marker system yet described. This value has already been exploited in parentage investigations, but there is little work as yet on the determination of HLA antigens in dried blood.

No review as brief as this one can do justice to the history, development and complexities of HLA. Many excellent reviews are available, however, including those of Mayr (1969), Walford (1969), Amos and Ward (1975), Albert (1976), Albert and Götze (1977), Götze (1977), Miller (1977), Albert and Scholz (1978), Bodmer (1978a), Bender (1979) and Perkins (1979). Beginning in 1964, a series of eight international conference/workshops has been held periodically, at which the specialists have exchanged information and reagents, and have reached agreements about the status of the HLA system. A nomenclature committee made up of internationally recognized specialists, and functioning under

the auspices of the World Health Organization (WHO) and the International Union of Immunological Societies (IUIS), has met after the last few of these conferences, and set forth the current standardized nomenclature. The proceedings of the eight conferences, held at Durham, Leiden, Torino, Los Angeles, Evian, Aarhus, Oxford and again in Los Angeles, (Histocompatibility Testing, 1964, 1965, 1967, 1970, 1972, 1975, 1977 and 1980) provide an accurate picture of the prevailing information about HLA during its evolution and up to the present time.

46.2 Definition and Function of HLA Specificities

Detailed genetic characterization of the H-2 region in the mouse has come about largely through the use of inbred strains of animals developed for the purpose by the pioneering investigators. Human populations are, however, highly outbred, and contain a large number of haplotypes representing a large number of recombinations. Accordingly, population and statistical genetic methods have played a major role in the analysis of the human serological data. Thus, although there are clear analogies between human and mouse MHC genetics, different methods have been required to arrive at a detailed understanding of them.

It became clear early in the development of HLA serology that leukoagglutinating, and later cytotoxic, antibodies are found in the sera of pregnant and parous women (Payne and Rolfs, 1958; van Rood *et al.*, 1958). A substantial percentage of pregnant or parous women turn out to have in their sera lymphocytotoxic antibodies against the HLA antigens of the child which were inherited from the father (Wolf, 1971; Escolar and Mueller-Eckhardt, 1971; Nymand *et al.*, 1971; Bertrams *et al.*, 1971). In the first years of HLA research, it was not possible to find two antisera which gave exactly identical reaction patterns against defined panels of donor cells. This behavior was recognized as being indicative of an extraordinary antigen diversity and structural complexity, but it also made the task of sorting out the specificities more difficult. At the beginning, large numbers of cell samples form unrelated donors were tested against large numbers of different undefined sera. The resulting reaction patterns were then analyzed by a complex series of 2×2 comparisons of pairs of antisera, in which each antiserum is eventually compared with all others. These analyses, done in a number of different laboratories, were made more manageable with the help of computers. Details of the 2×2 comparison method will be found in Cavalli-Sforza and Bodmer (1971). Briefly, such an analysis is designed to show similarity or dissimilarity between members of a pair of antisera, and combinations of these analyses leads to definitions of groups of sera which are highly associated. Associations between sera indicate that they share antibodies directed against the same antigen. Cell samples that react with all the sera of a highly associated group are inferred to possess the "antigen" corresponding to the "antibody" common to all the sera in the group. In this way "antigens" can be defined even when the only antisera against them are polyspecific, although these definitions are operational. Operationally defined antigens have frequently been found to be of a compound nature as more refined analysis with more and different antisera, and different cell panels, became possible. The revisions in the HLA nomenclature over the years largely reflect these changes in the operational definitions of the antigens.

The involvement of cell surface antigens in transplant rejections was clearly shown in animals, and these antigens were known to be present on a variety of tissues and on leucocytes. van Rood *et al.* (1964) and Dausset *et al.* (1965) carried out studies on the relationship of the leukocyte antigens to skin transplantation survival. These studies have been extended by van Rood *et al.* (1966) and by many others, and it was soon clear that HLA represents the major histocompatibility complex in the human species (Bach and Amos, 1967).

46.3 Genetics and Nomenclature of the Serologically Defined HLA Loci

46.3.1 HLA-A and HLA-B Antigens

As noted in 46.1 above, it was clear, at least by 1964, that there appeared to be two different genetic loci controlling the lymphocyte surface antigens. These were originally called "4" ("FOUR") and "LA" and both were multiple allelic. The antigens controlled by the multiple alleles were inherited in a simple Mendelian codominant fashion (e.g. Payne and Hankel, 1961). Dausset *et al.* (1965) proposed a unifying genetic model in which these two loci were seen as part of the same system (called "Hu-1", but later known as "HLA"). Population and family studies indicated that these two loci were autosomal and closely linked (Dausset *et al.*, 1965; Bodmer *et al.*, 1966; Ceppellini *et al.*, 1967). The first unequivocal report of crossing over between the *LA* and *FOUR* locus genes was that of Kissmeyer-Nielsen *et al.* (1969).

The Nomenclature Committee in 1967 combined the FOUR and LA loci into a system that was called "HL-A". The different alleles were assigned numbers, in the sequence of their discovery or of their final definition. Rapid developments in the field resulted in a large number of newly discovered antigens, and of antigens whose status was not immediately clear. In 1970, an important new agreement was reached. Most of the newly discovered antigens, which had a variety of local and unsystematic designations that were quite confusing, were given provisional HL-A numerical designations. The provisional designation was indicated by a "w" (or "W"), which stood for "workshop", according to Bender (1979). The "w" designations were chosen so that the conversion to a recognized specificity could be accomplished simply by omitting the "w". In 1976, the Nomenclature Committee set forth the rules which, with minor modification, still form the basis of HLA nomenclature (WHO-IUIS Terminology Committee, 1976). The system was to be called "HLA" (the dash in HL-A was dropped). The genetic loci of the system would be designated by upper case letters, A, B, C, D, etc., as necessary.

The "A" locus would thus be called "HLA-A", the "B" locus "HLA-B", and so on. Antigens would continue to be designated by numbers, assigned by international agreement. Provisional specificities would continue to be indicated by the prefix "w" or "W". These would continue to be assigned so that "promotion" to a fully recognized specificity could be achieved by dropping the "w". A lower case "w" was preferred for the provisional designation, but the upper case "W" was permitted because it was realized that most computer output devices are incapable of printing lower case letters. The loci previously called "LA" (first locus) and "FOUR" (second locus) would henceforth be called 'HLA-A" and "HLA-B", respectively. For historical reasons, specificities belonging to HLA-A and HLA-B are numbered jointly, so that there is no overlap in numbers between them. Thus there are antigens HLA-A1, HLA-A2 and HLA-A3, but there are no "HLA-B1", "HLA-B2" or "HLA-B3", and so forth. There are certain criteria for the recognition of specificities, and for the recognition of provisional specificities. These have been somewhat refined as time has gone along. Each major workshop has resulted in the recognition of some new provisional specificities, and the conversion of previously provisional specificities to fully recognized ones.

By the time of the sixth workshop in Aarhus in 1975 (Histocompatibility Testing, 1975), there were 8 fully recognized HLA-A specificities and 12 additional "w" or provisional ones. There were eight specificities at HLA-B and 12 provisional ones (WHO-IUIS Terminology Committee, 1976). In the next report (WHO-IUIS Nomenclature Committee, 1978) 10 established and 10 provisional specificities at HLA-A, and 12 established and 21 provisional ones at HLA-B were listed. Most recently (WHO-IUIS Nomenclature Committee, 1980), there were no changes in the HLA-A locus antigens. At HLA-B, there were nine new provisional specificities, HLA-Bw55 through Bw63.

It should be noted that the HLA-A, HLA-B and HLA-C (see below) antigens are defined principally by serological reactions, and are sometimes referred to as "SD" ("serologically detected" or "serologically defined") antigens. The loci have been called *SD1*, *SD2*, and *SD3* at times. In section 46.4 we will briefly discuss HLA antigens that were defined originally by other than serological reactions.

46.3.2 Cross reactivity of antisera and "splits"

The phenomenon usually called cross reactivity (the term is not altogether accurate according to Albert and Götze, 1977) has been a major problem in fully understanding HLA serology. Cross reactivity was recognized by Svejgaard and Kissmeyer-Nielsen in 1968, and is a fairly general phenomenon among certain combinations of HLA antigens. When immunization experiments were done in which the donor differed from the recipient for only one known HLA antigen, it was observed that the resulting antiserum reacted not only with cells positive for the antigen that was different (and to which the antibodies were expected to be directed), but also with cells positive for other antigens. For example, an antiserum produced by immunizing an HLA-A2,A3,B8,B12 recipient with HLA-A2,A3,B7,B12 cells reacted with all HLA-B7 positive cells (as expected), but also reacted with cells positive for HLA-B27 or Bw22, even though the immunizing cells did not carry these antigens. Absorption of this antiserum with cells positive for HLA-B27 or Bw22, but negative for B7, removed all the activity. It was thought for a time that this phenomenon was due to overall biochemical similarity of the antigens, but the idea is contradicted by the existence of anti-HLA-B7 sera which cannot be absorbed by HLA-B27 or Bw22 cells. This kind of cross reactivity was found to be a generalized phenomenon among certain groups of antigens, e.g. HLA-A1, A3, A11, or HLA-A2, A28 at the HLA-A locus, and HLA-B7, B27, Bw22, or HLA-B5, Bw35, Bw15, B18 at the HLA-B locus. The patterns of cross reactivity were seen not only in antiserums resulting from planned immunizations, but in those obtained from unselected multiparous women as well. The groups of cross reacting antigens, it may be noted, always involve the products of the same locus. Titration experiments with antisera directed at a group of cross reacting antigens indicate that most of them have one "main" specificity that is relatively high titered in comparison with the activity toward the other specificities. This behavior has led to the subdivision of some antigenic specificities into what Bender (1979) called "subtypes". The terms "broad" and "narrow" specificity are also used in this context. Thus, HLA-Aw23 and Aw24 are regarded as "subtypes" of HLA-A9. Another way of putting it would be to say that A9 is the "broad" specificity, while Aw23 or Aw24 are "narrow" ones. Similarly, HLA-A25 and A26 are subtypes of HLA-A10. The divisions of HLA-A9 into Aw23 and Aw24, or of A10 into A25 and A26, are referred to in the literature as "splits", and there are quite a number of them known at HLA-A and HLA-B. The nomenclature rules allow for specification of the broad specificity in parentheses next to a narrow one, e.g. HLA-Aw23(9).

46.3.3 Formal genetics and chromosomal localization

Many population and family studies have been carried out on the HLA system (e.g. Svejgaard *et al.*, 1970; Mayr, 1971 and 1977; Colombani and Degos, 1972; Spielmann *et al.*, 1974; Greenacre and Degos, 1977). A child inherits an HLA *haplotype* from each parent. The haplotype is, essentially, the series of HLA genes on the chromosome that is inherited, and consists of an HLA-A allele, an HLA-B allele, and one allele each from the additional loci to be discussed below. The sum of the two inherited haplotypes is the individual's genotype, although it is not always possible to discern the genotype from the phenotype, since one phenotype may be dictated by a number of different genotypes. If the paternal chromosomes are designated P/P′ and the maternal ones M/M′, four types of children are possible: P/M, P/M′, P′/M and P′/M′, where P, P′, M and M′ represent haplotypes (or chromosomes). If the HLA-A, -B and -C loci are all considered, up to six different alleles could occur in a person, e.g. HLA-A2,A3,B12,Bw35,Cw4,

Cw5, representing the haplotypes HLA-A2, B12,Cw5 and HLA-A3,Bw35,Cw4. Haplotypes cannot be determined from phenotypes without doing family studies. Typing results often yield information about five, four or even fewer alleles, and it is not possible to distinguish between homozygosity for an allele, and the presence of a serologically undefined allele (the latter often called a "blank"). In a case, for example, of an HLA-A2,A9,B12 phenotype, the genotype could be A9,B12/A2,B12 or A9,B12/A2,BX or A9,BX/A2,B12, where BX represents a serologically undefined allele at HLA-B. The probable genotype of the person could be calculated on the basis of haplotype frequencies. The situation is reminiscent of Rh system genetics (section 22). Suppose the HLA-A2,A9,B12 person in the example above were a mother with three children, all of whom had inherited an HLA-A9,B12 haplotype; this result would show that she had an HLA-A9,B12 chromosome, but it would not distinguish between the genotypes HLA-A9,B12/A2,B12 and HLA-A9, B12/A2,BX. In surveying populations, and even families, therefore, statistical methods must often be used to estimate genotypic and haplotype frequencies. There are fairly complex methods for estimating gene frequencies (e.g. Yasuda and Kimura, 1968) as well as comparatively simpler ones (e.g. in Albert and Götze, 1977; Bender, 1979). All the methods give generally similar results.

