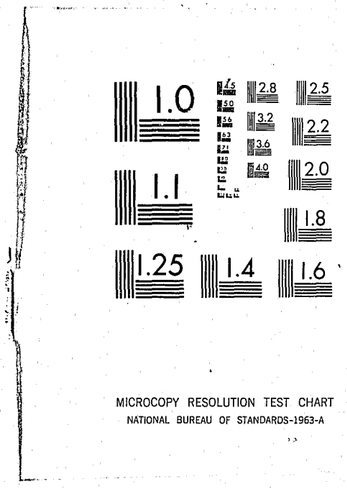

MICROCOPY RESOLUTION TEST CHART
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FINAL REPORT

NEW METHODOLOGY FOR THE DETECTION OF NARCOTICS

GRANT NO. NI 71-088G
NATIONAL INSTITUTE OF LAW ENFORCEMENT AND CRIMINAL JUSTICE
LAW ENFORCEMENT ASSISTANCE ADMINISTRATION
UNITED STATES DEPARTMENT OF JUSTICE
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PREFACE - PURPOSE OF STUDY

The specific aim of this study was to isolate bacteria or fungi capable of synthesizing adaptive enzymes with metabolic activity versus the morphine substrate. Evidence for the production of such induced enzymes was to be the ability of the organisms to grow in a minimal medium in which morphine sulfate was the sole carbon and nitrogen source. Quantitative evidence for morphine utilization was also to be provided by the development of a sensitive chemical procedure for measuring the progressive disappearance of the morphine substrate from the culture medium in which the microbial growth was obtained. If successful results were obtained in this initial study, subsequent funding was to be requested for additional studies leading to the overall goal of development of a rapid method for the detection of narcotic levels in body fluids of suspected addicts.

SUMMARY

The purpose of this study was to isolate bacteria or fungi capable of synthesizing adaptive enzymes with metabolic activity versus the morphine substrate..

Sixty-two experimental lots of culture media were prepared in order to investigate all facets of the adaptive process. A wide variety of sources was utilized in order to maximize the liklihood of the successfull recovery of organisms possessing the requisite qualifications. Pure cultures of organisms known to possess the ability to adapt readily to environments containing only low levels of nutrients were used as inocula as were ATCC bacterial and fungal stock cultures, selected for their metabolic activities versus similar substrates as reported in the literature. Other sources included ornamental poppy plants, cesspool and manure samples, pond and river water, and dust from narcotic cabinets. Soil samples from opium poppy fields, were received at three different times and tested by two main experimental approaches.

The test methodology was developed as the experimental phase progressed. A direct approach was used in which microbial isolates from the various sources outlined above were inoculated directly into culture media in which morphine sulfate was the sole carbon-nitrogen source. This experimentation did not lead to the recovery of candidate organisms.

The major approach used throughout this study was that of co-induction of enzyme production. By this procedure the morphine substrate was incorporated into culture media containing yeast extract as a nutritive source. The theory behind this method is that while growth of the organisms is primarily due to their metabolism of the main nutrient (yeast extract) there is simultaneous degradation of the morphine molecule. By sequential passage in media containing successively lower concentrations of the main nutrient, the ability to degrade the morphine substrate is enhanced. Such an approach has been successfully used in an atropine test system. The results of co-induction experiments are listed below:

1. No candidate organisms were recovered from the sample group that included the ornamental poppy plants, water and dust samples, cesspool and manure extracts and pure cultures of environmental isolates.
2. When ATCC bacterial and fungal isolates were investigated, evidence for morphine utilization was obtained. The contract period expired before further testing and verification of these results was possible.
3. No candidate organisms were isolated from the LE and HRC-E soil samples from Turkish opium poppy fields.
4. The HRC-B Turkish soil samples, received late in the contract period provided isolates that showed promise of adaptive enzyme activity following a number of passages, particularly in media adjusted to pH 8.2. Again the expiration of time prevented pursuit of these findings.

In the final month of the contract period, another experimental approach was initiated. Morphine sulfate solutions were "fed" to portions of the opium poppy field soil samples at 10 day intervals in an attempt to induce enzymes in response to the presence of the morphine substrate. The initial samplings from these soils following three "feedings" provided isolates which gave preliminary evidence for morphine utilization. This was the status of this phase at the time of the expiration of the contract.

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EXPERIMENTAL PLAN

Initially, two main experimental approaches were designed to obtain candidate organisms for the study. Pure cultures of organisms known to possess the ability to adapt readily to environments containing only trace levels of nutrients were to be used as inocula for culture media in which morphine sulfate was the sole carbon and nitrogen source. Soil samples from various sources (including soil from opium poppy fields) were to be collected, microorganisms isolated from them, and these isolates similarly processed.

A co-induction approach was also contemplated in which a nutritive substrate (yeast extract) would be incorporated into the culture medium in addition to morphine sulfate. The amount of nutrient would be progressively deminished during serial passages with the morphine concentration being either kept constant or being simultaneously increased. This would provide for continuous exposure of the microorganisms to morphine and enhance the possibility of adaptive enzyme production while still providing a minimal level of general nutrient substrate so that higher metabolic levels are initially maintained.

An experimental plan was also devised for the development of a chemical assay to monitor morphine consumption in the culture media. The method was to be based on a chemical oxidation of non-fluorescent morphine by ferricyanide to form the highly fluorescent oxydimorphine (pseudomorphine).

MATERIALS AND METHODS

A. Pure Cultures of Microorganisms

Twenty-nine strains of Pseudomonas species were used in the initial growth phases. These organisms were recent environmental isolates from faucets, sink drains and similar high moisture sites.. They were designated by the code letters HIP followed by code numbers. Additionally two strains of Pseudomonas putida, ATCC 12633 and ATCC 17390, were also included in the study.

Certain ATCC strains of bacteria and fungi were also selected for trials as candidate organisms. These organisms were assigned the following code letters which appear as their designations in the various Tables of Data.

Code Letter	Organism	ATCC Number
A	Cladosporium cladosporoides	6721
B	Pseudomonas sp.	11922
C	Polyporus sanguineus	11934
D	Arthrobacter oxydans	14358
E	Stachybotrys chartarum	16275
F	Oidiodendron echinulatum	16287
G	Pseudomonas putida	17484
H	Polyporus sanguineus	20160

B. Other Sources of Microorganisms

Ornamental poppy plants were collected. Extracts of the seeds, leaves, and soil were prepared and used to inoculate various culture media lots. Cesspool samples were similarly processed. Water samples from three locations in the Jones Falls Waterway system served as inocula as did pond water and manure from a local farm. Also tested were dust samples from the narcotics cabinets of a Baltimore hospital.

Soil samples from poppy fields in Turkey were received during the contract period on three separate occasions. Negotiations were also initiated to obtain soil from poppy growing areas in India. Because of the Indian-Pakistani conflict, delivery of these soil samples was delayed until after the expiration of the contract period. Two groups of soil were received in January-February 1972, one group being collected when the plants were 6" to 9" high and the second one at the time of capsule formation.

C. Culture Media

Sixty-two lots of culture media were prepared, including both agar and broth types. The following four basal salts formulations were used.

