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Microchemical Methods of Assay for Erythrocite Isoenzymes in Dried Blood through Autoradiography

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ABSTRACT

This study attempted to develop an autoradiographic method for the detection of isoenzymes in blood. It was expected that this would simplify, make more sensitive and add to present techniques of blood comparisons.

The initial approach explored the feasibility of finding an alternative medium to hydrolyzed starch and assaying the material utilizing both electrophoresis and classical partition chromatography. This method was unsuccessful and attempts were made to utilize bioluminescence in a gel medium. This method too was unsuccessful.

An Outline of the Organization of this Report:

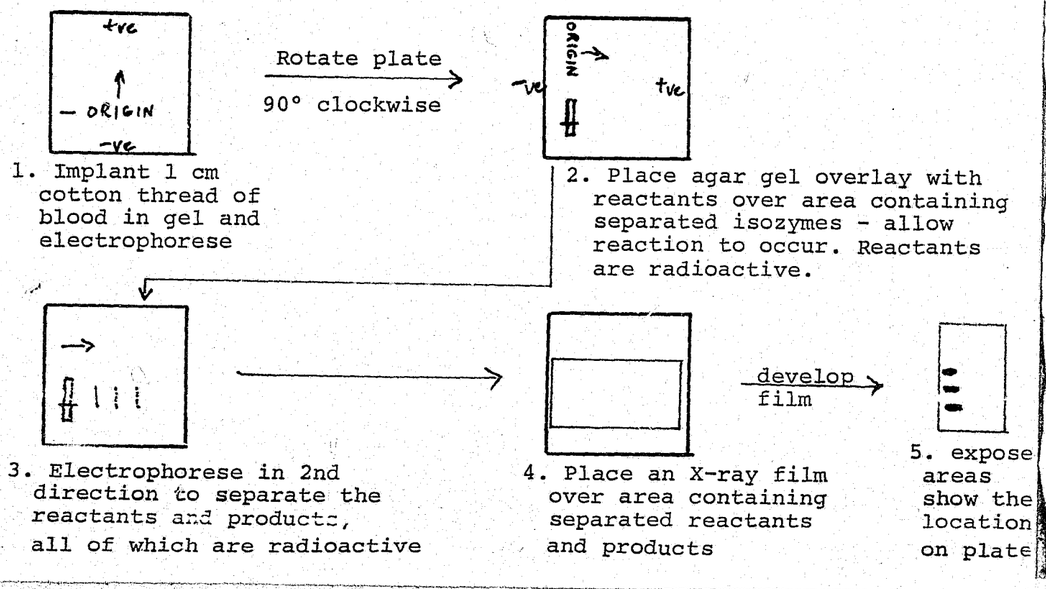
I. Introduction

Early in 1971, after having had modest experience with the typing of several isoenzymes of the human erythrocyte, we proposed to attempt to modify the existing procedures. The modifications consisted of: (a) find: an alternative separation medium to hydrolyzed starch; (b) making use of this medium, which would be suitable for both electrophoresis and classica partition chromatography, to attempt to assay the genetically determined isoenzyme red cell types for adenylate kinase (AK), and perhaps, phospho-glucomutase (PGM). The justification for the approach was, very simply, that (a) starch gel electrophoresis is cumbersome in comparison with othe types employing different media; and (b) an assay based on radioactive substrates might be much more sensitive than classical biochemical assays.

While it is not necessary to have a more sensitive assay when working with fresh blood in unlimited supply, it would be extremely useful to have a more sensitive assay for bloodstains found at crime scenes, particularly in cases where material was limiting.

We began by exploring the possibility of carrying out electrophoresis on cellulose thin layers and on cellulose acetate thin layers. We made some effort to group blood lysates for AK type on these support media following electrophoretic separation using the classical enzyme-coupling assay method. These efforts did not meet with success. We therefore decided, in the interests of time, to use thin starch gels for separation of the AK isoenzymes and attempt to develop an autoradiographic assay on starch gels. This decision precluded the use of partition chromatography as a method for separating reactant and product, but, since in the case of AK, both reactant and product are charged, we felt that we could bring about their separation by electrophoresis.