As with other blood group, enzyme and serum group polymorphisms, HLA gene frequencies are very differently distributed in different racial and ethnic groups. Summaries may be found in Albert and Götze (1977) and Bender (1979). Among the most frequent HLA-A alleles in European Caucasians are HLA-A2, A1, A3 and A9; in African Blacks, Aw30 is frequent along with A2 and A9, while in Japanese, A9 is very frequent with A2 and A11 somewhat less so. Similar differences are seen in the alleles at other loci. The most common Caucasian haplotypes are HLA-A1,B8, A2,B12 and A3,B7.

Many of the HLA haplotypes occur much more frequently than would be expected on the basis of the gene frequencies for the alleles involved (e.g. Hiller *et al.*, 1978). In the case of two loci like HLA-A and HLA-B, linked but separable by recombination, it is expected that large numbers of recombinations between the loci will eventually bring the alleles into random association with each other, i.e., into "linkage equilibrium". The situation that is actually found in some haplotypes, where associations between certain alleles differ significantly from what would be expected on the basis of random association, is called "linkage disequilibrium". Linkage disequilibrium can be found at any pair of linked loci. Some alleles at the closely linked loci determining salivary polymorphic proteins (see in section 47) exhibit this phenomenon, for example. Details of linkage disequilibrium analysis may be found in Cavalli-Sforza and Bodmer (1971). The reasons for the disequilibria are unknown, and a number of different explanations are possible. The recombination frequency between HLA-A and HLA-B is sufficiently low (Svejgaard *et al.*, 1971; Belvedere *et al.*, 1975; Bijnen *et al.*, 1976), however, (about 0.8%) that once linkage disequilibrium is established, it will take many generations to reestablish equilibrium again.

Pious and Soderland (1977) estimated the mutational rate for the HLA-B locus, and said that their data were consistent with most of the variants having arisen by mutational events.

In 1970, Lamm *et al.* found a hint of linkage between PGM_3 and *HLA*. Somatic cell hybrid studies then enabled Jongsma *et al.* (1973) to assign PGM_3 to chromosome 6. *HLA*, PGM_3 and *SOD-B* were then shown to be syntenic by further somatic cell hybrid studies (van Someren *et al.*, 1974). Mayr *et al.* (1975) confirmed the PGM_3:*HLA* linkage. A peculiar inversion allowed the absolute assignment of *HLA* to chromosome 6 by Lamm *et al.* (1974). Thus, the human MHC, which includes the HLA loci, is on chromosome 6, and is linked to PGM_3 as well as to *GLO* and *Bf* (Weitkamp, 1976) and to a number of the polymorphic complement component loci (section 45.2). Albert and Götze (1977) said that the likely order for all the loci clustered on the short arm of chromosome 6 is PGM_3. . . . . GLO. . . . C2. .HLA-D. .Bf. .HLA-B. .HLA-C. . . HLA-A, with C4 somewhere near Bf or HLA-B.

46.3.4 The HLA-C locus

Sandberg *et al.* (1970) found an antiserum which detected an antigen that would not fit into the two locus model. A third locus, called "AJ", was postulated and was soon proven to exist (Solheim and Thorsby, 1973; Mayr *et al.*, 1973). It has also been called the third serologically defined locus, or "SD3", but is now known as *HLA-C*. The HLA-C locus is closely linked to HLA-A and -B, and crossovers between HLA-A and -C alleles and between HLA-B and -C alleles have been reported (Löw *et al.*, 1974; Hansen *et al.*, 1975; Bijnen *et al.*, 1976; Waltz and Rose, 1977).

The third locus antigens have been studied in part by a special technique called "capping" (Taylor *et al.*, 1971). A number of cell surface proteins, including HLA antigens, can, after binding to specific antibodies in the absence of complement, redistribute to form aggregates on the cell surface. They may then form a "cap" at the cell pole (capping). The aggregated surface proteins may then be taken up by the cell, or shed to the medium. Lymphocytes treated in this way show a temporary loss of the antigen involved. The finding by Bernoco *et al.* (1972) that HLA specificities can be individually capped provided a clever method for the study and characterization of these antigens.

At present, there are 8 alleles at *HLA-C* (WHO-IUIS Nomenclature Committee, 1980). They are designated HLA-Cw1 through Cw8, and the first five are considered fully established specificities. Because of the close association of the complement component polymorphic loci *C2* and *C4* with the HLA locus region, and the possibility of resulting ambiguity and confusion in the nomenclature between the alleles of *C2*, *C4* and *HLA-C*, the committee has recommended retaining the "w" designations for all

HLA-C alleles for the time being. Of the antigens defined thus far, HLA-Cw3 and Cw4 are the most frequently occurring in Europeans.

46.4 Lymphocyte Defined Antigens—A Further HLA Locus

As has been noted, the HLA-A, -B and -C locus alleles and antigens were defined by serological reactions, primarily by the lymphocytotoxicity test (discussed further below) using antibodies directed at the specificities. The antigens coded for by these alleles have thus been referred to as "serologically defined" ("SD").

Another group of antigens belonging to HLA, but detected by different methods, has also been found. These were detected by cellular reactions involving lymphocytes, and are referred to as "lymphocyte defined" ("LD"). The distinction may soon be mainly methodological, since it appears that the "LD" antigens can be detected serologically now.

When lymphocyte suspensions from unrelated individuals are mixed and incubated in culture, the cells may enlarge and transform into dividing blast cells (Hirschhorn *et al.*, 1963; Bain *et al.*, 1964). The dividing cells must replicate their DNA, and the lymphocyte stimulation reaction can thus be followed by monitoring the incorporation of radiolabelled DNA precursors (e.g. ^{3}H-thymidine). One of the two cell populations can be inactivated by treatment with radiation or mytomycin so that they cannot divide and proliferate, but can still stimulate the untreated cells in the culture to do so. This finding provided the basis for a one-way or unidirectional stimulation test (Bach and Voynow, 1966). A two-way mixed lymphocyte culture could, therefore, be analyzed as the sum of the two one-way reactions. The rationale for mixed lymphocyte culture studies was the finding (Bach and Hirschhorn, 1964) that the degree of blast transformation observed in the mixed culture was related to the degree of incompatibility for transplantation antigens, and an *in vitro* measure of transplantation incompatibility was clearly desirable. The test was developed (Bach *et al.*, 1967) into what is now called the "mixed lymphocyte culture" ("MLC"). Absence of stimulation in this procedure is interpreted to mean identity of the "MLC" antigens in the two kinds of cells. At first, MLC was thought to be just an alternative way of typing HLA antigens, because Bach and Amos (1967) observed that the lymphocytes of HLA-identical siblings did not stimulate each other in MLC, while those of HLA-nonidentical siblings did so. It was soon found, however, that the cells of unrelated HLA-identical individuals stimulated each other in MLC (Kissmeyer-Nielsen *et al.*, 1970; Schellekens and Eijsvoogel, 1970; Schellekens *et al.*, 1970; and others). These observations could be understood in two different ways: (1) that the HLA antigens were still "heterogeneous", i.e., that available typing serums did not allow for complete type determination; or (2) that the stimulation in MLC was not being brought about by HLA antigens as such, but by products of one or more "MLC" genes at a locus closely linked to the known HLA loci. Families were soon found (Amos and Yunis, 1971; Yunis and Amos, 1971; Mempel *et al.*, 1972) in whose members the HLA types and MLC behavior provided convincing evidence for a separate "MLC" locus, closely linked to HLA-B.

Recognition that the MLC tests were detecting antigens coded for by a separate locus led to efforts to devise methods for identifying different individual allelic products. MLC stimulation is a measure of difference, but not of the exact type of difference, between the antigens of the cell populations. Individuals can be selected, however, who are homozygous for certain MLC alleles. Their cells, homozygous typing cells (HTC), do not provoke stimulation in one-way MLC if the responding cells carry the same MLC allele. If a difference exists, however, a stimulation reaction is seen. The HTC technique was developed by Mempel *et al.* (1973), Dupont *et al.* (1973), Jørgensen *et al.* (1973) and van den Tweel *et al.* (1973). Another procedure for typing MLC determinants was developed by Sheehy *et al.* (1975) and Sheehy and Bach (1976), among others, and is called "primed lymphocyte typing" (PLT). This method is based on the observation that stimulated T lymphocytes kept in culture for a matter of days will give a strong secondary (or "second set") stimulation response when stimulated by cells possessing the same MLC determinant as the stimulating cells in the original culture ("priming" cells).

Following the Aarhus workshop (Histocompatibility Testing, 1975), the nomenclature committee designated the "MLC" determinant locus as "HLA-D" (WHO-IUIS Terminology Committee, 1976). The locus had previously been designated variously as "MLR-S1", "LD-1" and "MLC-1". Six specificities were provisionally recognized at that time (Dw1 through Dw6). There are presently 12 HLA-Dw specificities (WHO-IUIS Nomenclature Committee, 1980). Further information on cellular typing may be found in the reviews by Albert and Götze (1977) and Bender (1979), and in Bach *et al.* (1977), van Rood *et al.* (1977) and Bradley and Festenstein (1978).

It was found by Ceppellini *et al.* (1971) that the MLC reaction can be inhibited by certain HLA antisera, and this observation provided the beginning of a serological definition of the B-cell alloantigens. The antibodies in the HLA antisera causing the inhibition are not HLA antibodies, because they cannot be absorbed by appropriate platelets. They can be absorbed out by appropriate leucocytes, however, and eluates from these leucocytes are also active (Revillard *et al.*, 1973; van Leeuwen *et al.*, 1973). These antibodies have been better characterized by van Leeuwen *et al.* (1973), and are detecting serologically antigens on B lymphocytes that are identical or very similar to the "MLC" determinants. These antigens are also referred to as "Ia" antigens, by analogy to the findings in the mouse. Sera containing strong HLA antibodies are often found to contain strong Ia-antibodies as well (Jones *et al.*, 1975). The complex subject of human Ia antigens has been reviewed by Wernet (1976), Barnstable *et al.* (1977), Bodmer (1978b) and Winchester and Kunkel (1979). It is not yet completely clear

whether the same polymorphic antigens are being detected by the cellular (HTC and PLT) techniques, and the Ia-serological tests. The WHO-IUIS Nomenclature Committee (1978) has assigned the symbol "DR" (for "D-related") to the HLA-D antigens detected serologically. The HLA-DR designations correspond to the relevant HLA-D designation. At present, there are six fully recognized HLA-DR specificities, and an additional four provisional ones (WHO-IUIS Nomenclature Committee, 1980).

46.5 HLA Testing and Typing Procedures

Methods for HLA typing are unlike any of the methods used for the typing of blood or serum groups or polymorphic enzymes. HLA antigens are routinely typed on leucocytes, and HLA typing generally tends to be done in a relatively few specialized laboratories. Typing of HLA-A, -B and -C antigens is carried out fairly universally using some variation of the complement mediated lymphocytotoxicity test on peripheral blood lymphocytes. Special procedures which were briefly discussed in section 46.4 are used for the definition of HLA-D and -DR specificities.

The microdroplet lymphocyte cytotoxicity test introduced by Teresaki and McClelland in 1964 has become universally accepted as the method of choice for HLA antigen typing. The principle of the test lies in the fact that lymphocytes containing a particular antigen will, after reaction with specific antibody to that antigen followed by the addition of complement, exhibit a cytotoxic reaction detectable by the penetration of the cell by an added dye. Test results are evaluated microscopically. If the lymphocytes being tested lack the antigen to which the antiserum is directed, there is no cytotoxic reaction, and the added dye is not taken up by the cells. This procedure can be employed using very modest amounts ($\mu\ell$) of cells, antisera, and complement (rabbit serum is usually the complement source). The procedure has been refined and modified over the years (Mittal *et al.*, 1968; Teresaki *et al.*, 1978a). A detailed review of the developments will be found in Teresaki's Philip Levine Award lecture (Teresaki *et al.*, 1978a).