Formulation #I

Chemical	Grams Per Liter
K_2HPO_4	1.0
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.2
NH_4NO_3	1.0
$CaCl_2$	0.02
$FeCl_3$	trace

Formulation #II

Chemical	Grams Per Liter
NH_4NO_3	10.0
K_2HPO_4	20.0
KH_2PO_4	6.0
$MgSO_4 \cdot 7H_2O$	1.0
$MnSO_4$	0.1
$Zn\ SO_4$	0.01
$Fe\ SO_4$	0.1

Formulation III

Chemical	Grams Per Liter
Na_2HPO_4	11.29
KH_2PO_4	3.0
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.5
NH_4Cl	1.0

HRC Formulation (Modification of Earle's Balanced Salt Solution)

Chemical	Grams Per Liter
NaCl	6.8
KCl	0.4
$CaCl_2$	0.2
$MgSO_4 \cdot 7H_2O$	0.2
NaH_2PO_4	0.125

The first three of these formulations were those reported in the literature for use in inducing enzyme production in various enzyme-substrate systems. The fourth one was a modification of Earle's Balanced Salt Solution, a medium used in tissue culture work. Hank's Balanced Salt Solution (containing dextrose) was used in two of the experimental formulae.

To these basal salts solutions, a variety of substances were incorporated prior to pH adjustment and sterilization of the media. In the co-induction experiments, yeasts extract was most frequently used as the nutritive source although in several lots of media, trypticase peptone or dextrose played this role. For solid culture media, either agar-agar (BBL Purified Agar or Difco Noble Agar) or agarose, a galactose polymer produced by chemical treatment of agar-agar, was used as the gelling component. The latter substance was tested since initial results indicated that the organisms were attacking the agar molecule. It was postulated that agarose might be more resistant to microbial degradation. Distilled water was used as the diluent for all media except for one experimental batch in which well water was investigated.

The majority of the media formulations were adjusted to a pH close to neutrality. However, after determining that the pH of the soil samples from the Turkish poppy fields was slightly above pH 8.0, a number of media were adjusted to pH 8.2 just prior to sterilization.

Morphine sulfate was incorporated into culture media in concentrations ranging from 0.1 - 0.4%. 10% stock solutions of the morphine were prepared in distilled water. These solutions were either used without sterilization or after membrane filteration. Very early in the experimental phase, other narcotics (codeine, dilaudid, methadone, and papaverine) were substituted for morphine, but this approach was subsequently terminated.

In one phase of the study, the gradient plate technique which has been successfully used in bacterial mutation studies was employed. By this method, a range of concentrations of morphine was established across the surface of the agar plate thereby exposing the inocula to different levels of morphine. The theory behind this method was that the organisms may grow in certain zones of the plate in response to an optimal concentration of the narcotic.

The composition of the sixty-two media lots are listed below:

Code	Basal Salts Formulation	Type of Agar	% Morphine	Enrichment Type	%	Other Additives Type	%
M-1	I	Purified	0.1	-	-	-	-
M-2	I	"	0.1	-	-	-	-
C-1	I	"	-	-	-	Codeine	0.1
D-1	I	"	-	-	-	Dilaudid	0.1
ME-1	I	"	-	-	-	Methadone	0.1
A-1	I	"	-	-	-	-	-
A-2	I	Noble	-	-	-	-	-
M-3	I	"	0.1	-	-	-	-
A-3	II	"	-	-	-	-	-
M-4	II	"	0.1	-	-	-	-
BB-1	I	-	-	Trypticase	0.5	-	-
BB-2	II	-	-	"	0.5	-	-
MB-1	I	-	0.1	-	-	-	-
MB-2	II	-	0.1	-	-	-	-
A-4	H-BSS	Noble	-	Dextrose in H-BSS		-	-
M-5	H-BSS	"	0.2	Dextrose in H-BSS		-	-
A-5	E-BSS (HRC)	"	-	-	-	-	-
M-6	"	"	0.4	-	-	-	-
M-6/A-5	"	"	0.4→0 G*	-	-	-	-
A-6	"	Agarose	-	-	-	-	-
M-7	"	"	0.4	-	-	-	-
M-7/A-6	"	"	0.4→0 G	-	-	-	-
AE-1	"	Noble	-	Yeast Extract	0.5	-	-
M-6/AE-1	"	"	0.4→0 G	"	0.25	-	-
AE-2	"	Agarose	-	"	0.5	-	-
M-7/AE-2	"	"	0.4→0 G	"	0.25	-	-

Code	Basal Salts Formulation	Type of Agar	% Morphine	Enrichment Type	%	Other Additives Type	%	pH
A-7	I	Noble	-	-	-	-	-	
M-8	I	"	0.4	-	-	-	-	
P-1	I	"	-	-	-	Papaverine	0.4	
M-8/A-7	I	"	0.4>0 G	-	-	-	-	
P-1/A-7	I	"	-	-	-	Papaverine	0.4 O G	
BB-3	I	-	-	-	-	-	-	
MB-3	I	-	0.1	-	-	-	-	
MB-4	I	-	0.4	-	-	-	-	
MB-5	I	-	0.1	-	-	-	-	
MB-6	III	-	0.1	-	-	-	-	
MB-7	III	-	0.15	-	-	-	-	
MBE-2	III	-	0.15	Yeast Extract	0.5	-	-	
A-8	III	Noble	-	-	-	-	-	
M-9	III	"	0.2	-	-	-	-	
MB-8	III	-	0.15	-	-	-	-	
MBE-3	III	-	0.15	Yeast Extract	0.5	-	-	
M-10	III	Noble	0.2	-	-	-	-	
MB-9	III	-	0.2	-	-	-	-	
MBE-4	III	-	0.2	Yeast Extract	0.5	-	-	
MB-10	III	-	0.2	"	"	-	-	
MBE-5	III	-	0.2	Yeast Extract	0.4	-	-	
MBE-6	III	-	0.2	"	0.2	-	-	
MBE-7	III	-	0.2	"	0.1	-	-	
AE-3	III	Noble	-	"	0.5	-	-	
EM-1	III	"	0.2	"	0.5	-	-	
A-9	III	"	-	-	-	-	-	
M-11	III	"	0.2	-	-	-	-	
MB-11	I	-	0.25	-	-	-	-	8.2
MBE-8	I	-	0.2	Yeast Extract	0.4	-	-	8.2
MBE-9	I	-	0.25	"	0.2	-	-	8.2
MBE-10	I	-	0.3	"	0.1	-	-	8.2
MB-12	I	-	0.25	-	-	-	-	7.1
MB-13	I	-	0.2	-	-	-	-	8.2
MB-14	I	-	0.2	-	-	-	-	7.1
MB-15	III	-	0.2	-	-	-	-	8.2
MB-16	III	-	0.2	-	-	-	-	7.1

G = Gradient Plate

TEST METHODOLOGY AND RESULTS

Phase #1 - Initial Work With a Wide Variety of Pure Cultures and Environmental Isolates

Early investigations during the contract period dealt with the pure cultures of twenty-nine Pseudomonas species as well as with isolates from portions of ornamental poppy plants, cesspool and manure samples, pond and river water, and dust from narcotic cabinets.

The Pseudomonas species were collected over a period of time from environmental sites in local hospitals. These sites included high moisture areas such as sink drains, faucets and rubber tubing of suction and humidifier bottles. The pseudomonads were surviving in these locations in an atmosphere devoid of extraneous nutrients and thus are capable of synthesizing essential metabolites from very simple organic materials. For this reason, these strains were chosen as candidate organisms for inclusion in this study.