Thus, in broad outline,our plans were,after initial exploratory experiments, to carry out experiments designed to perfect the scheme diagrammed below:



1. Implant 1 cm cotton thread of blood in gel and electrophorese

2. Place agar gel overlay with reactants over area containing separated isozymes - allow reaction to occur. Reactants are radioactive.

3. Electrophorese in 2nd direction to separate the reactants and products, all of which are radioactive

4. Place an X-ray film over area containing separated reactants and products

5. expose areas show the location on plate

Almost the entire time spent on the project dealt with the above scheme. In subsequent sections, it will be made clear that what seemed a disarmingly simple task at the outset was to be fraught with technical difficulties.

As time progressed, it began to be clear that it was not going to be technically feasible to develop a reasonable autoradiographic assay on starch gels. At the suggestion of a colleague, therefore, we began to explore an alternative possibility for accomplishing the same end, viz. the development of a more sensitive assay for AK in red cells or stains. Since ATP is a product of the AK reaction (when ADP is substrate), and since ATP reacts with luciferin under certain conditions to give off light, (bioluminescence) and since extremely minute quantities of ATP can be detected in solution by this method, we thought it might have an application here. Although, not very much time was spent on this part of the work in comparison to the other parts, it is clear at this time that an assay for closely spaced isoenzymes in a gel medium based on bioluminescence cannot be developed for technical reasons that will be dealt with in subsequent sections.

II. Materials and Methods

Autoradiographic studies:

All electrophoretic separations were carried out on thin (1 mm) starch gels as described in detail in (1) and (2). Nucleotide separation experiments were carried out using AMP, ADP, and ATP obtained from Sigma Chemical Co., St. Louis, Mo. Ordinarily single cotton threads were soaked in 0.1M solutions of nucleotides which had been adjusted to pH 7 with NaOH and which contained equimolar amounts of $MgCl_2$. Other concentrations used are indicated in legends to figures. Cotton threads were 1 cm long.

The initial experiments were designed to find out, simply, whether or not magnesium-nucleotides could be separated in the same buffer systems used for isoenzyme separation. Later, for purposes of applying the magnesium-nucleotide, Mg-ADP, as a reaction substrate, it was necessary to apply the substance with a 1 cm by 2 to 3 mm piece of Whatman #3MM filter paper. This latter was necessary to assure that substrate contacted all the separated isoenzymes within the gel.

Hydrolysed starch used in gel preparation was obtained from Connaught Medical Laboratories, Toronto, Canada. The buffer system used for the electrophoretic separations consisted of 0.1M citric acid adjusted to pH 7 with NaOH for the tank buffer (or dilutions of this buffer as indicated in "Results") and 0.005M Histidine-NaOH, pH 7, for the gel buffer. Note that adenylate kinase can also be determined in a pH 5 buffer system, but pH 7 is much closer to the pH optimum for the reaction we hoped to effect within the gel.

All thin starch gels were prepared in glass plates according to references (1) and (2). Electrophoresis was always carried out in Shandon flat-bed tanks using thin sponges as wicks and Shandon low voltage power supplies (400 V DC maximum).

Radioactively labelled substrates ^{14}C-AMP and ^{3}H-AMP were obtained from New England Nuclear Corp., Boston, Mass. Films for the detection of radioactivity were Kodak Type M Industrial X-ray films, double-sided emulsion. Films for the detection of light from luciferase reactions were Kodak RP-Royal X-Omat Medical X-ray films, double-sided emulsion. Developers for these films were obtained from local photographic supply houses and other chemicals and supplies were obtained from local suppliers. All purified enzymes used as well as the luciferase-luciferin preparation were obtained from Boehringer-Mannheim Corp., New York, N.Y.

Polaroid photography was carried out using a Polaroid 440 camera equipped with a close-up lens attachment. When photography was done using an ultraviolet light source, the camera was also equipped with a Polaroid Yellow cloud filter for detection of fluoresced light.