There are a number of technical problems associated with HLA typing that must be appreciated if correct results are to be obtained. These problems are discussed by Teresaki *et al.* (1978a) and by Joysey and Wolf (1978). In addition, the complexities and idiosynchrasies of HLA system antigens must be taken into consideration. Skill and experience in lymphocyte typing, and the use of carefully and thoroughly evaluated reagents, are both essential.

Efforts to standardize the lymphocytotoxicity tests used for routine HLA typing have been quite successful, although there are some variations in procedure in different laboratories (Teresaki *et al.*, 1978a; Ray, 1979). Small differences in technique can significantly affect the sensitivity of the test, however (Joysey and Wolf, 1978).

The biggest problems in HLA serology probably have to do with the antisera. Antisera must be procured, and then exhaustively evaluated in terms of specificity and cross reactivity using large panels of highly selected cells of well defined specificity. The well known cross reactivity of HLA antisera, and the fact that many serums contain a number of different antibody specificities, can cause serious difficulties of interpretation in inexperienced hands. As one example, the cross reactivity of HLA-B7 and HLA-B27 can lead to typing errors (Joysey and Wolf, 1978; Larsen, 1979). A tissue type cannot be reliably diagnosed until it has been confirmed by a battery of well authenticated antisera. Likewise, antisera must be thoroughly evaluated against large panels of cells of selected known specificity. Panels for evaluating most antisera can be smaller in number if they are highly selected (Kissmeyer-Nielsen and Kjerbye, 1972).

Since around 1965, the NIH [National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services] Serum Bank has been a major coordinating center for the exchange of HLA antisera. Catalogs of these reagents are produced periodically, and the sera are available to qualified investigators (see in Amos and Ward, 1975). Many exchanges of serums and cells take place among specialized HLA testing laboratories as well. These exchanges, and the fairly frequent international workshops, have helped considerably in the standardization of typing reagents, techniques, and nomenclature. Because of the relative scarcity of thoroughly evaluated reagents, and the complexities associated with accurate HLA typing, this work will probably continue to be restricted to a relatively small number of specialized laboratories. It may be noted that the microdroplet lymphocyte cytotoxicity test is now applicable to the determination of some HLA-D specificities as well (Teresaki *et al.*, 1978a).

46.6 Biochemical Studies on HLA Antigens

The HLA antigens are membrane-associated, and the usual problems connected with efforts to dissociate and study membrane bound proteins have faced biochemists engaged in HLA antigen characterization. Solubilization and isolation of these molecules has been carried out using a variety of techniques and extraction media, including mechanical disruption, hypotonic lysis, freezing-thawing, various salt or proteolytic enzyme (especially papain) treatments, and detergent treatments. The development of continuous lymphoid cell lines derived from normal donors has contributed to the biochemical studies by providing relatively large amounts of genetically uniform cellular material. Reviews of the biochemical work may be found in Albert and Götze (1977), Albert and Scholz (1978) and Barnstable *et al.* (1978).

There are conflicting reports in the literature about the carbohydrate content of purified HLA antigens. Some of the solubilization techniques apparently yield preparations which lack detectable carbohydrate. The consensus appears to be that there is some carbohydrate associated with the native antigens, but its exact composition and the mode of its attachment to the protein moiety have yet to be established.

Isolated HLA antigen molecules have a general structure

consisting of one heavy and one light chain. The chains are not covalently bound to one another. The heavy chain is the one coded for by the HLA locus gene, and contains the alloantigenic portion of the molecule. The MW of the heavy chain varies from 34,000 to 55,000 depending on the method used to solubilize the antigen. Strominger *et al.* (1977) presented a model of the native structure of HLA-A and -B antigens based on studies in cultured lymphocytes. The N-terminal end of the heavy chain is the one that protrudes to the outside of the cell membrane. This protruding part of the chain appears to represent a large portion of the whole molecule. A hydrophobic portion of the heavy chain was postulated to occupy the membrane itself, with a comparative short portion of the chain (the C-terminal end) protruding to the inside of the cell membrane. There are two intrachain disulfide bridges in the heavy chain. Amino acid composition studies on a number of solubilized HLA antigens from various HLA-A and -B types indicate considerable similarity. It appears that the amino acid residue at position 9 is locus-specific (Phe in the HLA-A products; Tyr in the HLA-B ones), but the nature of the differences giving rise to the alloantigenic specificities have not yet been clarified. Most of the data suggests that the carbohydrate moiety is not involved in determining antigenic structure or specificity.

The light chain (MW 11,600) of the HLA-A and -B molecules is associated with the N-terminal end of the heavy chain, according to the structure proposed by Strominger *et al.* (1977). The light chain is apparently not involved in determining alloantigenic specificity, and the light chain has been identified as β_2-microglobulin. β_2 microglobulin is found in small amounts in various body fluids and was first isolated and purified by Berggård and Bearn (1968). It has 100 amino acid residues and a single disulfide bridge formed by Cys residues at positions 25 and 81. There is no carbohydrate in the purified preparation. There is some sequence homology of β_2-microglobulin with the constant region of the IgG heavy chain (Smithies and Poulik, 1972). β_2-microglobulin synthesis is controlled by a gene on chromosome 15 (Goodfellow *et al.*, 1975; Zeuthen *et al.*, 1977), and its structural gene is completely separate from the HLA complex locus, therefore.

Although there are fewer detailed studies on the HLA-C antigens than on the -A or -B molecules, the data indicate that at least the general structure is very similar.

Berg (1971b) reported the curious observation that there are amino acid compositional similarities between HLA antigens, the Lp(a) protein and the LDL of serum (section 45.1). Charlton and Zmijewski (1970) found that HLA-A7 people had a soluble HLA-A7 in their plasma which occurred as a low density lipoprotein. Orr *et al.* (1979) recently noted that an 88 amino acid residue fragment of the HLA-B7 heavy chain showed significant structural homology with the constant domains of immunoglobulins.

The structure of the HLA-DR antigens (Ia-antigens) is somewhat different from that of the HLA-A, -B and -C molecules. The data thus far indicate that the DR antigens resemble mouse and guinea pig Ia antigens. Snary *et al.* (1977) have presented a model for the structure of these molecules with which the data of Klareskog *et al.* (1977) appear to be consistent. The molecule is a glycoprotein consisting of two polypeptide chains of MW about 33,000 and 28,000. The chains are not covalently linked, and both of them have associated carbohydrate. The 33,000 peptide is suggested to be the one coded for by the HLA-DR genes, and accordingly, the one containing the allotypic site. Synthesis of the 28,000 peptide might well be controlled at a completely different locus.

46.7 Medicolegal Applications

46.7.1 Disputed parentage

The use of the HLA polymorphism in disputed parentage represents its only significant medicolegal application at the present time. The extraordinary extent of the polymorphism and its widespread distribution in populations make HLA the most singularly powerful system for parentage investigations. This fact may be appreciated by examining Table 18.1. The probability of exclusion of a falsely accused father using HLA typing is very high, and values ranging from about 80% to over 90% are quoted by various authors. The PE is dependent, of course, on how many specificities can be typed, and on the distribution of haplotypes in the population in question. The system is so powerful in making distinctions in the population because of the large number of haplotypes and their widespread distribution in the population. With 17 alleles at HLA-A and 28 at HLA-B, Bender (1979) said that 17 homozygous and 136 heterozygous A types, and 28 homozygous and 378 heterozygous B types were possible. Since the antigens are inherited as haplotypes, it must be considered that there are then 476 different A,B haplotypes which allow for about 113,500 combinations! The most frequent phenotype occurs in only about one of 100 persons. Bender (1979) also said that at least two-thirds of the German population have two different A and two different B alleles (a "full house" in laboratory jargon).

Application of HLA typing to parentage cases has been practiced for some years, and application of HLA to paternity cases is discussed by Mayr (1971 and 1974), Soulier *et al.* (1974), Spielmann and Seidel (1974), Spielmann *et al.* (1974), Speiser (1975) and Miller, in AABB (1978). Calculations of PE were discussed by Mayr and Pausch (1975), and the principles underlying calculation of the probability of paternity where no exclusion is obtained were given by Mayr (1972). Hummel and Conradt (1978) noted that very low probability of paternity values could be obtained in some cases, even when the PE value for exclusion of a falsely accused father was high, and they tended to take the view that the probability of paternity calculation was the only meaningful one in a given case.

In this country, Dr. Teresaki has been a leader in the application of HLA typing in paternity cases (Teresaki, 1978; Teresaki *et al.*, 1978b). If ABO and Hp are typed in addition

to HLA, probabilities of paternity are at least 95% in the non-exclusion cases. In a number of states, blood grouping evidence is not admissable unless it is exculpatory (i.e. results in excluding the putative father), but this situation is changing somewhat as members of the legal community become more aware of the present status of blood testing (e.g. Seider, 1980). Lee (1975) surveyed 24 countries, and found that HLA typing was used in paternity cases in just under half of them. In this country, it is one of the seven systems recommended by the joint AMA–ABA Committee (Joint AMA–ABA Guidelines, 1976), along with six blood group systems (ABO, Rh, MNSs, Kell, Duffy and Kidd). For some reason, this committee chose to overlook isoenzyme and serum group systems altogether. Polesky and Krause (1977) found in their survey of American laboratories that about 17% of AABB referene laboratories could do HLA testing, but less than 2% of other laboratories surveyed had this capability.

The applicability of HLA typing in some uncommon situations has been described. Speiser *et al.* (1974) discussed the situation in which the putative father could not be tested. Teresaki (1978) had a case in which the mother could not be tested. In both situations, exclusions could be obtained. Two very rare cases of so-called superfecundation have recently been diagnosed by HLA typing. In both, fraternal twins were shown to have different biological fathers (Teresaki *et al.*, 1978c; Bertrams and Preuss, 1980).

46.7.2 HLA Antigen typing in dried blood

Only a few studies have been carried out on bloodstain typing. Rittner and Waiyawuth (1974) obtained a high percentage of correct results using an inhibition of lymphocytotoxicity test. They then (1975) compared microlymphocytotoxicity and microcomplement fixation procedures on bloodstain material, and concluded that the latter would be better suited. A number of specificities could be reliably detected, but relatively large amounts of dried blood were used in the tests. Newall (1979) evaluated two different versions of microlymphocytotoxicity inhibition for the typing of HLA-A2 and B5 in dried bloodstains of varying age. The two-stage extraction-absorption test was not found to be very satisfactory. Better, more reliable results were seen with a one-stage procedure. Antisera varied in their ability to give unequivocal typing results, and stains from different persons that contained the same antigen gave differing degrees of inhibition, especially with older stains. Stains up to 10 weeks old could be typed correctly, however, if three different antisera were used in each test. Hodge *et al.* (1979 and 1980) recently investigated HLA-A1 typing in 4 cm long bloodstained threads using inhibition of microlymphocytotoxicity procedures. Three antisera were used in parallel. Most stains could be typed correctly even in stains over 100 days old. Still, a few false positive and negative typings were observed in the samples. The well known HLA-A1-A11 cross reaction could be seen in HLA-A2,A11 stains with one of the anti-HLA-A1 sera known to be cross reactive with HLA-A11. While these initial results are encouraging, more groundwork remains to be done before this powerful polymorphic system can be exploited in bloodstain typing.

Hillman and Shaler (1981) have recently explored procedures for the recovery of leucocytes from dried bloodstains. Using special extraction techniques, relatively high recoveries are possible. The leucocytes thus isolated from bloodstains might enable HLA typing to be carried out much more readily than is possible with whole bloodstains. Such leucocytes can be used for cytologic sexing studies (section 48), and for the determination of genetic markers that are expressed in white cells but not in red cells.

It may eventually be of medicolegal interest that HLA antigens appear to be present in semen (Singal *et al.*, 1971; Singal and Berry, 1972) and on sperm cells (Halim and Festenstein, 1975; Festenstein *et al.*, 1977).