The ornamental poppy plants were processed in lieu of opium poppies, which were unobtainable due to the rigid restrictions placed on their growth. It was our intent to recover organisms from the various parts of the plant and soil in close association with the roots; and test them for their ability to produce the requisite enzymes.

It was thought that organisms from cesspool and manure would possess the ability to ferment a wide variety of substrates due to their normal role in the breakdown of organic matter in these types of biological matter. Pond and river water should contain bacteria with broad metabolizing capabilities.

Narcotic injections are routinely prepared in the nursing station drug areas of hospitals. During this process, air bubbles are expelled from the syringes resulting in the production of an aerosol of narcotic in the immediate environment of the drug cabinet. Dust in crevices in this area is thus constantly exposed to minute showers of narcotic solutions. For this reason dust was collected from these narcotic cabinets and subsequently cultured for the presence of microorganisms possessing the unique ability sought in our study.

This phase of our study represented the initial experimentation with media preparation, organism manipulation, and isolation techniques. A number of observations were thus made at this stage that affected the later experimental approaches. It was found that the majority of the pure cultures and fresh isolates, while capable of growing on media consisting of basal salts, agar-agar, and morphine as sole carbon and nitrogen source, would also grow equally as well on the identical medium minus the morphine. The organisms therefore appeared to be capable of attacking the agar molecule since theoretically there was no other carbon or nitrogen source in the medium. The possibility of the presence of trace amounts of

nutrients either in the distilled water or as carryover from the inocula was investigated. Several sources of distilled water were tested in parallel without altering the above results. Nutrient transmission in the inocula for both broth and agar media was prevented in all further transfers by the institution of a centrifuging and washing procedure. The only exception to this rule involved initial transfer stages directly from soil extracts and cultures. This observation of growth on agar-agar alone resulted in emphasis on the broth approach throughout the study although use of agar media was not completely eliminated. Poor growth on media containing basal salts formulation #II resulted in the elimination of this salt solution from subsequent investigations As can be noted by study of the ingredients of this formula, the salt concentration is extremely high, a fact which possibly accounts for its poor performance.

Results obtained from this phase of the work are summarized in Tables 1-7. A study of Tables 1-3 reveals the fact that in most cases growth of the organisms did occur on the solid media containing nutrients. The problem was that there was either no growth on both the basal media control and the corresponding morphine-containing media or growth was observed on both media types. Those strains which were exceptions (i.e. the three Pseudomonas strains that did not grow on the agar control plates) were further passed on both types of solid media. This secondary passage resulted in growth

on the plain agar as well, again suggesting the probability of degradation of the agar molecule (Table 4.) Broth media were used for growth of the complete group of Pseudomonas species. Nine of the strains yielded minimal growth in morphine broth. These were passed in the same media and failed to grow. See Tables 5A and 5B for this data. Broth to agar transfers of the same nine strains were also made. The results of this passage are presented in Table 6. In no instances was growth obtained on morphine agar without growth on the analogous medium minus the morphine.

The results of testing of the poppy plants, water samples and cesspool and manure extracts are also presented in Tables 3, 6, and 7. The inocula from poppy plant extracts in Table 7 represent secondary passages. No candidate organisms were recovered from these specimens.

In another experimental approach, a variety of inocula were used on an assortment of sixteen agar media. Dry swabs of dust from the narcotic cabinets were shaken in sterile water and the resultant suspensions used to inoculate culture media. Additionally several stock organisms were employed. Culture media included freshly prepared media falling into several groups; namely agar media without morphine or any other carbon or nitrogen source, agar media containing morphine, agar media containing yeast extract, agar media containing yeast extract and morphine, and gradient plates of these combinations. Test results are presented in Table 7.

Essentially no growth was obtained from the dust samples. The other organisms showed a wide variation of growth patterns. Luxuriant colonial growth occurred on media supplemented with yeast extract while the majority of growth on agar with and without morphine consisted of pinpoint colonies representing bacteria growing under a state of stress. Transfers of isolates from this series of plates did not result in the recovery of candidate organisms.

Phase #2 - Investigations Employing ATCC Bacterial And Fungal Strains

The American Type Culture Collection (ATCC) stock strains used in this phase were listed in the section on Materials and Methods. The rationale for the selection of these organisms was either based on literature reports in which members of these genera were used in substrate degradation studies or on descriptions of their activity given in the ATCC catalogue. The former approach was necessitated by the failure of attempts to obtain specific strains of organisms from the laboratory investigators. The latter approach resulted in the inclusion of five ATCC strains selected because of their activity versus ring-structured compounds. All organisms were received as lyophilized preparations, which were reconstituted and grown in nutrient broth. Prior to use as inocula, all broth cultures were centrifuged and washed three times so as to eliminate the possibility of nutrient carryover.

Series A

The initial cultural results with this group of organisms are presented in Table 8. Sixteen freshly prepared agar media were inoculated with the eight test strains. Growth on agar without an added carbon-nitrogen source was approximately equal to that on morphine-containing agar. Since the variable of nutrient carryover was eliminated, the results would seen to provide evidence for the ability of these organisms to attack the agar molecule. Alternative hypotheses for growth in a menstrum devoid of nutrients would be that there were trace nutrients in the water used in the medium or even that the organisms could utilize CO_2 from the air.

Table 9 presents data obtained following the agar to broth transfer of growth from the plates described in the preceding paragraph. The broth media inoculated were of two types, namely morphine broth with and without nutrients. As can be noted from the Table, only one organism produced turbidity in the media minus added nutrients (yeast extract). Aliquots from those few tubes were used as inocula for both agar and broth media. Results of this serial passage are given in Table 10. Following transfer, the organisms failed to grow in unsupplemented morphine broth but did grow on unsupplemented morphine agar (and on agar minus a carbon or nitrogen source as well).

Since growth of the eight organisms was obtained in broth media MBE-2 and MBE-3 (Table 9), positive tubes were centrifuged and the growth washed twice with sterile water to again eliminate nutrient carryover. It is possible that by the process of co-induction these organisms while deriving most of their growth requirements from the yeast extract, also produced adaptive enzymes capable of attacking the morphine. The washed preparations were passed to fresh tubes of morphine broth with and without yeast extract. Results are presented in Table 11. Luxuriant growth of all six organisms occurred in yeast extract containing media while three organisms grew in plain morphine broth. Positive cultures were centrifuged, washed and passed to fresh tubes of the same media. As can be seen in Table 12, no growth was obtained in the morphine broth minus nutrients.

Series B

Fresh agar slant cultures of the ATCC organisms were prepared. Growth was washed off the slants with sterile distilled water and inocula were prepared by the normal centrifuge-wash procedure. Flasks containing enriched medium (MBE-10) and tubes containing two lots of morphine broth (MB-11 and MB-12) and one of enriched medium (MBE-9) were inoculated. Results of this testing are presented in Table 13. Four strains grew in un-enriched morphine broth. Growth from all positive tubes was processed by centrifuging and washing. Identification of the cultures was by the Roman Numerals listed in Table 13 and by the letter E if growth originated from enriched medium. Tubes of MB-11 (morphine broth) and MBE-10 (enriched morphine broth) were inoculated. Table 14 presents the data generated in this testing. As can be noted the results indicate growth of certain strains in media in which morphine sulfate was the sole carbon-nitrogen source. Further testing of these candidate organisms was not pursued under the contract due to the expiration of the contract period.

Phase 3 - Turkish Soil Samples

Three groups of soil samples collected from opium poppy fields in Turkey were received during the contract period. The methodology used and the results obtained on each group will be presented separately.