Luciferase studies:

Luciferase experiments were carried out on thin starch gels as follows For ATP detection, ATP was placed at the origin and electrophoresed a short time to move it off the origin. A reaction overlay was then prepared in agar gel medium (1% final) consisting of 40 mg $MgCl_2$, Tricine-NaOH, pH 8, buffer (0.1M), and variable amounts of luciferase (0.2 to 2 mg) in a final volume of 20 ml. This overlay was poured over the area of the gel of interest, allowed to gel, and incubated at 37° or at room temperature for varying amounts of time from 12 hours to 3 days. Before incubation was begun, an X-ray film was placed over the reaction area in total darkness and the entire plate and film was wrapped in light tight aluminum foil for incubation. After incubation, the X-ray film was removed in total darkness and developed.

Luciferase experiments designed to detect purified myokinase within the gel were similar to the above except that the overlay mixture contained 5 mg of ADP in addition to the other materials (per 20 ml final volume).

III. Results:

In order to construct an autoradiographic assay as outlined above, it was necessary to establish the following things experimentally and approximately in this order: (a) that Mg-nucleotides could be separated by electrophoresis in the appropriate buffers (i.e. those used for the enzyme separations); (b) that the AK reaction would occur within the gel medium; and (c) that radioactive materials embedded within the gel could be readily detected by X-ray films.

It is well known that AMP, ADP, and ATP are readily separable on the basis of charge differences at virtually any pH. However, in the presence of Mg^{++} ions, the situation is entirely different. Magnesium ions are known to chelate to the phosphate oxygens of the nucleotides; since the negative charge resides on the phosphate-oxygens, there is a diminution of the net charge on the molecule according to the equilibrium constant for formation of the chelate for the various nucleotides. This has the net effect of equilizing the charges on ATP, ADP, and AMP, and that is why the experiments were necessary. We could not operate in the absence of magnesium, because it is absolutely required for the reaction.

Separation was extremely poor under a number of different conditions tried. Fig. 1 shows a representatively poor separation. 13% starch gels, 11% starch gels and 10% starch gels were tried, as well as different concentrations of citric acid-NaOH tank buffer. Using cotton threads as application vehicles, a good separation could be obtained using 10% starch gels and 0.01M citric acid-NaOH, pH 7, tank buffer. The results of such a separation are shown in Fig. 2. In addition, voltages and times used for electrophoresis were varied in an effort to optimize conditions. We also found that single cotton threads saturated with 5mM Mg-nucleotide represented the limit of our ability to detect nucleotide by fluorescent light with short-wave UV activation.

Because we wanted to apply the nucleotide substrate in such a way as to assure that it contacted all the previously separated isoenzymes, the cotton thread application was not suitable for further work. Instead, we applied the magnesium nucleotides by placing small pieces of filter paper saturated with the proper solutions onto the gel, incubating at 37° for 30 min, and thus allowing diffusion of the material into the gel. The filter paper applicator was then removed.

To our dismay, it turned out that the larger the width of the "band" of magnesium nucleotide applied to the gel, the worse became the separations. Fig. 3 shows the "bands" of nucleotides immediately after filter paper application but before electrophoretic separation. Fig. 4 shows the results of the electrophoretic separation, and it is obvious that the separations are much poorer than in the case of the cotton thread vehicle. The usual difficulty, under all conditions tested, was that ADP and ATP did not separate, but that AMP separated moderately well from the other two.

It occurred to us that it was not, in fact, necessary to have a good separation between ADP and ATP, but only between AMP and the other two nucleotides. The reason for this is as follows: The AK reaction may be represented as 2 Mg-ADP → Mg-AMP + Mg-ATP. Thus, ADP is the reactant and AMP and ATP are products. Now we only needed to locate ONE product in order to assure that reaction had occurred. And the system consisting of 0.01M citric acid-NaOH tank buffer and histidine gel buffer with filter paper application appeared to be adequate for this purpose. Electrophoresis was carried out at 150 V DC for 50 minutes to achieve these results.

The next part of the study dealt with finding out whether the AK reaction would occur within the gel. Before proceeding to that problem, however, we chose to check whether we would be able to detect radioactive nucleotides in the gel by means of our X-ray film. To assure that the film was responsive to the β radiation, and to check our development procedures, we impregnated a piece of filter paper with known quantities of ^{14}C-AMP and apposed the paper to an X-ray film at room temperature overnight. We had, by this time, chosen to use AMP + ATP as substrates (reactants) and look for ADP product, as the reaction is reversible and the equilibrium favors ADP by several orders of magnitude. The results were quite good and are shown in Fig. 5.