SECTION 47. POLYMORPHIC PROTEINS OF HUMAN SALIVA

47.1 Introduction

Saliva contains a variety of proteins and enzymes, many of which it shares with other body fluids and tissues. Some of the antigens, enzyme and serum group system proteins that exhibit genetic polymorphism and occur in saliva as well as in other tissues and fluids have been discussed elsewhere in this book. Soluble ABH and Lewis blood group substances (sections 19.8.1, 19.8.3.1 and 20.4) are found in saliva as well as in many other body fluids and tissues. Likewise, the antigen Sd^a (25.8) can be found in saliva. Some immunological studies which included identification of some of the proteins of saliva were discussed in section 11.4. Saliva contains a few proteins which appear to exhibit inherited variation (presence or absence) that have been detected by their reactions with various lectins. One of these, which was called "L", was detected by its reaction with a lectin from *Lotus tetragonolobus* (section 19.8.3.1).

In 1964, Niswander *et al.* detected a component in saliva by immunoelectrophoresis and reaction with a rabbit antiserum raised against human parotid saliva. It was called "SC_1" (for "salivary component" migrating most anodally by immunoelectrophoresis at pH 8.6). The component migrated in the "albumin" position, but was not albumin, nor was it identical to or associated with $ABHLe^a$. It did not occur in serum. It was inherited, but appeared to be subject to post-translational modification, and the inheritance pattern was not fully clear. The relationship of SC_1 to the proteins discussed in section 47.2 is not clear. Two more saliva antigens were detected by Balding and Gold (1973). They were studying bacterial hemagglutinins from *Clostridium botulinum* types C and D. It was found that some but not all salivas from different people would inhibit these reactions. The antigen inhibiting the type C agglutinin was called CbC, and that inhibiting type D activity was called CbD. The characteristic was inherited, and the responsible genes, called Sal^{CbC} and Sal^{CbD}, respectively, had frequencies of about 0.73 and 0.27 in a Caucasian population. In 1975, Wiener and Moon described an antigen called "Cl" on red cells and in saliva. The antigen was defined by a lectin prepared by absorbing extracts of the seeds of *Clerodendron trichotomum* Thumberg (from Korea) with selected group O red cells, and was said to be associated with the ABHLe macromolecule.

Saliva contains a number of enzymes, the most important of which for forensic purposes is probably amylase (at least at the present time). The use of amylase as an identification marker was discussed in section 11.3. The polymorphism of the salivary amylase (AMY_1), as well as that of pancreatic amylase (AMY_2) was discussed in section 37.3. The use of salivary alkaline phosphatase as an identification marker was covered in section 11.2. Pini Prato (1970) noted two alkaline phosphatases in human saliva following polyacrylamide gel electrophoresis. The relationship of these isozymes to the other human alkaline phosphatases (section 37.2) is not yet clear. Detection of the PGM_1 isozymes in the cellular fractions of saliva has been reported (section 27.4.3), although PGM should probably not be regarded as a salivary enzyme as such. The immunoglobulin markers of the Gm and Km system can be detected in saliva, and this matter was covered in section 44.6.3.

Several systems of salivary proteins and enzymes, which have not yet been discussed, exhibit genetically controlled variation by electrophoresis or isoelectric focusing. The rest of section 47 is devoted to a discussion of these systems. Most of the work is comparatively recent, and only a handful of laboratories appear to be engaged in these studies. The most comprehensive review of this material was given by Azen (1978). I am indebted to his review in helping to organize some of the material in this section. A useful and comprehensive review of methods employed in studying salivary polymorphisms will be found in Tan and Teng (1979).

47.2 Polymorphic Salivary Protein Systems

47.2.1 Acidic protein (Pa system)

Polymorphism in an acidic protein in saliva (as against the basic ones of the Pb system discussed below) was described by Friedman *et al.* (1972), and studied further by Friedman *et al.* (1975). The Pa protein, which is either present [Pa(+)] or absent [Pa(−)] was detected using acid urea starch gel electrophoresis, and a stain designed to detect argninine-rich proteins. Other electrophoretic systems were used to study Pa further, and to compare it with other salivary proteins. Pa can be typed in submandibular or parotid saliva, but something like half the whole saliva samples were found to be unsuitable for typing, even if frozen immediately after collection in preparation for lyophilization. Saliva preparations are usually frozen, and lyophilized, and the lyophilizate later reconstituted for electrophoretic study (Friedman *et al.*, 1975). The locus controlling the Pa protein was designated *Pa*, and the protein was clearly inherited as an autosomal dominant as indicated by studies on a number of families. Friedman *et al.* (1975) call the phenotypes Pa(+) and Pa(−) and the genes Pa^+ and Pa^-. Azen (1978) calls the phenotypes Pa 1 and Pa 0, and the genes Pa^1 and Pa^0, respectively (because there is now a "Pa^2"). Pa(−) × Pa(−) matings did not give rise to any Pa(+) children. Gene frequencies for Pa^- (Pa^1) were approximately 0.21, 0.14 and 0.42 for American Whites,

American Blacks and Orientals, respectively. In 1977, Azen described a rare variant of Pa, called Pa 2. Pa 2 is a separate protein which is seen on the typing gels, and was accounted for by a rare allele Pa^2. The finding of the Pa^2 accounts for the change in nomenclature noted above.

Friedman and Merritt (1975) partially purified the Pa (Pa 1) protein, and found that it was rich in Pro, Glu and Gly, low in Thr, and that it had no Tyr or Cys. The pI was 3.9–4.5. Azen and Denniston (1974) and Azen (1977) found, however, that Pa 1 was a disulfide-bonded dimer, using disulfide bond splitting agents and ^{14}C-iodoacetamide to identify the products. Friedman and Merritt (1975) mentioned that there were some associated acidic proteins in saliva, called "Pa II", to distinguish them from "Pa I", the polymorphic Pa.

47.2.2. The Pr and Db systems and their relation to Pa

The Pr system polymorphism was originally described by Azen and Oppenheim (1973). The proteins exhibiting this polymorphism had previously been characterized to some extent by Oppenheim *et al.* (1971). Four proteins made up the system, and were detected by alkaline slab polyacrylamide gel electrophoresis using parotid saliva which had been concentrated 5–10 fold by lyophilization and reconstitution in gel buffer. The proteins are visualized by means of acidified 3,3′-dimethoxybenzidine. The proteins were negatively stained, i.e., the background changed color while the protein zones did not. More contrast could be obtained if a second staining step was done with a peroxide solution. Apparently, some of the DMB precipitated in the gel in the first step, everywhere except at the protein zones, and could then react with the peroxide in the second step to give a brown background. The basis for this unusual staining reaction is not known (Azen and Oppenheim, 1973; Azen, 1978). Three patterns could be distinguished, and family studies showed that these could be accounted for by two alleles, called Pr^1 and Pr^2. The Pr^1 gene was responsible for the protein bands called Pr 1 and 3 (originally I and III), while Pr^2 was responsible for Pr 2 and 4 (originally II an IV). Frequencies of the Pr^1 allele were about 0.73 in Whites, 0.8 in Blacks and 0.84 in Chinese.

The Pr proteins are especially rich in Gly, Glu and Pro and have pI in the range of 4.09–4.71. Pr 1 has a MW of 12,300 and Pr 3 of 6,100 by sedimentation ultracentrifugation (Oppenheim *et al.*, 1971). Immunological evidence suggests that the Pr proteins separable by isoelectric focusing are very similar if not identical in antigenic structure (Friedman and Karn, 1977).

Azen and Denniston (1974) studied the Pr proteins more closely, and found that two additional proteins at the so-called "X" position (slightly slower than but close to Pr 1) could be distinguished by changing the pH or gel concentration for electrophoresis. One of these proteins had a disulfide bond, and is now known to be identical to Pa 1 (Azen, 1977 and 1978). Some people have the "X" proteins while others do not. One of the X proteins is usually found associated with Pr^2 products, and only rarely with Pr^1 products. The other X protein is always associated with a product of Pr^1. These findings led Azen and Denniston (1974) to suggest that there were at least two additional alleles at the *Pr* locus, called $Pr^{1'}$ and $Pr^{2'}$. The data also suggested that the gene locus responsible for the Pa 1 protein, *Pa*, and the *Pr* locus genes have undergone occasional recombination. In addition, all Pr 2′ individuals are Pa 1, and most Pr 1′ people are Pa 0, but an occasional Pr 1′ individual is Pa 1. This suggests a significant linkage disequilibrium between *Pr* and *Pa*. There is now no doubt about the close linkage between the two loci (Yu *et al.*, 1978).

Azen and Denniston (1974) found another pair of polymorphic proteins, called "Db" (for "double band"). These, like Pr, were detected by negative staining with dimethoxybenzidine after electrophoresis on polyacrylamide gels. The double bands, one of which is between Pr 2 and Pr 3 and the other of which runs behind (cathodal to) "X", are either present or absent. The phenotypes are denoted Db+ and Db−. The locus controlling the variation is called *Db*, and the gene determining Db+ is dominant. Approximately 12% of Whites, 56% of Blacks and 7% of Chinese were Db+. *Db* is very closely linked to *Pr* (Azen and Denniston, 1974; Friedman *et al.*, 1975; Azen, 1977).

Extensive studies on the linkage relations of *Pa*, *Pr* and *Db* have been carried out by Azen and Denniston (1974), Friedman *et al.* (1975), and Yu *et al.* (1978 and 1980a). The evidence suggests that there are three separate, but closely linked, loci, *Pr* and *Pa* being closer than *Pr* and *Db*. Definite recombinations have been seen in the family material of Yu *et al.* (1978), thereby proving the existence of separate loci. Azen (1978) said that the *Pr* locus had three definite alleles, Pr^1, $Pr^{1'}$ and Pr^2, that the *Pa* locus had Pa^0, Pa^1 and Pa^2, and that *Db* had Db^+ and Db^-. A reasonable (but not yet proven) order for the loci is *Pa. . .Pr. . .Db* (Yu *et al.*, 1980a).

47.2.3 Basic protein (Pb)

Polymorphism in basic proteins of parotid saliva was detected by Azen in 1972, using acid urea starch gel electrophoresis and staining for argining-rich proteins. Three patterns are seen, and called 1, 2-1 and 2. The proteins show asymmetric patterns in the homozygous types, and there is variation in band intensity in different samples of the same type. Pb 1 homozygotes show four bands, called "a", "b", "d" and "e", while Pb 2 homozygotes show only a "c" band. The letters represent the order of the bands on the plate. The patterns can be explained by a pair of codominant alleles, Pb^1 and Pb^2, at an autosomal locus (Azen, 1973). Pb^1 frequency is about 0.84 in Blacks, but over 0.99 in Whites; the Pb system is thus polymorphic principally among Blacks.

The Pb proteins have been purified and partially characterized (Peters and Azen, 1977). The MW ranged from 5,800 to 7,200, and the proteins are extremely basic (pI > 9.5). Approximately 45% of the amino acid residues are the basic His, Lys and Arg ones. The proteins contained no carbohydrate, and lacked Cys, Pro, Thr, Val, Met and Try.

There is evidence from the biochemical and amino acid composition and sequence studies (Peters and Azen, 1977) that the polymorphism is due to a combination of allelic differences and post-translational modifications involving proteolysis and deamidation. There is probably a single amino acid difference (Glu vs Gln) in the primary gene products. This model helps to explain the peculiar asymmetric banding patterns in the homozygous types. Peters *et al.* (1977) found a larger MW protein in parotid saliva, called "post-Pb protein", which most closely resembled the Pb "e" band protein in MW. It was immunologically cross-reactive with an antiserum raised against the Pb proteins, and was first thought to be a possible precursor of the Pb proteins. It is now believed that this is not the case, however (Azen, 1978), and this protein is probably determined at a different genetic locus.

Despite a considerable amount of effort by the Wisconsin group, the Pb proteins have not been assigned a physiological function thus far.

47.2.4 Other polymorphic salivary proteins

47.2.4.1 The major parotid salivary glycoprotein (Gl). In addition to the acidic proline-rich proteins which have been shown to exhibit genetic polymorphism (Pa, Pr and Db systems—see above), parotid saliva contains various basic proline-rich proteins. Unlike their acidic counterparts, many of them are glycosylated. Earlier studies (e.g. Friedman *et al.*, 1971) have shown that they are heterogeneous, differing in size, charge and MW, and there was speculation that this variability might have a coherent genetic basis. In 1979, Azen *et al.* carried out extensive studies on the major glycosylated protein in parotid saliva, and showed that it exhibits discrete, genetically controlled molecular variants.