Group #1 - Soil Obtained via Law Enforcement Administration (Code - LE)

This group consisted of two soil samples received through the efforts of Law Enforcement Administration personnel. The soil was moist and impacted. No description of the collection sites accompanied the samples. Aliquots of the soil were collected from fourteen different locations within the soil blocks. Each portion taken weighed approximately 200 grams. Numbers 1-14 were assigned randomly. Each portion was stored in a glass beaker in a moist chamber at room temperature. The remainder of the soil was stored in new plastic bags.

Series #1A

Aliquots of each of the fourteen samples were added to individual 250 ml. flasks containing 100 ml. of sterile phosphate buffered saline (PBS). The resultant soil suspensions were incubated and shaken on an incubator-shaker at 28°C. at 150 rpm for six hours. Supernatants of these soil extracts were used to inoculate a variety of agar media (Table 15). Poppy seeds were obtained from a local bakery and extracts of these were also used as inocula. Large numbers of many different types of organisms were recovered on this series of plates. These have now been separated into pure cultures and will be used in further inoculations. Before pure cultures were achieved, passage of isolates to other media was effected.

The same soil extracts in PBS were used as inocula for tubes of morphine broth and basal salts broth minus morphine. For growth results, see Table 16. Growth from many of the soil isolates was obtained in broth containing 0.1% morphine. There was evidence of an inhibition of growth in broth media containing the higher level of morphine (i.e. 0.4% morphine sulfate). The poppy seed extracts yielded organisms which grew luxuriantly in morphine broth. In addition, however, these same isolates grew equally well in basal broth media containing no added nutrients.

Broth to agar passage of the MB-3 cultures of soil extracts and of the MB-4 cultures of poppy seed extracts was performed in order to obtain isolated colonies for the preparation of pure cultures. Eight different bacterial and five different fungal isolates were established in pure culture and used to inoculate morphine broth (Table 17). No growth of any of these isolates was obtained.

Series #1B

The same soil extracts in PBS (Series #1A above) were used as inocula for flasks of morphine broth MB-5 flasks were inoculated with soils #1-6. MB-6 flasks were inoculated with soils #7-13. These cultures were shaken on the incubator-shaker at 28°C. and 150 rpm for 72 hours at which time transfers were made to agar media. Results of these transfers are listed in Table 18. Agar colonies were picked and used to inoculate morphine broth lot MB-8 and enriched morphine broth lot MBE-3. Perusal of Table 19 discloses the fact that although growth was obtained in the enriched morphine broth, no growth occurred in the identical medium minus the yeast extract enrichment. Three passages of the organisms in media containing progressively less yeast extract plus a constant concentration of morphine failed to demonstrate effective co-induction activity.

Group #2 - Soil Obtained Via HRC-England

(Code = HRC-E)

Series 2A

This group consisted of four soil samples obtained through the efforts of our English division. Information as to the source of these soils is presented below:

Bag No. 1:

Name of the owner of the field: Abdullah Güçlü

Province: Afyon

District: Merkez

Sub-district: Izcehisar

Village: --

Previous cultivation: Poppy

Bag No. 2:

Name of the owner of the field: Hasan Genç

Province: Afyon

District: Merkez

Sub-district: Çobanlar

Village: Ataköy

Previous cultivation: Barley

Bag No. 3:

Name of the owner of the field: Fevzi Kara

Province: Afyon

District: Çay

Sub-district: --

Village: Pazarağaç

Previous cultivation: Poppy

Bag No. 4:

Name of the owner of the field: Halil Ibrahim Kulak

Province: Afyon

District: Bolvadin

Sub-district: --

Village: --

Previous cultivation: Poppy

Initial processing of these four soil samples was by direct inoculation into morphine broth MB-9 and into the same medium to which sterile yeast extract solution was added to a final concentration of 0.5%. Incubation on the shaker apparatus was for a six day period with passage to fresh flasks of the same media. The second group of flask cultures was incubated for six days. Broth to agar passes from all sixteen flasks were made as well as broth to broth transfers. Growth results appear in Table 20. Predominant organisms from the agar plates were isolated in pure culture and growth from the MB-4 broth centrifuged and washed. These were used as inocula in a series of four successive transfers in media containing successively lower concentrations of yeast extract nutrients. No successful co-induction was achieved. (Tables 21-24).

Series 2B - Reprocessing of HRC-E and LE-Soil

Flasks of enriched morphine broth (MBE-4) were inoculated directly with soil samples HRC-E #1-4 and LE #1-8 and shaken at 27°C. - 150 rpm. for 5 days. No growth was obtained from LE 1 and 4. Twenty-five (25) ml. of the others were centrifuged at 3000 rpm. for 5 minutes to remove the soil particles. The supernates were collected in sterile tubes and utilized in one of two ways. (Parts A and B below).

Part A

Eight (8) mls. of each supernatant were centrifuged and washed twice with Basal Salts Medium III (with recentrifuging each time at 10,000 rpm. for 20 minutes). The final pellets were dissolved in 10 ml. of BSM III and the resultant suspensions used to inoculate the following culture media.

1. Flasks of MB-10 and MBE-5 were inoculated with growth suspensions from HRC 1-4 and LE 2 and 6.
2. Tubes of MB-10 and MBE-5 were inoculated with growth suspensions from LE 3, 5, 7, 8.
3. Plates of M-11 and A-9 were inoculated with growth suspensions from all ten soils.

This experimental phase was designed to be a co-induction series in which the yeast extract nutrient concentration was to be successively decreased. The initial results obtained are tabulated in Table 25. These are the growth readings recorded on the seventh day post-inoculation. Growth had appeared after 48 hours incubation in the broth medium containing 0.4% yeast extract (MBE-5) and aliquots of all flask cultures were removed, processed by the centrifuge-wash method, and used as inocula for morphine broth with and without yeast extract. (Table 26). This identical procedure was followed on the seventh day. (Table 27) This sequential sampling was performed due to the fact that the time required by these organisms to produce adaptive enzymes under these conditions was not known. It was postulated that

it might be desirable to pass organisms, which have been adapted to morphine utilization through co-induction, to media minus the added nutrient (yeast extract) before many growth cycles occur. Therefore, sampling was performed both early and late within the incubation period.

Study of Tables 25-27 reveals that no growth occurred in the morphine broth minus yeast extract. Subsequent sequential transfers from the tubes of enriched media listed in these tables to morphine broth with and without yeast extract were made without any evidence of co-induction. This data is not presented in tabular form.

Part B

The supernatants following the low speed centrifugation were used as inocula without further centrifuge-wash treatment. Tubes of MB-10 and MBE-5 and plates of M-11 (morphine agar) and EM-1 (enriched morphine agar) were inoculated and incubated for 7 days. Results are presented in Table 28.

The growth from the M-11 plates was pooled by combining loopsfull of each in 10 ml. of BSM III and using the resulting suspension to inoculate one flask each of MB-10 and MBE-6 and agar media as listed in Table 29. Subsequent transfers of the three organisms recovered from the enriched agar medium EM-1 and of two different organisms isolated from the MBE-6 flask failed to result in the development of candidate organisms.

Group #3 - Soil Obtained Via HRC-Baltimore

(Code = HRC-B)

Two soil samples from opium poppy fields in Turkey were obtained through contacts developed by HRC-Baltimore personnel The samples were coded Afyon and Bayat for purposes of our study as these were the names of the Turkish provinces from which they were obtained.