It was also clear, however, from the size of the exposure spots that ^{14}C was going to be too high-energetic an emitter for this work, because we wanted to be able to discriminate between sites of reaction on the plate that we knew would be quite close together. We suspected that the solution to this problem was simply to use tritiated AMP. We also discovered that it was going to be necessary to add quite a bit of radioactive material in order to be able to detect it in the gel with film. Fig. 6 and Fig. 7 show the results of an experiment in which we simply incubated all the separate nucleotides, two mixtures of nucleotides, along with ^{14}C-AMP as controls, and, in addition, incubated purified myokinase with radio-AMP and unlabelled ATP under proper reaction conditions in test tubes. After time for reaction had elapsed, we removed reaction mixture by soaking three thicknesses of filter paper in each and applying these to the gels as usual. Electrophoretic separation was carried out and the separated nucleotides photographed. X-ray film was then overlayed and left for 24 hrs at room temperature in the dark. The film was then developed. As can be seen, the separation was extremely poor in this experiment and the expected 2 radioactive spots in sample #4 were not observed; but the film failed to be exposed by the radioactive carbon in samples #1 and #5. Thus, more radio-carbon would have to be added to get good exposure. And since we had a separate problem with radio-carbon and were intending to change to ^{3}H-AMP, even more tritiated material would have to be present because it is a weaker emitter.

At this point in the work, we had reached the maximum possible point in the experimental development of the system. The reason is a technical one, and, although seemingly trivial, disallows the development of the system any further. Both ^{3}H-AMP and ^{14}C-AMP (as well as the similarly labelled ATP) are stable only in 45 to 50% ethyl alcohol solutions.

It is known that concentrations of ethanol higher than about 1% (v/v) inhibit most enzymes including myokinase (AK). Thus, adding the amount of radioactive substrate necessary for detection would have resulted, as a secondary effect, in adding large amounts of ethanol, much larger in fact than is required for enzyme inhibition. The obvious solution to this problem is to remove the ethanol from the solution and use a strictly aqueous solution of isotopic compound. We checked with New England Nuclear and several other large suppliers to determine the feasibility of this treatment of the labelled AMP. As it turned out, the labelled AMP or ATP is extremely unstable in the absence of ethanol, and the suppliers were quite certain that removing the ethanol would result in chemical breakdown of the labelled material through reactions caused by the emitted radiation. Since our experiments take quite a bit of time from start to finish, we could then not be certain whether the labelled material we thought we were adding was, in fact, present, or whether breakdown had occurred.

Thus, it is technically out of the question under present circumstances to develop the type of measuring system for the isoaymes which he had hoped and intended to do.

We carried out some studies toward the end of the project on the possibility of detecting, within the gel, the bioluminescence caused by the luciferase reaction in the presence of ATP. ATP is, of course, one product of our reaction with ADP as substrate. We first made an effort to see if we could detect, simply, ATP within the gel using a luciferin and luciferase containing overlay in the presence of $MgCl_2$ and buffer. We tested our system in solution and found that bioluminescence was present. In the gel, however, we failed to detect light at the points in the gel where ATP was located (we knew by fluorescence under UV). We attempted to use a far faster (more light-sensitive) X-ray film to see if it would make a difference.

With the faster RP Royal X-Omat film it was possible to see points of exposure corresponding more or less to the ATP spot locations on the plate. We found this encouraging, and proceeded to see if we could detect purified adenylate kinase within the gel. This experiment was performed by applying a cotton thread containing known myokinase to the origin of the gel, electrophoresing for about 2 hours to move the enzyme off the origin and into the gel, and then applying a luciferin-luciferase containing overlay in agar gel to the starch gel which also contained the other materials required for both the myokinase and the luciferase reaction