Parotid saliva was extensively studied using a polyacrylamide gel electrophoretic system and acidic (pH 2.4) buffers. Fairly high gel concentrations were required (routinely 9% and sometimes 13.5%). Thick (6 mm) gels were employed, and sliced prior to staining for carbohydrate. The optimal resolution of the bands was obtained with samples that had been subjected to neuraminidase treatment. Other staining overlays served to distinguish the proteins of the Pa, Pr and Db systems from those under study here.

Eleven phenotypes of the major glycosylated proteins were observed, five of which appeared to represent homozygosity at the controlling locus. The system was termed "Gl", and the homozygous phenotypes Gl 1, 2, 3, 4 and 0. In addition, heterozygous phenotypes 1-2, 1-3, 1-4, 2-3, 2-4, and 3-4 were seen. There are four band positions in the system used, and they represent (in order from origin to cathode, in the direction of the run) the 4, 1, 2, and 3 bands. Gl 0 has no bands. Five codominant alleles at an autosomal locus account for the phenotypes, and they were called Gl^1, Gl^2, Gl^3, Gl^4 and Gl^0. Gene frequences seen in 143 Whites were $Gl^1 = 0.742$, $Gl^2 = 0.04$, $Gl^3 = 0.155$, $Gl^4 = 0.017$ and $Gl^0 = 0.046$, while in 82 Blacks they were $Gl^1 = 0.459$, $Gl^2 = 0.05$, $Gl^3 = 0.337$, $Gl^4 = 0.044$ and $Gl^0 = 0.11$. Homozygous types could not be distinguished from the heterozygous condition in which one of the alleles was Gl^0 (e.g. Gl^1Gl^1 and Gl^1Gl^0 people type as Gl 1, and so forth). Studies on 41 families with 146 children were completely consistent with the postulated mode of inheritance. Studies on informative families strongly suggested linkage of *Gl* with *Pr* and *Db*, thus placing it in the *Pa–Pr–Db* region. Products of *Gl* show evidence of strong linkage disequilibrium with those of *Pr*, *Db* and *Pa*. Biochemical studies on the Gl 1 and Gl 4 proteins indicated similarity in amino acid composition, and similarity to the composition of Pr 1. The polymorphic forms of Gl proteins showed microheterogeneity due to differences in size and charge, and the polymorphism seemed to be accounted for on the basis of MW differences in primary gene products.

47.2.4.2 Pm. In 1977, Ikemoto *et al.* described another polymorphism in parotid saliva using a modification of the acid urea starch gel electrophoretic system described by Azen (1972 and 1973). The Pm protein migrates in between Pa and Pb proteins (the designation "Pm" stood for "salivary parotid middle band protein"). The Pm protein is either present or absent in a given individual, and its presence or absence can be determined in whole saliva, suggesting that it is one of the more stable of the salivary parotid proteins. Family studies indicated that the protein is simply inherited as a Mendelian dominant, and the *Pm* locus is autosomal. *Pm* is apparently not linked to *Pa* or *Pb*. In a sample of 195 Japanese, the Pm^+ frequency was about 0.38. 20 Chinese were also typed, and although the number is small, Pm^+ was about 0.3 by my calculations. Azen and Denniston, in a personal communication to Yu *et al.* (1980b), have found that *Pm* is closely linked to *Pr* and *Db*.

47.2.4.3 Ph. In 1979, Ikemoto *et al.* reported on the polymorphism of a further protein in parotid saliva, detected by SDS-polyacrylamide gel electrophoresis (Ikemoto *et al.*, 1979a). This protein differed from Pb, Pa, Pr, Db and Pm, and was designated "Ph", for "salivary parotid heavy protein", because it appears to have a larger MW than the other parotid proteins (this observation had been previously reported by these authors in the Japanese literature). The MW of the Ph protein was determined to lie between 68,000 and 155,000 by SDS-polyacrylamide gel electrophoresis. Ph, like Pm, is either present or absent, and the Ph+ condition is simply inherited as a Mendelian dominant at an autosomal locus. Among 218 Japanese subjects, Ph^+ frequency was about 0.026 with Ph^- about 0.974. *Ph* was not associated with *Pr*, *Db* or *Pm*, but was associated with *Pa*. Ph was a glycoprotein, and the authors said that its relationship to Gl would require further study.

47.2.4.4 Sal I and Sal II. In 1974, Balakrishnan and Ashton described two polymorphic proteins in parotid saliva, detected by polyacrylamide gel electrophoresis at pH 8. They said that this work had been reported at the 4th International Congress of Human Genetics in Paris, and that these proteins did not correspond to the Pb proteins of Azen (1972 and 1973). The polymorphism was detectable in whole or in parotid saliva. The complex pattern of bands

could be divided into six regions, and the variation was observed in region 4. Two bands were involved; both could be present or absent. In addition, either one could be present in the absence of the other one. The proteins were inherited, and the most viable genetic explanation postulated two loci called *Sal I* and *Sal II*, each of which had a dominant and a recessive allele. *Sal I* controlled the "fast" band, and its alleles were designated *F* and *f*; the alleles of *Sal II* were correspondingly designated *S* and *s*. The "null" condition (no bands) was attributed to the *ffss* genotype. Azen (1978) said that examination of the patterns in the photograph of Balakrishnan and Ashton (1974) suggested that the two bands might represent two of the Pr proteins. However, the genetic analysis, and particularly the finding of the "null" phenotype, would not fit with this explanation.

47.3 Polymorphic Salivary Enzyme Systems

The best studies of polymorphic enzymes in saliva is probably amylase, and this system has been discussed elsewhere, as noted in section 47.1. In this section are discussed salivary acid phosphatases (Sap), esterases (Set or Set_1), Glc-6-phosphate dehydrogenase (Sgd) and peroxidase (SAPX).

The presence of an acid phosphatase activity in saliva was noted by Giri in 1936. It had a higher activity with hexose diphosphate than with glycerophosphate, and the pH optimum was 2 to 3 units higher with the former. Using polyacrylamide gel electrophoresis, Tan and Ashton (1976a) found five patterns of salivary acid phosphatase, and one deficient type. These patterns could be explained on the basis of two separate genetic loci, called *Sap-A* and *Sap-B*. *Sap-A* had three alleles, $Sap\text{-}A^{A}$, $Sap\text{-}A^{A'}$ and $Sap\text{-}A^{0}$, while *Sap-B* had two alleles, $Sap\text{-}B^{B}$ and $Sap\text{-}B^{0}$. Operation of these loci gave rise to the phenotypes AB (which was common), B, AA', A'B, AA'B, A and O. The relationship of these isozymes to other human acid phosphatases (section 29.3) will eventually want some further explanation.

In 1976, Tan found polymorphism among a pair of carboxylesterase bands in saliva using polyacrylamide gel electrophoresis and detection with α-naphthyl acetate and Fast Blue RR salt. Of the two bands, a fast one and a slow one, both or either could be present in a given person, giving the phenotypes F, FS and S. The locus controlling this polymorphism was called *Set-1*, and the observations could be understood in terms of two alleles, $Set\text{-}1^{F}$ and $Set\text{-}1^{S}$. $Set\text{-}1^{F}$ had a frequency of about 0.61 among Caucasians and about 0.5 among Japanese, both in Hawaii. As with the case of Sap isoenzymes noted just above, the relationship of Set-1 isozymes to the other carboxylesterases of human tissues and their genetic loci (section 31.3.6) will require additional study.

Tan and Ashton (1976b) described a fairly well distributed polymorphism in salivary glucose-6-phosphate dehydrogenase, denoted "Sgd" (it may be recalled that the locus denoting the red cell Glc-6-PD is often denoted *Gd*—see in secton 33.1). Polyacrylamide gel electrophoresis was used to separate the isoenzymes, and three phenotypes called 1, 2-1 and 2, were observed. The best genetic explanation for the data was an autosomal locus *Sgd*, with a pair of codominant alleles Sgd^{1} and Sgd^{2}. The fact that *Sgd* is autosomal shows that it is separate and distinct from *Gd*, which is well established as being X-linked. The Sgd^{1} frequency was about 0.75 in Caucasians, 0.66 in Japanese and 0.7 in Chinese, all living in Hawaii.

Genetic polymorphism in salivary (parotid) peroxidase (SAPX) was described in detail by Azen (1977). The isoenzymes were detected in concentrated parotid saliva samples by polyacrylamide gel electrophoresis in Tris-lactate buffers at pH 2.4. The gels were then washed in alkaline buffers to bring the pH to the 8.0-8.4 range prior to detection of activity. Detection was carried out using a modification of the procedure described by Uriel (1958) for Cp detection (section 45.5). It may be recalled that Cp is often detected by taking advantage of its oxidase activity. Azen (1977) found two comparatively common and one rare SAPX phenotypes. SAPX 1, the most common type, had a major fast ("F") band, a faster minor "f" band (probably a conversion product of F), and several slower bands. SAPX 2, the next most common type, showed a major slow ("S") band, and several associated slower bands, the first of which ("s") probably represents a conversion product of S. SAPX 3 was seen in one family. These phenotypes could be accounted for on the basis of three alleles, $SAPX^{1}$, $SAPX^{2}$ and $SAPX^{3}$. One informative family in the study showed that there was an intimate relationship between SAPX and Pa. This finding implied that SAPX would also show a relationship with Pr and Db as well, and this suspicion was confirmed. Extensive biochemical studies on the proteins and genetic studies on the interrelationships of the determining genetic loci showed that there is a perfect correlation between *SAPX* and *Pa* products. $SAPX^{1}$ behaves as a recessive, while $SAPX^{2}$ and $SAPX^{3}$ behave as complete dominants. The SAPX 2 and 3 products appear to be genetically controlled modifications of SAPX 1, rather than different primary gene products. Pa 1 is always associated with SAPX 2, and Pa 2 is always associated with SAPX 3. The *Pa* locus may be regarded, on the basis of these data, as a kind of "modifier" locus, whose products modify the SAPX 1 type. When the Pa type is Pa O, the SAPX type is 1. It is probable that Pa 1 or Pa 2 monomers complex with SAPX 1 through disulfide bonds to yield SAPX 2 or SAPX 3.

47.4 Typing Salivary Polymorphic Proteins

Polyacrylamide gel electrophoretic methods for typing the salivary proteins and enzymes were collected and organized by Tan and Teng (1979) in a useful summary paper. Typing some of the proteins in a reproducible way is obviously somewhat involved (Azen, 1978). More than one electrophoretic system must be employed to discern all the variants in some systems. Azen (1978) gave a thorough discussion of the most optimal typing methods for the various systems, based upon his considerable experience.

Friedman and Allushuski (1975) described a special electrophoretic system for defining Pr-Db-Pa types. It appears that the typing of the polymorphic isoenzymes, leaving aside SAPX which is closely associated with Pa, may be more straightforward.

Most workers have used parotid saliva for this work, at least in the case of the nonenzymatic proteins. This material is collected in a special way in a little cup, quickly frozen, and then lyophilized. Reconstitution of the lyophilizate for typing results in a several-fold concentration of the proteins. Many authors have noted that these proteins seem to be especially subject to proteolytic degradation, which changes the electrophoretic patterns and results in the inability to determine the type. Whole saliva is considered unsuitable for typing many of the proteins, apparently because of the rapid proteolytic activity that occurs in the oral cavity. The apparent sensitivity of the proteins to degradative change, and the necessity to concentrate even fresh material in order to diagnose the types in several of the systems, indicate that the nonenzymatic proteins may not be very suitable candidates for saliva stain identification work. There is no reason why specialized laboratories could not employ some of the systems, which have simple and clear cut inheritance patterns, in disputed parentage cases. Indeed, some of the systems are beginning to find their way into medicolegal work already (see immediately below).