Just prior to the receipt of these samples, an experiment was performed in which the pH of the soil samples received to date was determined. Upon receipt of the new samples, their pH was also measured. Results appear in Table 30. Since the majority of the soil samples gave a pH reaction of above 8.0, new culture media preparations were prepared in which the final pH was adjusted to pH 8.2. (See media #MB-11, MBE-8, MBE-9, and MBE-10 in materials section at beginning of this report). A control medium (MB-12) was prepared at the same time. The pH of this medium was adjusted to 7.1.

Initial processing of the HRC-B soil samples was by the following procedure. Twenty grams of the two soils were added to flasks of MBE-8 broth. These flasks were incubated for 7 days on the shaker apparatus. The soil particles were removed by low speed centrifugation and the resulting supernatants processed by the normal centrifuge - wash procedure. The washed cells were then inoculated into flasks of MB-11, MB-12, and MBE-10. Following a 7 day incubation period, growth was obtained in the enriched medium only (Table 31). The two positive cultures were processed by the centrifuge -wash method and used to inoculate MB-11, MB-12 and MBE-9 media. After two passages in

enriched morphine broth, growth was obtained in the two non-enriched morphine broth. Mininal growth occurred in the medium adjusted to pH 7.1. A greater amount of growth was obtained in the medium adjusted to pH 8.2. See Table 32 for these results.

Growth from the positive morphine broth and enriched morphine broth tubes was used to inoculate fresh media after the normal centrifuge-wash processing. Some growth was obtained in morphine broth without added nutrients. (Table 33). The contract period expired before continued work with these organisms could be undertaken.

Phase #4 - Morphine-Fed Soil Approach

Aliquots of the two LE soils were combined as were aliquots of the four HRC-E soils. Each pool was packed in large glass beakers, which were stored in a moist chamber. Morphine sulfate solution was added to each pool at 10 day intervals. After three morphine "feedings", soil samples were taken from three sites in each pool. Area in which dried morphine had concentrated were selected. Flasks of MB-11 and MBE-8 were inoculated and shaken for 6 days prior to harvesting the growth by the centrifuge-wash method. The resulting organism pellets were suspended in MB-12 and used to inoculate tubes of MB-11, MB-12 and MBE-8. Results in Table 34 show that moderate amounts of turbidity did occur in some of the non-enriched morphine broths. The contract period expired before further pursuit of these findings could be initiated.

SUMMARY OF RESULTS

The data generated during the contract period is summarized in the Summary Section immediately preceding the Table of Contents at the beginning of this report.

TABLES OF RESULTS

Pages 26-60

Table 1.- Growth Results of Pseudomonas Strains with and without Narcotics

Organisms HIF#	Culture Media*									
	A-1		C-1		M-2		D-1		ME-1	
Day	3	7								
2	-	+**	-	+	-	+	-	+	-	+
3	-	±	-	-	-	+	-	+	-	±
4	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	-	+
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	+	-	+	-	+	-	+	-	+
9	-	-	-	-	-	-	-	-	-	-
10	-	+	-	±	-	+	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	+	-	+	-	+	-	+	-	-
13	-	-	-	-	-	-	-	-	-	-
14	-	+	-	+	-	+	-	+	-	+
15	-	±	-	-	-	-	-	±	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	+	-	+	-	+	-	+	-	±
18	-	±	-	+	-	-	-	-	-	±
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	+	-	+	-	±	-	±	-	+
26	-	-	-	-	-	-	-	-	-	-
27	-	±	-	+	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-
30	-	±	-	-	-	-	-	-	-	-
31	-	±	1 colony+		-	+	-	-	-	-
32	-	+	-	+	-	+	-	+	-	+

* Key to culture media designation in all tables

A = Agar medium without added narcotic
M = Agar medium containing morphine sulfate (0.2%)
C = Agar medium containing cocaine (0.2%)
D = Agar medium containing dilaudid (0.2%)
ME= Agar medium containing methadone (0.2%)

Numbers refer to various batches of these media.

** Growth Symbols

+ = luxuriant growth
± = minimal growth
- = no growth

Table 2 Growth Results of First Passage of Pseudomonas Growth from Primary Plates.

Note: All positives obtained on plates of the primary series (Table 1) were transferred to homologous media. Growth results of this passage are presented in Table 2.

Organism HIP#	Culture Medium											
	A-1		C-1		M-2		D-1		MB-1			
Day	3	7										
2	+	+*	-	-	-	-	-	-	-	-		
3	-	-			-	-	-	-	-	-		
5	+	+	+		+	+	+	-	-	-		
8	-	-	-	-	+	+	-	-	+	+		
10	-	±	-	-	+	+						
12	-	-	-	-	-	+	-	-				
14	-	+	-	+	+	+	-	+	+	+		
15	+	+					-	-				
17	+	+	-	±	-	±	-	+	-	-		
18	+	+	+	+					+	+		
25	+	+	+	+	+	+	+	+	+	+		
27	+	+	+	+	+							
30	-	-										
31	+	+	+	+	+	+						
32	-	+	+	+	+	+	+	+	+	+		

* Growth Symbols

+ = Luxuriant growth
± = Minimal growth
- = No growth

Table 3
Growth Results of a Variety of Microorganisms on New Agar Media Formulations

Organisms	Culture Media																	
	A-2		H-3		A-3		H-4		A-4		M-5		Blood	
Days	4	7												
HIP-2	1	2*	1	2	-	-	-	-	2	4	±	4	4	
3	±	1	±	1	-	-	-	-	2	2	±	2	4	
4	±	1	±	1	-	-	-	-	2	3	±	3	4	
5	2(2)	3	2	3	-	-	-	-	2	4	1	4	4	
6	-	1	-	1	-	-	-	-	2	3	-	1	4	
7	-	1	-	1	-	-	-	-	2	3	-	2	4	
8	+	2	1	2	-	-	-	-	1	3	±	3	4	
9	±	2	-	1	-	-	-	-	1	3	±	1	4	
10	±	2	±	1	-	-	-	-	1	3	1	4	4	
11	±	2	±	2	-	-	-	-	1	4	±	4	4	
12	1	2	1	2	-	-	-	-	1	3	1	2	4	
13	-	-	-	-	-	-	-	-	-	-	-	-	4(Not Pseudo)	
14	1	2	1	2	-	-	-	-	1	3	±	2	4	
15	±	2	1	2	-	-	-	-	±	4	1	2	4	
16	-	1	-	1	-	-	-	-	1	3	-	1	4	
19	1	3	1	3	-	-	-	-	1	4	-	2	4	
20	1	1	Isol Col.	1	-	-	-	-	1	2	1	3	4	
25	1	2	1	2	-	-	-	-	2	3	2	4	4	
26	1	2	1	2	-	-	?	-	2	4	1	4	4	
27	1	2	1	1	-	-	?	-	2	3	2	4	4	
31	1	2	1	2	-	-	-	-	2	3	2	4	4	
33	1	2	±	2	-	-	-	-	2	4	2	4	4	
Ps putida 12633	-	-	-	-	-	-	-	-	-	-	-	-	-	
" 17390	-	-	-	-	-	-	-	-	-	-	-	-	4	
Ornamental Poppy Seeds	2	4	2	3	-	-	-	-	3	4	3	4	4)	
Soil	1	3	1	2	-	-	-	-	3	4	2	3	4)	mixed
Water	1	2	1	2	-	-	-	-	3	4	2	3	4)	
Leaves	2	3	2	3	-	-	-	-	3	4	3	4	4)	
Kramer Cesspool A	1	2	1	2	-	1	±	1	2	2	1	1	4	
" B	1	1	1	2	-	1	±	1	2	2	1	1	4	
Jones Falls Water - X	-	3 col.	-	-	-	-	-	-	±		-	-	Spreader	
Y	-	30 Col.	-	Few Col.	-	-	-	-	-	10 Col.	±	50 Col.	4))	mixed
Z	2	2	2	3	-	Few Col.	1	1	3	4	2	4	4))	
Inoc. 6/7/71														
Days	2													
Number	3		4		3		4		4		4		4	
Pool P	Isol. Col.		1 Isol. Col.		-		-		-		-		-	
Pool TP	Col. 2		Isol. Col.		-		-		Isol. Col.		Isol. Col.		4	