Although positive results of a sort were obtained in the sense that there was, in fact, considerable bioluminescence, the exposure spots on the X-ray film were enormous compared with the area of the gel occupied by the enzyme. An example of this type of result is indicated in Fig. 8. The photograph is taken of a piece of X-ray film which was 6 X 5 inches. Two explanations for this behavior were possible: (1) the luciferase preparation was contaminated with adenylate kinase and therefore the entire reaction overlay was luminescing; or (2) there was a light scattering by the gel which caused far more of the film to be exposed than the area immediately next to the enzyme and reaction site on the gel. We checked with Boehringer-Mannheim technical personnel and received credible assurances that there was in fact no significant adnylate kinase activity in the luciferase preparations. Thus, the possibility of light scattering within the gel remained the only tenable explanation. It is, in retrospect, not terribly surprising that starch gels or agar gels or a combination of the two should scatter light quite considerably, since their internal structure must necessarily involve rather large particles characteristic of colloidal type gels. Unfortunately, this phenomenon renders it impossible to get the fine resolution that would be required to detect the different red cell adenykate kinase types.

Several experiments involving chaging conditions and concentrations to attempt to minimize the scattering effect did not result in significant improvement in the results. If enough luciferase is employed to allow any detection at all, the scattering of the light prohibits close resolution of reaction centers that are close together as is the case in adenylate kinase types 1 and 2-1 from red cells.

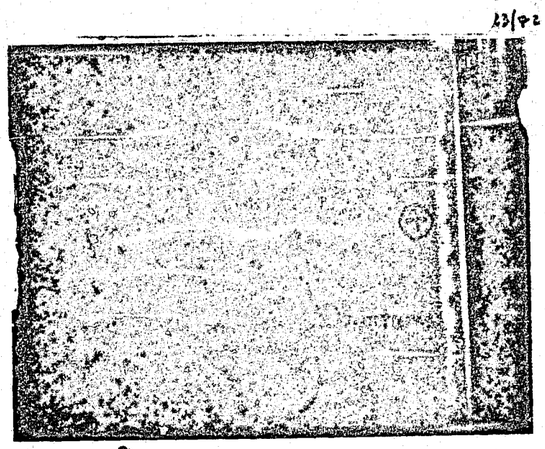


Figure 1: Nucleotide-Mg Separations on 13% Starch Gel
Solutions of nucleotides and $MgCl_2$ were prepared in water to 20mM in nucleotide and 20mM in $MgCl_2$. Cotton threads, saturated with each solution were applied to the gel and electrophoresis was carried out at 200 V DC for 1 hour.
1=ATP 2=ADP 3=AMP 4=$MgCl_2$ 5=ADP+ATP 6=ADP+AMP 7=ATP+AMP 8=AMP+ADP+ATP

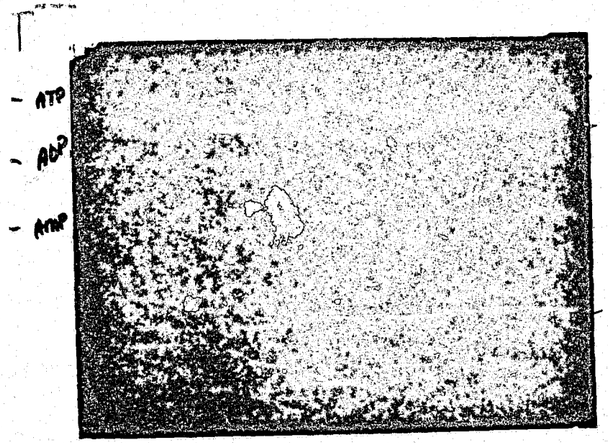


Figure 2: Nucleotide Separations Under Optimal Conditions
Single cotton threads were applied to a 10% starch gel saturated with the indicated nucleotide concentrations. $MgCl_2$ was equimolar in each case. Tank buffer was 10 mM citric acid-NaOH, pH 7.
1=10mM ATP,ADP,AMP 2=7.5mM AMP,ADP,ATP 3=5mM AMP,ADP,ATP
4=ATP 5=ADP 6=AMP 7=ATP,ADP