47.5 Medicolegal Applications

With a single exception, to be noted below, the few papers on medicolegal applications of the salivary polymorphic proteins have been concerned with disputed parentage. Ikemoto *et al.* (1979b) carried out extensive population studies on the Pa, Pr, Db, Pm, Ph and Amy_1 systems in Japanese. The data were compared with published data for other populations from various sources. The probability of excluding a falsely accused father using each of the systems, and for the Pa, Pr, Db, Pm and Ph systems combined, was calculated from the Japanese population data. They noted that the combination of bloods groups, isoenzymes, and serum group systems currently employed for paternity testing (which did not include HLA) had a combined PE of about 0.92. The five salivary systems gave a combined PE of 0.305, and when combined with all the other marker systems, raised the overall CPE to 0.944. The value calculated for the combined PE (CPE) of the five salivary systems is, however, almost certainly in error on the high side. The Japanese workers assumed independent segregation for all the loci in doing the computation, and it is now clear, as Yu *et al.* (1980b) pointed out and as was discussed above, that Pa, Pr, Db and Pm are all closely linked, and that some allelic combinations at these loci show significant linkage disequilibria. Accordingly, independence of the loci cannot be correctly assumed in computing the PE for any combinations of these systems.

Pronk *et al.* (1979) briefly noted some of the problems associated with changes in the Pb patterns in saliva between the time of collection and that of actual analysis. These alterations are of a proteolytic nature.

Hayashi and Hayashi (1979) have described a polyacrylamide disc gel electrophoretic procedure for Set phenotyping, which was applicable to whole saliva and to saliva stains up to 6 weeks old. The conditions used to examine the stains were slightly different from those used for fresh material (which was frozen at once after collection, and thawed just prior to typing). The polymorphism was recommended for paternity investigations and as an identification marker in salivary stains.

SECTION 48. POLYMORPHIC PROTEINS IN HAIR

A number of genetic markers have been detected in hair, which shares them in common with blood and other tissues. These markers have been discussed in previous sections devoted to the various systems.

ABO grouping of hair was discussed in 19.10.7.1. ABO is the only blood group antigen marker system that has been detected in hair, and the only marker that is present in the hair shaft. There seems to be no doubt that the glycoproteins which possess ABH antigens are present in the hair shaft. Yet, while some workers have reported entirely reliable results in typing hairs, others have found that the type corresponding to that of the blood cannot always be determined correctly.

A number of isoenzyme markers which are expressed in red cells as well as in tissues can be diagnosed in the cells of the hair root sheath. These markers appear to be determinable about as reliably in hair roots as they are in blood (though, perhaps, many are determinable longer in dried blood than in plucked hairs). Among these are PGM_1 types and subtypes (27.4.3), ESD (31.4.4), PGD (33.2.4) and GLO (34.5.2). PGD aside, these systems give reasonably good discrimination in most populations, and would yield considerable individualizing information if they were all used on the same sample. A root sheath is required, however, in order to make typing possible.

Hair is common physical evidence, and techniques other than morphological comparisons that might help to individualize human hair would be most welcome in the forensic science laboratory. Many hairs encountered in case work lack root sheaths, so the enzyme markers cannot be used. And, since everyone is not in agreement about the reliability of ABO typing, morphological comparison of questioned and known hairs is usually the only remaining approach. While such comparisons have indisputable value, especially in excluding common origin, less subjective procedures would be desirable.

Dr. Baden and his collaborators at Harvard have recently carried out extensive studies on the structural proteins of hair. The data indicate that there are detectable differences in these proteins in hairs from different individuals, and that some of these differences are simply inherited. The major structural proteins of hair (keratins) consist of fibrous proteins and the amorphous matrix proteins. The fibrous proteins consist of a number of different polypeptides, having many CySSCy residues and MW in the range of 40,000–58,000. These proteins can be separated and examined by disc polyacrylamide gel electrophoresis if they are first reductively denatured in urea, and the S-carboxymethyl derivatives prepared. Baden *et al.* (1975) reported a genetic variant in one of the peptides of the fibrous proteins using this technique. The variant type occurred in about 5% of Caucasians. Hairs from any body site in variant persons showed the variant pattern. Four families were studied, and indicated that the trait was autosomal and dominant. Interestingly, the characteristic variant pattern could also be detected in nails.

The matrix proteins are a little more difficult to separate, but Lee *et al.* (1978) reported a procedure for doing so. In about 300 people, two variant proteins were detected that showed as extra bands in the usual pattern. The extra variant bands were called V_1 and V_2. V_1 was inherited in one mother-child pair. The inheritance of V_2 was not studied. Another variation was seen in which one of the usual bands was significantly reduced. This pattern was reproducible in people who exhibited it, and it appeared to have been inherited in one family. These data were regarded as preliminary, suggesting further family and population studies as well as searches for additional variation. While this work is at an early stage, the results do seem to offer hope that detectable polymorphic protein systems may well be present in the structural proteins of hair. Further study will be needed before these systems can be exploited for medicolegal work.

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UNIT VIII. DETERMINATION OF SEX OF ORIGIN, NONGENETIC MARKERS AND BLOOD COMPONENT PROFILING

SECTION 49. DETERMINATION OF SEX OF ORIGIN

49.1 Introduction

For some years, there has been interest in developing procedures which would enable the reliable determination of sex of origin of bloodstains and of certain other human tissues such as saliva and hairs. In some cases, such a determination could be informative and useful. Three general approaches have been used for this purpose, two of them cytological, and the third having to do with the measurement of sex hormone levels. The cytological techniques are concerned with the determination of either sex chromatin (Barr bodies), or of so-called F-bodies (Y-bodies) in cells.

49.2 Determination of Sex Chromatin

Sex chromatin bodies (Barr bodies) were discussed in section 1.2.4.4. These structures are characteristic of nuclei in certain cells from female mammals, including the nuclei of smooth muscle, adrenal cortex, and various epidermal cells. The structures may also be seen in polymorphonuclear leucocytes from peripheral blood smears of females, where they are sometimes called "drumsticks". These structures were first observed in cat neurons (Barr and Bertrand, 1949), and later in other cells and other species (Barr, 1957 and 1960). The nature of sex chromatin in cells is discussed by Moore (1966a), and he has also described its discovery and the earlier work in the field (Moore, 1966b). Davidson and Smith (1954) first noted sex chromatin structures ("drumsticks") in the neutrophil leucocytes of peripheral blood from females. These structures should be called "sex chromatin", or "X chromatin", though the latter term has not gained wide acceptance. Dr. Barr himself has discouraged the use of the term "Barr bodies".

Reports on the frequency of sex chromatin have varied, depending upon the tissue being studied. At least 100 cells must be examined according to most authorities, and some have recommended from 300 to 500. The variations may be due to tissue differences, and to differences in various technical factors. Other nuclear appendages may be mistaken for sex chromatin bodies by inexperienced observers as well. The somewhat subjective nature of sex chromatin determinations has been noted by Grob and Kupperman (1961), and they emphasized the importance of using reproducible techniques and experienced scorers.

In the earlier literature, a low frequency of occurrence of sex chromatin bodies was reported in cells from normal human males, but the general consensus of opinion some years ago seemed to be that normal male cells do not in fact exhibit the structures (Grob and Kupperman, 1961). As was noted earlier, there is evidence (Ohno, 1966) that the sex chromatin body structure represents an inactivated X chromosome (see in section 1.2.4.4). Thus, in people suffering from abnormalities of sex chromosome composition, the correlation between phenotypic sex and sex chromatin body frequency in nuclei may not hold up. These cases are comparatively rare in the population, but could lead to misinterpretation in medicolegal work. Discrepancies between phenotypic sex and chromatin body frequency may be observed in (1) chromatin negative females; (2) chromatin positive males; and (3) genetic mosaics. Phenotypic females who are chromatin negative are mainly of two types, those with Turner's syndrome, and those with testicular feminization syndrome. The former have various abnormalities, including ovarian agenesis, and karyotype analysis reveals that they have only 45 chromosomes, the missing one being an X. They are sometimes called "XO females". The latter group are phenotypically female, although they exhibit various abnormalities of sexual development. Karyotype analysis shows that they are chromosomally male, i.e., that they are XY and have 46 chromosomes. They are sometimes called "XY females". Males who are chromatin positive are often those who have so-called Klinefelter's syndrome. They have 47 chromosomes and an XXY sex chromosome composition, and they show a variety of abnormalities of sexual development. Genetic mosaics may be of either phenotypic sex, and different tissues of the same individual may show differences in sex chromatin pattern. Cytologic sexing by means of chromatin bodies has been reviewed by Grob (1970).

49.2.1 Sex chromatin determination in blood and bloodstains

Postmortem blood does not appear to be very suitable for sex chromatin determination, apparently because the leucocytes undergo lysis and degradative changes quite soon after death. Dixon and Torr (1956 and 1957) said that they had had no success with this technique in post mortem blood. Schilling (1960) reported that post mortem blood was good for up to about 6 hours after death for this determination.

Some reports have indicated limited success with bloodstains. De Bernardi (1959) studied bloodstains on hairs with some success. He said that at least 200 nuclei needed to be examined. Davidson (1963) described a procedure for the isolation of white cells from bloodstains, and examined a number of slides for chromatin bodies. Diagnosis of sex could not be made in every case, and he thought that the results of the determination should be taken only as an indication of possible sex. Ishizu *et al.* (1973) suggested that chromatin body determinations coupled with F-body determinations (see below) on the same material provided a more definitive conclusion than either cytological method by

itself. Caroff and Breton (1966) reviewed chromatin body sexing from a medicolegal point of view.

49.2.2 Sex chromatin determination in epithelial cells and hair

From a forensic point of view, the ability to determine sex in epithelial cells is probably most applicable to post mortem tissues and to exfoliated epithelial cells in saliva stains and traces. Dixon and Torr (1956 and 1957) noted that chromatin bodies could survive for a few weeks in post mortem epithelial tissues, and thought that this determination might be of value in some cases, such as for example with a body part which had been recovered and submitted for identification. They buried fetal tissue from a female in soil, and could still determine sex chromatin for about 4 weeks. Holzer and Marberger (1957) obtained somewhat similar results in their tests on post mortem tissues. Edwards and Cameron (1964) said that excellent preparations could be obtained from the mucosal epithelium of the urinary bladder, but that bodies even a few days old could give misleading results.

Sanderson and Stewart (1961) described a rapid, easy method for sex chromatin determination in buccal epithelial cells using aceto-orcein staining. Radam (1965) discussed the preparation of buccal epithelial cells for chromatin body determination. Renard (1971) described techniques for the preparation of epithelial cells from salivary and vaginal materials and for chromatin body determinations. Generally, 40% of the nuclei of cells of female origin recovered from various stains were chromatin positive. Ando (1973) examined buccal epithelial cells from salivary stains and traces for both Barr bodies and F-bodies (see below). The chromatin bodies were found to be less stable over the course of time than the F-bodies.

Hair root sheath cells have been found to be suitable material for chromatin body sex determinations in many cases. Dixon and Torr (1956) found that hair root sheath cells provided excellent material in post mortem examinations. Schmid (1967) described a technique for sexing single head hairs from living persons. It was best if the hairs were examined at once, and not stored, in his experiments. Culbertson *et al.* (1969) also obtained reliable determinations from hair root sheath cells. In 30 people, 15 males and 15 females, Montanari *et al.* (1967) found that Barr bodies occurred in $29 \pm 5\%$ of the nuclei of these cells from females, while the values were very much lower in preparations from males ($6 \pm 2\%$). Bassett (1978) described procedures for sex chromatin determination in hair root sheath cells, and reported generally reliable and satisfactory results. He noted the importance of having experienced examiners score the nuclei, and the possibility of subjective error. He said further that preparations with sex chromatin frequency in the range of 10 to 40% of scorable nuclei should be considered inconclusive. Males and females showed sex chromatin in 5% and 57%, respectively, of their scorable nuclei.

Moore (1966c) discussed the determination of sex chromatin in connection with medicolegal problems.