*Growth Symbols
Number refer to quantitative estimates of growth.
4 = Largest amount of growth
1 = smallest amount of growth
+ = Questionable growth
Col. = Isolated colonies

TABLE 4

GROWTH RESULTS OF SECOND PASSAGE OF PSEUDOMONAS GROWTH

NOTE: Since growth was obtained on morphine agar with 3 Pseudomonas strains which did not grow on the agar control plates, this growth was transferred to fresh plates of both morphine agar and agar control plates. Results are presented in this Table.

	Culture Media	
Organism	A-1	M-2
	Day 3	
HIP - 8	+	+
HIP - 10	+	+
HIP - 12	+	+

Comments: These results indicated that the organisms were utilizing the agar molecule as a carbon source.

TABLE 5A

GROWTH RESULTS OF PSEUDOMONAS STRAINS IN BROTH MEDIA

	Culture Media			
Organism	BB-1	MB-1	BB-2	MB-2
HIP-2	4*	-	4	-
3	4	-	4	-
4	4	-	4	-
5	4	-	4	-
6	4	-	4	-
7	4	-	4	-
8	-	-	-	-
9	4	-	4	-
10	4	-	4	-
11	4	-	4	-
12	4	-	4	-
13	1	-	1	-
14	4	-	4	-
15	4	1	4	1
16	4	1	4	1
19	4	-	4	1
20	1	-	1	1
25	1	-	1	1
26	4	-	4	1
27	4	-	4	1
31	4	-	4	1
33	4	-	4	1

*Growth Symbols
4 = Maximum turbidity
1 = Slight turbidity

Comment: Those tubes of morphine broth showing turbidity will be used to inoculate additional culture media. (see Table 5B)

TABLE 5B

GROWTH RESULTS OF SECONDARY PASSAGE

Organism	From Broth	Culture Media MB-1	MB-2
HIP-15	MB-1	-	-
HIP-15	MB-2	-	-
HIP-16	MB-1	-	-
HIP-16	MB-2	-	-
HIP-19	MB-2	-	-
HIP-20	MB-2	-	-
HIP-25	MB-2	-	-
HIP-26	MB-2	-	-
HIP-27	MB-2	-	-
HIP-31	MB-2	-	-
HIP-33	MB-2	-	-

TABLE 6

Broth to Agar Transfers of Pseudomonas Strains

Broth Medium (Source of Inoculum)	Organism Designation	Agar Media A-2	A-3	M-3	M-4
		Day - 7			
MB-1	HIP-15	2*	-	2	2 colonies
	16	1	-	1	-
MB-2	15	2	1	2	1
	16	1	-	1	-
	19	1	1	2	1 colony
	20	1	1	1	1
	25	2	1	2	1
	26	1	1	1	1
	27	1	1	1	1
	31	1	1	1	1
	33	1	1 colony	1	5 colonies
	Poppy root	3	2	1	3
	Poppy water	3	1	1	3
	Poppy seed	2	1	1	3
	Poppy leaf	3	2	3	2
	Kramer A	2	1	1	2
	Kramer B	1	1	1	1

* Numbers refer to quantitative estimate of growth.

TABLE #7
GROWTH RESULTS ON NEW MEDIA FORMULATIONS
USING A VARIETY OF INOCULA

ORG # / Day	MEDIUM NO.															
	A - 5 4 18	A - 6	A - 7	M - 6	M - 7	M - 8	P - 1	M-6/ /A-5	M-7/ /A-6	M-8/ /A-7	P-1/ /A-7	M-6/ /AE-1	M-7/ /AE-2	AE-1	AE-2	H-5
1	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
2	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
3	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	4 4 Col. Col.	- -	- -	- -
4	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	1 1	- -
5	1* 1	- -	2 4	- -	- -	1 4	3 3	- 1	- -	2 2	2 4	4 4	4 4	4 4	4 4	1 3
6	2 3	2 2	3 3	2 2	1 2	3 3	2 2	2 2	1 1	3 3	2 2	4 4	4 4	4 4	4 4	3 4
7	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	- -	1 1	1 1	4	4	4	4	2 2
8	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2	1 2	1 1	1 1	4	4	4	4	3 3
9	2 2	2 2	2 2	2 3	2 2	2 2	2 3	2 2	2 2	2 3	2 2	4	4	4	4	3 3
10	2 3	2 2	3 3	2 2	2 3	3 3	3 2	3 3	2 2	3 3	2 2	4	4	4	4	3 3
11	2 3	2 2	3 3	2 3	2 3	3 3	3 4	3 3	2 2	3 3	2 2	4	4	4	4	3 3
12	1 1 Col. Col.	- -	- -	2 2 Col. Col.	- -	2 3 Col. Col.	2 Col.	H Col. Col.	- -	2 7 Col. Col.	2 Col.	3 4	3 3	4	2 3	2 2

*Numbers refer to quantitative estimates of growth.

Code to Inocula

1 = Narcotic cabinet dust 4N-1
2 = " " " 4N-2
3 = " " " 5N-1
4 = " " " 5N-2
5 = Bacillus contaminant
6 = Arthrobacter oxydans
7 = Pseudomonas putida 17484
8 = Poppy plant seed (from M-3)
9 = Poppy plant root (from M-3)
10 = Poppy plant water (from M-3)
11 = Pond water - tadpole (from M-3)
12 = Cladosporium cladosporoides 6721

TABLE 8

GROWTH RESULTS OF ATCC CULTURES ON A VARIETY OF AGAR MEDIA

	Test Organism							
	A	B	C	D	E	F	G	H
Medium No.	6721	11922	11934	14358	16275	16287	17484	20160
	Day 7							
A-5	3 Grey	1*	2	1	2	2	1	2
A-6	2+ Grey	1	1	1	1	1	±	1
A-7	3 Grey	1	2	1				
M-6	3 Grey	1	2	1	2	1	1	2
M-7	1	-	±	±	2	2	±	1
M-8	3 Lt.Brown	1	2	2	2	2	1	2
P-1	3 Grey	2	1	1	2M	1	2	1
M-6/A-5	3 Grey	1	2	2	2M	2	1	2
M-7/A-6	2 Lt.Brown	±	1	1	1	±	1	±
M-8/A-7	3 Lt.Brown	1	2	2	2	2	±	2
P-1/A-7	3 Grey	1	2	2	2	2	1	2
M-6/AE-1	4+Brown	4	4	4	3M	3	3	3
M-7/AE-2	4+Brown	4	4	4	3+M	3+	3+	3+
AE-1	4+Brown	4	4	4	4	4	4	4
AE-2	4 Brown	4	4	4	4	4	4	4
H-5	4+Brown	3	3	3	2	2	1	2