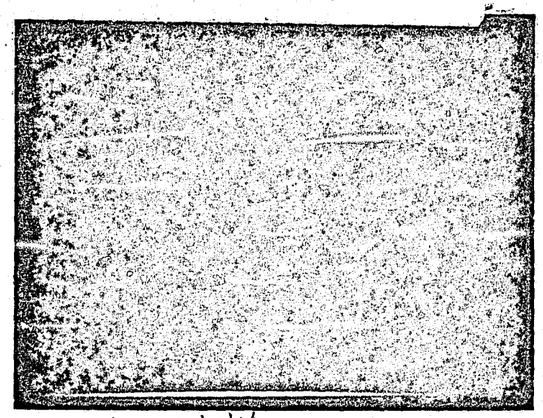


Figure 3: Nucleotide Bands after Filter Paper Application
7.5mM nucleotide solutions were prepared in buffer which were 7.5mM in $MgCl_2$ as well. Filter papers saturated with these solutions were placed on the gel, the gel incubated at 37° 30 min., and the filter papers removed.
1=ATP 2=ADP 3=AMP 4=ATP+ADP 5=AMP+ADP+ATP

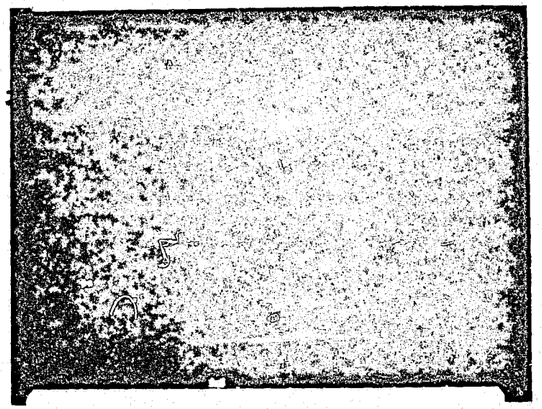


Figure 4: Nucleotides After Electrophoretic Separation Following filter paper application.
Conditions identical with those in Fig. 3. Electrophoresis was carried out at 150 V DC for 50 min at refrigerator temperatures.

Figure 5: Photo of exposed film. Applications of 0.4 and 0.8 µC of ^{14}C-AMP were made to filter paper. The film was pressed closely to the dried paper in darkness, exposed 24 hours and developed.

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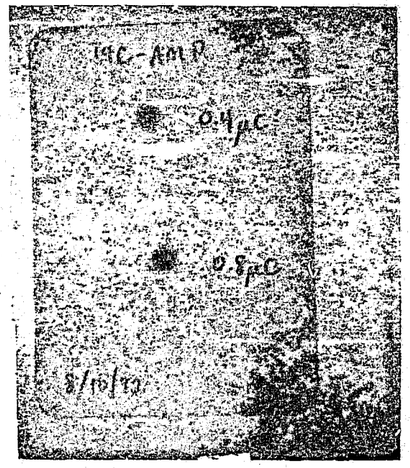


Figure 6.

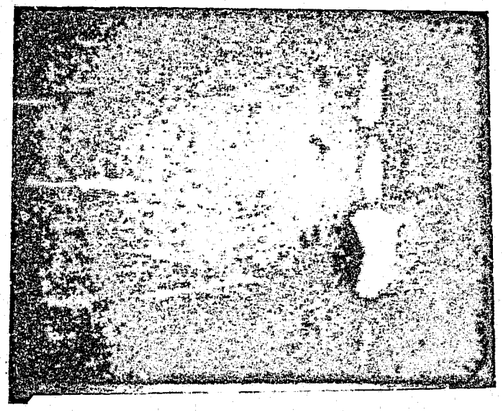
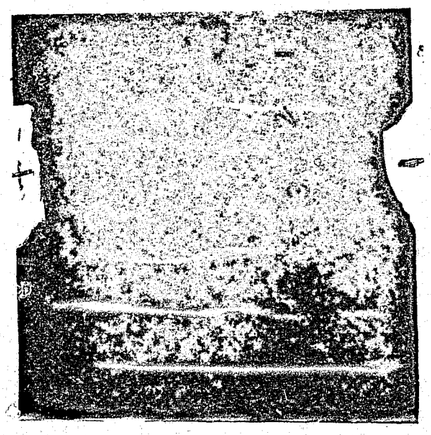


Figure 7.



Figures 6 and 7. Autoradiographic detection of the products of the AK reaction by starch gel separation.