49.3 Y-Body (F-Body) Determinations

In 1968, Caspersson *et al.* reported that metaphase chromosomes exhibited various fluorescence patterns after staining with fluorescent dyes, and it was suggested that the technique might provide a useful tool for chromosome mapping. These experiments were done on nonhuman material. Certain loci of the chromosomes were observed to bind quinacrine diHCl preferentially. In 1969, Zech found that the long arm of the human Y chromosome fluoresced brightly after staining human chromosome preparations with quinacrine mustards. The ability to observe a small fluorescent body in human cell preparations containing a Y chromosome, after staining with quinacrine diHCl, was quickly shown to be a property of a number of types of cells. In buccal epithelium, leucocytes and cultured skin fibroblasts, a large proportion of the cells from males (usually 25–50%) exhibited the fluorescent body (or F-body) after suitable preparation and quinacrine di-HCl staining (Pearson *et al.*, 1970; George, 1970).

49.3.1 F-Body determinations in dried blood and bloodstains

The current of opinion in the literature appears to be that F-body determinations can provide a highly reliable method of dried blood sexing if the procedures are carried out skillfully and if a sufficiently large number of leucocytes can be obtained for counting. A number of different techniques have been reported for these determinations. There is some variation in the estimate of the maximum age of a bloodstain that is still suitable for sexing by F-body counting. In addition, storage temperature and conditions as well as the nature of the substratum appear to affect the results obtained.

Phillips and Gaten (1971) reported successful results with bloodstains up to 10 days old on solid (nonabsorbent) substrata. About 86% of the leucocytes from male blood smears showed F-bodies, while the figure was only about 0.5% with female samples. In 1972, Phillips and Webster gave an improved technique in which 2 mM $MgCl_2$ was employed as an extraction medium instead of McIlvaine's buffer. In 1974, Phillips and Gitsham gave a procedure for bloodstains on absorbent substrata, and reported the results of a series of blind trial studies. Extraction techniques were not found very satisfactory for F-body determinations. Scraping the stain so as to cause a "dust" of bloodstain to fall upon the slide was the preferred technique. Reliable sexing was not possible in all the stains tested, but could be done in many cases. At least 100 nuclei had to be scored, they said. It was important to recognize, too, that readers had to become experienced in examining these preparations, and that there was an element of subjectivity in the determination. Müller *et al.* (1971) tested stains up to 32 days old and always found a higher F-body score in male bloods than in female bloods.

Schwinger (1972) could detect F-bodies in stains up to 30 days old. Blažek and Bráza (1972a) were able to determine the sex of bloodstains by F-body technique in stains 32 days

old. They said that the decay in the percentage of F-body positive nuclei from male stains was not a linear function of stain age. Aragones and Egozcue (1973) described a method which was applicable to stains up to 90 days old on absorbent or nonabsorbent surfaces. Brinkmann and Jobst (1973) found that 12.5% to 50% of cells from male bloodstains up to 4 weeks old contained F-bodies. It was possible to diagnose stains stored for 28 months at room temperature as being of male origin. Ishizu (1973) reported finding from 49% to 88% F-body-containing cells in blood smears from males and only 0% to 4% in those from females. Similar results were obtained with blood smears from newborns, and with cord bloods. Blood smears up to 5 months old from males showed more than 30% F-body positive cells. Schaidt and Krüger (1973) found that blood smears could be sexed by F-body technique for up to 19 days when kept at room temperature, but for up to 63 weeks if kept in a refrigerator. Bloodstains on textiles could be sexed for 8 weeks. Tröger and Liebhart (1974) found that male bloodstains stored for up to 15 months still showed detectable F-bodies. Thomsen (1975) gave an improved procedure for F-body determinations, the improvement consisting of a more effective filter combination for the fluorescence microscope.

Kringsholm *et al.* (1976) noted that they had found no overlapping between the sexes in the percentage of cells showing F-bodies. The highest female counts were always lower than the lowest male counts. They had not, therefore, observed any "false positive" males. Occasionally, a male blood could give a deceptively low F-body count, however, and this situation would result in a "false negative". A low F-body count had to be interpreted with caution, therefore, and could mean (1) a stain of female origin; (2) an older stain that was of male origin; or (3) a stain of male origin from a male with an abnormally low percentage of F-body positive cells. Kringholm *et al.* (1977) found that most bloodstains stored at room temperature for up to 21 weeks could be sexed by F-body technique. They did observe a few false negative results. Thomsen (1978) extended these aging studies to include stains kept at 5° and at 55°. False negative results appeared earlier in stains kept at either of these two temperatures than in those kept at room temperature, but there were no false positives. It appeared, therefore, that bloodstains retain their Y-body counts better at room temperature than at 5°, while the opposite appeared to be the case in the studies of blood smears (Schaidt and Krüger, 1973).

Schwinger and Tröger (1977) carried out blind trial studies using two different, independent scorers. In 21 stains up to 4 months old, the results of the two readers were very similar. Nanko (1980) has noted that F-body count in male blood smears fixed in methanol declines over the course of time, but the reason for the decline was not clear.

Wigmore *et al.* (1979a) reported on their technique for F-body determination in stains and on a series of blind trial sexing experiments. The subjective nature of the technique was emphasized, and the importance of carrying out case sample and control examinations on a "blind" basis was stressed. Cotton was found to be a problemetical substratum for bloodstains for this procedure, when compared with glass or nylon. It was difficult to obtain good results from bloodstains on wood or leather as well. In contrast to other reports, there was no significant reduction in the Y index of bloodstains as a function of age up to 138 days. In blind trial studies on casework materials, 65% of male bloodstains could be correctly identified, while no stains of female origin were misidentified as being male. Similar blind trial studies, with similar results, were reported by Wigmore *et al.* (1979b).

Thomsen (1979) gave a brief review of bloodstain sexing by F-body technique, and said that it provided a useful technique in bloodstain analysis.

Curran (1976) reviewed a trial court case in New York in which opposing panels of experts disagreed over whether a bloodstain on a jacket could be reliably sexed by cytological techniques. The stain was about 22 months old when the tests were conducted. The trial court judge in this case held that the tests to determine the sexual origin of cells in the stain (sex chromatin and F-body) failed to meet the requirements for reliability and general scientific acceptance, and the testimony was not admitted.

49.3.2 F-Body determinations in other tissues

There are a number of reports on sex determination by F-body technique in hair root cells (Sakai, 1972; Ishizu, 1972; Brinkmann and Jobst, 1973; Tröger and Liebhart, 1974; Kringsholm *et al.*, 1977). Tröger and Liebhart (1974) found that hair root sheath cells were suitable only for up to about 5 days with stored hairs, but Ishizu (1972) reported successful results with hairs up to 150 days old, as did Kringsholm *et al.* (1977) with hairs up to 27 weeks old. Nagamori (1978) reported successful F-body sexing determinations in hairs without roots up to 4 weeks old, provided they were kept dry.

Cells from various tissues and organs, examined post mortem, have been found suitable for F-body sexing determination. Kovacs *et al.*(1972) obtained reliable results in looking at 100 cells from brain, heart, liver, spleen and bone marrow in 16 male and 10 female bodies. Blažek and Bráza (1972b) obtained similar results with various tissues, and they showed further (1972c) that another fluorescent dye called 1-[(6-chloro-2-methoxy-9-acridinyl)amino]-3-(dimethylamino)-2-propanol diHCl worked as well as quinacrine diHCl. F-body determination has also been described in the cells of cartilage, periosteum and marrow (Berghaus *et al.*, 1973) and in human tooth pulp (Sommermater, 1975).

Tröger *et al.* (1976) found that Y-bodies were detectable in significant percentages in buccal epithelial cells derived from cigarette butts smoked by males. 10% of the male cells still showed Y-bodies when the cigarette butts were 43 days old, while the female cells from the same source had never shown counts higher than 6%. Y-bodies can be detected in sperm cells (Sakai, 1972). Tröger and Eisenmenger (1977) did F-body analysis in vaginal epithelium to try and use their absence as a means of identifying the cells as being of female

origin. They said that stains had to be less than 4 weeks old, however, to justify such a conclusion.

Ishizu *et al.* (1973) described a technique for the consecutive determination of F-bodies and sex chromatin in the same sample material, which included saliva, hair roots and blood. The results of the two determinations, they said, were more definitive than those from either separate determination alone.

49.4 Sex Hormone Level Determinations

The use of pregnancy-associated hormones as bloodstain markers for blood derived from pregnant women was discussed in section 8.2.1. The idea of using male and female sex hormones (primarily testosterone and 17β-estradiol) as indicators of sex in dried bloodstains has been around for some years, but there appear to be very few published systematic studies on the subject. Among the most sensitive (and, hence, desirable for this purpose) methods of the assay of lower MW compounds such as steroids in complex mixtures is radioimmunoassay (RIA). This technique was discussed briefly in section 1.3.4.3.

Many RIA procedures have been described for steroid hormones, like testosterone, in serum (see, for example, Sheldon and Coppenger, 1977; Joshi *et al.*, 1979). In theory, these procedures should be adaptable to the analysis of bloodstains, and one could try to relate the sex of origin of bloodstains to the levels of one or a combination of the major sex hormones, especially testosterone, 17β-estradiol and progesterone.

The most extensive studies to date are those of Shaler (1975). He adapted serum RIA procedures for testosterone (T) and estradiol (E) to determinations in bloodstains, and then measured the "T/E" ratios in a selection of samples. The ratio was first determined on a "blind" basis in about 1,500 serum samples. The T/E ratio varied from 60 to 0.02. Analysis showed that values greater than 3.51 could be assigned as male with a high degree of probability. Predictability of sex based on the T/E was not 100% accurate, however; several male sera showed abnormally low ratios and several female sera showed abnormally high values. Unfortunately, nothing was known about the history or state of health of the donors. The ratio was next determined in about 250 bloodstains from 6 to 8 months old. Sex of origin was known, but the hormone level measurements were carried out on a blind trial basis. The predictability of sex was about the same as that found for the serum samples. It was noted that in the bloodstains, the T/E ratio separating males from females at the same probability level was 1.0, rather than the 3.51 seen with the serum samples. 98.9% of bloodstains from males had T/E ratios greater than 2.0, while 92.7% of stains of female origin had values below 1.0.

Recently, Szendrényi and Földes (1980) reported that they could successfully sex experimental bloodstains up to 6 weeks old by determining the testosterone concentration by RIA. Stains prepared from 32 males and 24 females were studied, and showed little change in testosterone concentration between 48 hours and 6 weeks of aging. The protein concentration of the samples was also determined, and it was recommended that the testosterone level be referred to the protein concentration when analyzing stains. Thus, testosterone levels expressed as pg/10 mg protein in stains correctly predicted the sex of origin in all cases in the limited number of stains analyzed.

Other hormones have been assayed in dried blood by RIA, but not for medicolegal purposes. Illig and Rodriguez de Vera Roda (1976) reported on an RIA technique for determining thyroid stimulating hormone (TSH) in dried blood spots. The method was apparently designed in order to enable samples from newborns to be forwarded to a distant testing laboratory in a convenient way.

SECTION 50. NON-GENETIC MARKERS AND BLOOD COMPONENT PROFILING

50.1 Introduction

Over the years there has been occasional interest in attempting to individualize blood using non-genetically determined components. Three general classes of components have been studied: (1) Components present in blood as the result of environmental exposures or of the ingestion of specific agents. This class includes primarily antibodies and drugs. The antibodies may be directed against blood group antigens, against specific pathogenic agents to which the individual has been exposed, or to allergens. This class also includes the hepatitis antigens. (2) Components present in blood as the result of normal metabolis processes. This class includes primarily metal ions and smaller MW metabolites. And (3) The serum proteins. Although serum proteins are genetically determined, and many are controlled by polymorphic loci, the kinds of studies being discussed here usually seek to find individual differences in the qualitative or quantitative patterns of a large series of the proteins.

Two general approaches have been taken in these studies. In the first, a specific antigen, antibody, drug or component is detected and/or quantitated. In the second, the pattern of a series of components (usually serum proteins) in different individuals is examined. The individual components are not necessarily identified. Quantitative immunoelectrophoretic techniques have been employed in a number of these kinds of studies.