* = Numbers refer to quantitative estimates of growth

M = mycelial growth

TABLE 9

AGAR TO BROTH TRANSFERS OF ATCC ORGANISMS

Inocula Source		Broth Media MB-7 Day 5	9	MBE-2	MB-8		MBE-3	
M-8	A	-	-	+ Black mold	-	-	+	
	B	-	-	+ Particulate growth	-	-	+	
	C	-	-	+ Slimy growth	-	-	+	As for MBE-2
	D	-	-	+ Thready growth	1	2	+	but much heavier
	E	-	-	+ 3 Turbidity	-	-	+	growth in these
	F	-	-	+ 2 "	-	-	+	MBE-3 tubes
	G	-	-	+ 4 "	-	-	+	
	H	-	-	+ 3 "	-	-	+	
M-6/AE-1	A	-	-		-	-		
	B	-	-	Same	-	-	Same	
	C	-	Slt.turbidity	as	-	-	as	
	D	1	2	above	1	1	above	
	E	-	-		-	-		
	F	-	-		-	-		
	G	-	-		-	-		
	H	-	-		-	-		

TABLE 10

RESULTS OF BLIND PASSAGE (BROTH TO AGAR) OF ARTHROBACTER OXYDANS, ATCC-14358

Culture Media Inoculated		MB-7 6G D	MB-8 6G D	MB-8 8 D
		Day 4		
Agar	A-8	4+ pinpoint	4+ pinpoint	4+ pinpoint
	M-9	4+ pinpoint	4+ pinpoint	4+ pinpoint
Broth	MBE-3	2+ turbidity	4+ turbidity	3+ turbidity
	MB-7	-	-	-
	MB-8	-	-	-

TABLE 11

RESULTS OF TRANSFERS FROM ENRICHED MORPHINE BROTH TO MORPHINE BASAL BROTH AND ENRICHED BROTH (ATCC ORGANISMS)

Inocula	Broth Media MB-7	MB-8	MBE-3
Day 3			
6 C	-	-	3
D	1	-	3
E	-	-	3
F	-	-	2
G	2	1	4
H	1	2	3
Contaminant	-	-	3

TABLE 12

RESULTS OF SECONDARY PASSAGE OF POSITIVE TUBES OF TABLE 11 TO MORPHINE BASAL BROTH AND ENRICHED BROTH (ATCC ORGANISMS)

(Numbers = Quantitative Estimates of Growth)

Inoculum		Broth Media	
Medium	Code	MB-7	MBE-3
MBE-3	6C	-	4
MBE-3	6D	-	4
MBE-3	6E	-	4
MBE-3	6F	-	4
MBE-3	6G	-	4
MB-7	6D	-	4
MB-7	6G	-	4
MB-7	6H	-	4

TABLE 13

FIRST PASSAGE OF ATCC ORGANISMS IN SERIES B

(Numbers = Quantitative Estimates of Growth)

Code	Organism	Broth Media MBE-10 Flasks	MB-11 Tubes	MB-12 Tubes	MBE-9 Tubes
I	Pseudomonas sp. 11922	4	1	3	4
II	Arthrobacter oxydans 14358 (MBE-3 8D)	4	-	-	4
III	Pseudomonas putida 17484	4	2	-	4
IV	Arthrobacter oxydans 14358	-	-	-	-
V	Polyporus sanguineus 20160	4	-	-	4
VI	Polyporus sanguineus 11934	4	1	2	4
VII	Cladosporium Cladosporioides 6721 (MBE-3 8A)	4	3	3	4
VIII	Cladosporium Cladosporioides 6721	4	-	-	4

TABLE 14

SECOND PASSAGE OF ATCC ORGANISMS IN SERIES B

(Numbers = Quantitative Estimates of Growth)

Organism Code	Broth Media MB-11	MBE-10
I	-	2
I-E	2	2
II-E	-	3
III	-	1
III-E	1	3
V-E	-	4
VI	-	3
VI-E	-	4
VII	-	4
VII-E	±	4
VIII-E	2	4

TABLE 15
RESULT OF INITIAL PLATING OF TURKISH SOIL AND
POPPY SEED EXTRACTS

		CULTURE MEDIA							
Sample #		A-5	A-6	M-6	M-7	M-8	P-1	AE-1	AE-2
Soil	Day 1	5/5*	-	5	1	10 (1 Mold)	10	20 Lg. Mix	25 Lg. Mix
	2	5	2	20	20	15	7	30 Lg. Mix	35 Lg. Mix
	3	25	30	150	90	40	150	200 Med. Mix	100 Mix
	4	1	1	5	1	1 Mold	-	20 Mix	4 Mix
	5	20	5	10	10 (1 Mold)	15	15	15 Lg. Mix	10 Mix
	6	25	15	5	10	35	25	25 Lg. Mix.	25 Lg. Mix
	7	5	10	10	1	10	-	20 Lg. Mix	10 Lg. Mix
	8	3	-	-	1	9	2 Mold	18 Lg. Mix	20 Lg. Mix
	9	100 (96% pure)	3 (pure)	13 (1 lg. Mold)	6 (sev. Mold)	6	3 (2 mold)	31 Lg. Mix	12
	10	6 (1 Mold)	6	6	1	12	8	20 Mix	30
	11	3 (1 Mold)	3	5	1	6	5	25 Mix	30
	12	13 (1 Mold)	25 (3 Mold)	15	6	12	20	20 Mix	30
	13	20 (1 Mold)	15 (3 Mold)	50	25	18	50	40 (Lg. & Spreader)	30
	14	3 (1 Mold)	1	1	2 (1 Mold)	5	3	8	15
Poppy Seed Extract	15	1	1	0	1 Mold	1	0	1	3
	16	0	0	0	0	0	0	0	0

*Numbers refer to number of colonies per plate.

TABLE 16

RESULTS OF DIRECT INOCULATION OF BROTH MEDIA WITH TURKISH SOIL AND POPPY SEED EXTRACTS

Inocula		Broth Media MB-1 (0.1% Morphine Sulfate)	MB-3 (0.1% M.S.	MB-4 (0.4% M.S.)	BB-3 (no M.S.)
	Day	10			
Soil Extract	1	1*	1	-	-
	2	1	2	-	-
	3	-	-	-	-
	4	1	2	-	-
	5	1	2	-	-
	6	2	2	-	-
	7	2	2	-	1
	8	-	2	-	-
	9	1	1	-	-
	10	-	-	-	-
	11	-	1	-	-
	12	-	1	-	-
	13	-	-	-	-
	14	1	1	-	-
Poppy Seed Extract	15	4** mold	4 mold	4 mold	4 mold
	16	2M	2M	2M	2M
	17	2M&T	2M&T	2M&T	2M&T
	18	2M	2M	3M	3M
	19	3M	3M	3M	3M
	20	4M	4M	4M	4M

* - numbers indicate turbidity without shaking
** - numbers indicate amount of mold growth

M = mold
T = turbidity

TABLE 17

GROUP OF PURE CULTURE ISOLATES FROM LE SOIL IN MORPHINE BROTH (10 DAYS INCUBATION)