Solutions contained 10mM nucleotides and $MgCl_2$ and 1 µC of ^{14}C-AMP where indicated and excess pure AK enzyme where indicated in Tris buffer. The mixtures were made in test tubes and allowed to incubate at 37° 30 min to allow reaction Filter paper applications of the mixtures were made to a 10% starch gel, the filter pape. removed, and electrophoretic separation carried out 1 hr at 150 VDC. Figure 6 shows the gel after separation. Figure 7 show a developed X-ray film after being overlayed on the gel 24 h. 1= AMP + ^{14}C-AMP 2= ADP 3=ATP 4=ATP+AMP+^{14}C-AMP+AK 5=ATP+AMP+^{14}C-AMP

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Figure 8: Photo of representatively "overexposed" piece of X-ray film following luminescence onto the film. Detectable quantities of purified AK enzyme was applied to a starch gel (10%) and electrophoresis was carried out at 200 VDC for 2 hours. The portion of the plate near the enzyme location was overlayed with melted agar containing 0.1M Tricine buffer, 5 mg ADP, 40 mg $MgCl_2$, in a final volume of 20 ml. A glass plate was placed over the gel and overlay, and a piece of X-ray film was placed onto the glass plate in darkness. The package was made light-tight and incubated at room temperature for 24 hours. The film was then developed.

IV. Discussion:

It passes almost without comment that the experimental results obtained in the study were quite disappointing in terms of the actual development of the system we had hoped for in the end. It is encouraging and somewhat rewarding to note, however, that while the system we desired could not be developed, there was in each case a quite reasonable and partially experimentally verifiable reason for the technical difficulty in each case. Thus, it would appear that, for the moment at least, it is not feasible to attempt autoradiographic analyses within gel media which require large amounts of labelled materials, and which contain inevitably and for stability reasons, an inhibitor of the reaction one wishes to detect. It is also unreasonable to hope for the development of a luciferase assay, sensitive though it may be, in a system that can scatter light apparently as well as these electrophoretic media.

Nevertheless, there have been a number of indirect effects of our having carried out this study which deserve some comment, as they may, in the final analysis, have more impact on law enforcement than any technique that might have been developed to precision. First, we are now capable of carrying out forensic blood grouping of either isoenzymes or antigens at John Jay College. As such, much graduate student research has been stimulated and is underway. Professor Gaensslen was in England in 1971 in Mr. Culliford's laboratory, and again in 1973, he worked with Dr. Barbara Dodd at the London Hospital on antigen determinations in bloodstains. Therefore, we are in an enviable position for teaching these techniques to graduate students who are to be tomorrow's criminalists and laboratory directors. We represent one of perhaps 2 or, at most 3, laboratories in the United States who are carrying out all the systems in blood grouping that are possible.

Perhaps the biggest single drawback to the routine useage of these systems in the U.S. by crime laboratories is the absence of meaningful population distribution data. We have, at the moment, made a modest but solid beginning in this direction. We have undertaken to survey the population of New York City for type distributions for glutamic-pyruvic transaminase as well as for adenylate kinase and phosphoglucomutase. These studies are expected to be completed by next fall (1973) and will involve about 10000 people, randomly selected, and of known ethnic origin. In addition, we are cooperating in a project with Dr. Simon Welsh in England on determining GPT types in stains.

We fully anticipate that since no new techniques need to be developed, we will finish and publish the results of these studies by late 1973 or early 1974. I shall in fact acknowledge this grant from LEAA as having supported those studies in part in whatever publications result from research that is current. The reason is, as pointed out, that our support from you in this respect has been rather a great stimulator of activity in terms of forensic serology at John Jay College.

Abbreviations Used in the Report:

AK = adenylate kinase or myokinase

ATP = adenosine triphosphate

ADP = adenosine diphosphate

AMP = adenosine monophosphate

Mg-nucleotides = ATP, ADP, or AMP in the presence of equimolar amounts of any magnesium salt; in this study, magnesium chloride

µC = microcuries

^{14}C = Carbon-14 or Carbon-14-labelled

^{3}H = Tritium or tritium-labelled

V DC = direct current voltage

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(1) Culliford, B.J. and B.G.D. Wraxall. Jour. For. Sci. Soc. 8 (2), 1968

(2) Culliford, B.J. The Examination and Typing of Bloodstains in the Crime Laboratory, Washington, D.C.: U.S. Government Printing Office 1971 publication of L.E.A.A., N.I.L.E.C.J., Dept. of Justice

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