A number of different factors are involved in determining whether non-genetic markers and blood component profiles are truly individualizing markers. In the case of specific blood components, these include: (1) whether the presence and/or quantity of the component is constant in an individual over time, and over what length of time; (2) whether the presence and/or quantity of the component is detectable in dried bloodstains in a way that can be related to a whole blood sample; (3) the extent to which the presence or quantity of the component declines in dried bloodstains over time; and (4) what fraction of the population might be expected to exhibit the presence and/or quantity of the component detected. In the case of blood component profiles, there are similar considerations: (1) whether inter-individual differences exceed intra-individual differences at a given time, and over the course of time; (2) whether patterns in bloodstains accurately reflect the pattern in whole blood from which the stain was derived; and (3) to what extent the different patterns actually vary in a population. Work on non-genetic markers is, for the most part, at an early stage of development in this field. Additional studies will be needed to establish the individualizing value of many of the markers. There is not much doubt that the finding of certain specific non-genetic markers, such as HBsAg for example, in a bloodstain would be valuable in an investigation, and could prove helpful if a comparison were possible with the blood of the suspected depositer of the stain soon afterwards.

50.2 Detection of Specific Components of Blood

50.2.1 Antibodies

Among the first studies on the detection of specific antibodies in dried bloodstains are those of Kirk and his collaborators in the early 1960's. In 1963, Thornton and Kirk conducted studies on the detection of incomplete (Coombs reactive) anti-D in dried bloodstains up to 28 days old. They found that the antibody was detectable, and that it persisted for at least several weeks in dried bloodstains was judged by its titer against Rh+ type O cells. Leister and Kirk (1963) reported on the detection of rheumatoid arthritis factor in dried bloodstains using gamma-globulin treated latex particles as a detection system. Dried, powdered blood kept in vials was used for these experiments. They found that the factor was detectable in dried blood up to 150 days old, and that blood with an initially high titer tended to retain its activity in the dried state. The relationship of the factor being tested for in these studies to the Gm system (section 44) is not altogether clear. In 1964, Leister *et al.* reported a series of experiments on the detection of syphilis antibodies in dried blood. A microflocculation test (Kirk and Bennett, 1939) was used in these studies. 520 dried blood samples up to 140 days old were tested, and the loss of activity was greatest in the first five days. Not every sample retained activity equally well, but a higher percentage of initially strongly reacting samples gave positive results after 140 days than of samples which had reacted more weakly at the beginning.

More recently, King, Whitehead and Werrett have done studies on the detection of various antiparasitic and allergen-associated antibodies in bloodstains. King (1974a) and King *et al.* (1975) described the detection of antibodies to *Mycobacterium tuberculosis, Treponema pallidum* (non-specific "syphilis"), *Vibrio cholera* (cholera), *Toxoplasma gondii* (toxoplasmosis), *Trichomonas vaginalis* (trichomoniasis), *Candida albicans* (candidiasis) and *Toxocara canis* (toxocariasis). There were technical difficulties with the *Toxoplasma* and *Toxocara*. Approximately 75 bloodstains were tested with the other five antigens using an indirect fluorescent antibody technique. The majority of samples gave consistent positive and negative results in

separate readings, but some gave ambiguous results. The antibodies were stable in bloodstains for at least several months. In the sample of bloods studied, the discriminating power for the five antigens was calculated to be 0.71, roughly comparable to the ABO blood group system. Antibodies to *Mycobacterium tuberculosis* could be demonstrated in semen if it was not more dilute than 1:10. It was noted that the antibody titer in a positive group of bloods behaves as a continuous variable, and that scoring samples as "positive" or "negative" was an oversimplification. It was also noted, however, that the procedure lends itself to the profiling of a large number of different antigens to which antibodies might be expected. In interpreting the results, caution must be exercised because there are some cross reactions with related antibodies in a large series.

In 1976, Werrett *et al.* extended the antibody profiling studies to include allergen-associated antibodies. These antibodies are of the IgE class (section 1.3.3.2), and require very sensitive detection techniques because the circulating levels of IgE are very low compared to most other serum proteins (see in section 39). Commercially available RIA test kits for four allergens were used in the studies to detect the antibodies in experimentally prepared bloodstains. It was found that the antibodies could be detected in bloodstains on a variety of substrata, but that antibody activity declined more or less linearly to about 50% after 6 weeks. Using the four allergen-associated antibodies, it was possible to discriminate six different blood stains on a blind trial basis, and the results correlated with the reported allergic conditions of the donors. The results were considered to be preliminary but promising, since the range of different antibodies which one assumes could be detected in bloodstains (and for which reagents are available) is quite large.

King *et al.* (1976) reviewed the studies on antibody profiling of bloodstains. The promise and potential value of this approach were discussed. A large number of antimicrobial and allergen-associated antibodies could potentially be employed for this kind of profiling. An antimicrobial antibody profile reflects an individual's environmental exposure and vaccination history. The blood of full-grown adults can be differentiated from that of younger children in this way (King and Whitehead, 1975). In addition, menstrual blood was found to have a much higher antibody level relative to hemoglobin than the circulating blood of the same donor. Allergen antibody profiles could give information about the geographical origins or history of an individual. Information of potential value to an investigation might thus be provided from the examination of a bloodstain at a crime scene.

50.2.2 Drugs and plasma metabolites

The same kind of RIA techniques used to detect drugs in serum can be adapted for measuring drug levels in bloodstains. As noted in section 49.4, Shaler and his collaborators adapted RIA techniques for steroid hormone determination to bloodstain analysis. They have extended these studies to include the detection of a number of drugs as well. Shaler (1975) said that dilantin, a drug used to treat epilepsy, could be detected in a fragment of bloodstain equivalent to 10 $\mu\ell$ of blood, and that this represented an amount approximately 100 times larger than what was needed for detection. This work has since been extended to include detection of morphine (Mortimer *et al.*, 1978a; Smith *et al.*, 1980), digoxin (Mortimer *et al.*, 1978b), and barbiturates (Smith, 1980) in bloodstains.

King (1974b) examined the possibility of profiling a series of plasma constituents of clinical importance in the individualization of bloodstains. Data on the levels of Na^+, K^+, Cl^-, Ca^{++}, Mg^{++}, PO_4^{3-}, protein, albumin, glucose, uric acid, urea nitrogen, cholesterol, SGOT (soluble glutamic oxaloacetic transaminase) and LDH (lactate dehydrogenase) were surveyed in this study. Generally, the intra-individual differences were too close to the population variances to make this "biochemical profile" of much individualizing value. He noted, however, that better analytical methods might improve the prospects for the use of this kind of profiling in forensic serology.

50.2.3 Hepatitis antigens

One of the best reviews of hepatitis serology may be found in the Nobel address by Blumberg (1977). The discovery of a detectable antigen associated with hepatitis B grew out of studies on the Ag lipoprotein polymorphism (section 45.1). A precipitating antiserum was discovered in the serum of a multiply transfused hemophiliac patient in New York which reacted differently from the known anti-Ag serums. Tested against a panel of sera, the antibody reacted with the serum of an Australian aborigine, and the antigen was given the name "Australia antigen", or "Au". The antigen was found to be very stable in stored sera, and was consistently positive or negative in the same person's serum collected in some cases over a number of successive years. It was noticed that all Au(+) sera came from transfused individuals, and by the end of 1966 it had been realized that Au was associated with acute viral hepatitis. Au could be transmitted by transfusion, and some of the people who received it in this way developed hepatitis. Some such people also developed anti-Au. This finding led to the mass screening of donor blood units for Au prior to any transfusions, and rejection of the Au(+) bloods. The virus responsible for hepatitis B is now known to contain the Australia antigen on its surface, and it is now termed hepatitis B surface antigen (HBsAg). The surface antigen can be removed to reveal a core antigen (HBcAg), and antibodies to both of these can be found in human sera. It turns out that HBsAg exhibits antigenic heterogeneity and a number of surface antigenic determinants (designated by various lower case letters) are known to be associated with the virus. Dmochowski (1976) has given a thorough and readable review of the entire subject of hepatitis, including information about the surface and core antigens and their serology. HBsAg may be detected by a variety of immunological techniques, immunodiffusion, crossed electrophoresis and complement fixation among them. Hemagglutination techniques, in which red cells or latex particles are

coated with anti-HBsAg and used as the test system, have also been described (see, for example, Archer, 1977).

HBsAg can be detected in dried blood. Hoste *et al.* (1977) described a series of studies on a number of experimentally prepared bloodstains using a "sandwich" RIA procedure for detection. HBsAg could be detected in stains up to 6 months old, though more material was needed for testing with the older stains. The presence of HBsAg is a fairly stable characteristic, and can persist for years in people who have it. The incidence of the antigen is fairly low in Western Europe and in this country, but much higher in some populations. HBsAg is sometimes found in so-called carriers, who appear to be healthy. Hoste *et al.* (1977) suggested that HBsAg could provide a useful nongenetic marker in bloodstains. Frazadegan *et al.* (1978) have described detection of HBsAg in blood dried on filter paper, although their interest was not in bloodstain testing *per se*. If HBsAg subtyping should become possible in bloodstains, an even greater discrimination among individuals will probably be possible. Richer *et al.* (1977) have shown, for example, that the frequency of the "e" antigen associated with HBsAg varies among different ethnic groups in the Montreal population.

50.3 Serum Protein Profiling

A number of workers have looked at serum protein profiles in different individuals using a variety of different techniques of separation and detection. The number of components resolved is dependent on the nature and resolving capability of the technique selected. In addition, some of the studies were carried out before the extent of serum protein polymorphisms was fully recognized (Unit VII). In any case, if the proteins being profiled are not identified, one can not be sure that one is not seeing an example of a known polymorphic serum protein when a difference is detected.

In 1953, Bernfeld *et al.* found differences in the serum protein profiles of a number of different individuals using moving boundary electrophoresis in a Tiselius cell (section 2.3.1). Brown and Kirk (1957) conducted similar experiments on dried blood using paper electrophoresis. They could distinguish some but not all the stains in a series prepared from ten different people. Laudel *et al.* (1963) could detect differences in the immunoelectrophoretic patterns in the sera of a small number of people after carrying out electrophoresis on cellulose acetate membranes and detecting the proteins with a horse immune anti-human serum.

Whitehead (1969) and Whitehead *et al.* (1970) explored the possibility of using antigen-antibody crossed electrophoresis (Laurell technique—see in section 2.4.3.2) as a means of finding individual differences in the serum protein patterns. Studies with bloodstain extracts indicated that individual differences could sometimes be seen. The technique was found to be especially valuable for distinguishing Hp phenotype (see in section 40), and the possibility of using the profiling technique to distinguish phenotypes in a number of different immunologically detectable polymorphic serum protein systems simultaneously was discussed. Phillips (1974) looked at a somewhat different approach to serum protein profiling. The quantity of various serum proteins in the sera of different individuals is known to vary. It was thought that these differences might be exploited as individualizing markers using specific antisera and immunochemical quantitation. Antigen-antibody crossed electrophoresis was considered too variable for this purpose, and a nephelometric quantitation procedure using an autoanalyzer was explored. The procedure was found to be unsatisfactory for bloodstain extracts and even to a great extent for older hemolyzed blood samples because of interference by hemoglobin. If suitable techniques could be devised for ridding the samples of hemoglobin, Phillips said, the technique might be very useful, and he recommended further study. Grunbaum and Hjalmarsson (1976) carried out preliminary experiments on aqueous extracts of bloodstains using isotachophoresis (section 2.5.2). There were differences in the patterns from four people, and these patterns did not change if the extracts were retested 24 hours later.

In 1976, Sweet and Elvins (1976a and 1976b) applied antigen-antibody crossed electrophoresis (which they call "crossed electroimmunodiffusion" or CEID—see in section 2.4.3.2) to the problem of distinguishing individual serum protein pattern differences. By selecting and scoring those peaks which showed completely different height ranges over several determinations among individuals being compared, it was possible to distinguish among ten persons in the study. There were five males and five females in the test group, and differences between males and females were noted in the patterns as well. There were a few serum proteins which showed significant differences in amount between the sexes, and it was suggested that analysis of these might provide a probable sex of origin estimate in bloodstains. Whitehead (1977), in a letter prompted by the work of Sweet and Elvins (1976a), pointed out some of the problems associated with serum protein profiling as a means of individualizing stains. He thought that considerably more work would be needed before profiling techniques would be suitable for use in actual casework, and that genetic markers would continue to provide the principal means of individualization available for some time. Sweet (1977) responded to Whitehead's comments in the same issue of *Science*, and did not, for the most part, disagree with them.

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