LE Soil Pure Culture No.		MB-3 (0.1% M.S.)	MB-4 (0.4% M.S.)
Bacterial	1	-	-
	2	-	-
	3	-	-
	4	-	-
	5	-	-
	6	-	-
	7	-	-
	8	-	-
Fungal	12	-	-
	2	-	-
	3	-	-
	4	-	-
	5	-	-

TABLE 18

BROTH TO AGAR TRANSFERS FROM MORPHINE BROTH INOCULATED WITH TURKISH SOIL EXTRACTS

Sample #	Culture Media: A-5 Day 8		M-8/A-7		M-9		M-10	
Soil	1	2* Mixed	1	Mixed	1	Mixed	1	Mixed
2	2	"	3	"	1	"	1	"
3	2	"	2	"	1	"	1	"
4	3	"	3	"	1	"	1	"
5	3	"	3	"	2	"	1	"
6	2	"	2	"	1	"	1	"
7	3	"	4	"	2	"	2	"
8	3	"	4	"	2	"	2	"
9	4	"	3	"	2	"	2	"
10	2	"	3	"	2	"	2	"
11	2	"	3	"	2	"	2	"
12	3	"	3	"	2	"	2	"
13	4	"	3	"	2	"	1	"

* Numbers refer to quantitative estimates of growth

TABLE 19

AGAR TO BROTH TRANSFERS OF ISOLATES FROM LE SOIL

Organism Source		Broth Media: MB-8	MBE-3
Soil	1	-	4
	2	-	4
	3	-	3
	4	-	4
	5	-	3
	6	-	4
	7	-	4
	8	-	3
	9	-	2
	10	-	4
	11	-	4
	12	-	2
	13	-	4

Numbers = Quantitative Estimates of Growth

TABLE 20

GROWTH OF HRC-E SOIL ON BOTH SOLID AND LIQUID MEDIA

AFTER ONE AND TWO PASSAGES IN SHAKER FLASKS

				Culture Media			
Code NO.	Passage No.	Soil No.	+ or - Yeast Extract	Agar M-8/A-7	Agar Blood Agar		Broth MB-4 Turbidity
1	1	1	+	4 Brown	4+	Mixed Bact. & Molds	4+ Dark Brown
2		2	+	3 Tan	4+	" "	2+ Lt. Grey
3		3	+	3 Tan	4+	" "	3+ Grey
4		4	+	3 White	4+	" "	4+ Lt. Brown
5		1	-	3 Tan	4+	" "	3+ Grey
6		2	-	3 White	4+	" "	2+ Lt. Grey
7		3	-	3 Tan	4+	" "	3+ Grey
8		4	-	2 White	4+	" "	3+ Grey
9	2	1	+	2+ Tan	4+	" "	-
10		2	+	2+ White	4+	" "	-
11		3	+	2+ White	4+	" "	-
12		4	+	2+ White	4+	" "	-
13		1	-	2+ Brown	4+	" "	-
14		2	-	2+ White	4+	" "	-
15		3	-	2+ White	4+	" "	-
16		4	-	2+ White	4+	" "	-

(Numbers refer to quantitative estimates of growth)

TABLE 21

FIRST PASSAGE OF PURE CULTURE AGAR ISOLATES AND MIXED CULTURE BROTH ISOLATES OF HRC-E SOIL

IN CO-INDUCTION SERIES

	Broth Media	
Inocula	MB-10 (Without Yeast Extract)	MBE-5 (With 0.4% Yeast Extract)
Agar Isolate #1	-	4
2	-	4
3	-	3
4	-	4
5	-	3
Broth Isolate #1*	-	4
2	-	3
3	-	3
4	-	2
5	-	3
6	-	4
7	-	3
8	-	3

* Note: Broth Isolates are from MB-4 broth cultures (Code #1-8) in Table 20.
(Numbers refer to quantitative estimates of growth)

Comment: Growth from the MBE-5 tubes was centrifuged and washed twice, then passed to MB-10 and MBE-6 (0.2% yeast extract). Results are presented in Table 22.

TABLE 22

SECOND PASSAGE OF PURE CULTURE AGAR ISOLATES AND MIXED CULTURE BROTH ISOLATES OF HRC-E SOIL IN CO-INDUCTION SERIES

	Broth Media	
Inocula *	MB-10 (Without Yeast Extract)	MBE-6 (With 0.2% Yeast Extract)
Agar Isolate #1	-	4
2	-	4
3	-	4
4	-	4
5	-	3
Broth Isolate #1	-	4
2	-	3
3	-	4
4	-	2
5	-	3
6	-	4
7	-	4
8	-	3

* Inocula = Washed growth from MBE-5 tubes in Table 21.

(Numbers refer to quantitative estimates of growth)

Comment: Growth from the MBE-6 tubes was centrifuged and washed twice, then passed to MB-10 and MBE-7 (0.1% yeast extract). Results are presented in Table 23.

TABLE 23

THIRD PASSAGE OF PURE CULTURE AGAR ISOLATES AND MIXED CULTURE BROTH ISOLATES OF HRC-E SOIL IN CO-INDUCTION SERIES

	Broth Media	
Inocula*	MB-10 (Without Yeast Extract)	MBE-7 (With 0.1% Yeast Extract)
Agar Isolate #1	-	4
2	-	4
3	-	3
4	-	4
5	-	4
Broth Isolate #1	-	4
2	-	4
3	-	3
4	-	3
5	-	4
6	-	4
7	-	4
8	-	3

*Inocula = Washed growth from MBE-6 tubes in Table 22.

(Numbers refer to quantitative estimates of growth)

Comment: Growth from the MBE-7 tubes was centrifuged and washed twice, then passed to MB-10 and MBE-7A (MBE-7 diluted with MB-10 to give final concentration of 0.01% yeast extract). Results are presented in Table 24.

TABLE 24

FOURTH PASSAGE OF PURE CULTURE AGAR ISOLATES AND MIXED CULTURE BROTH ISOLATES OF HRC-E SOIL IN CO-INDUCTION SERIES

	Broth Media	
Inocula*	MB-10 (Without Yeast Extract)	MBE-7A (With 0.01% Yeast Extract)
Agar Isolate #1	-	3
2	-	3
3	-	4
4	-	4
5	-	3
Broth Isolate #1	-	4
2	-	4
3	-	3
4	-	3
5	-	3
6	-	3
7	-	4
8	-	3

*Inocula = Washed growth from MBE-7 tubes in Table 23.

(Numbers refer to quantitative estimates of growth.

Comment: No evidence was obtained for the production of adaptive enzymes by this co-induction series of passages.

TABLE 25

INITIAL GROWTH RESULTS OF SERIES 2B SOIL EXTRACTS (GROWTH READINGS RECORDED AFTER 7 DAYS INCUBATION)

		Broth Media*		Agar Media	
Original Source of Inoculum		MB-10 Without Yeast Extract	MBE-5 (0.4% Yeast Extract	A-9 (Without Morphine)	M-11 (0.2% Morphine
HRC - 1	F	-	4	3	3
2	F	-	3	2	2
3	F	-	4	1	1
4	F	-	2	2	2
LE - 2	F	-	3	2	2
3	T	-	4	2	2
5	T	-	4	2	2
6	F	-	2	3	2
7	T	-	4	2	3
8	T	-	4	1	1

(Numbers refer to quantitative estimates of growth)

*Broth media were either in flasks or tubes (F or T)

Comment: Growth from the MBE-5 broths was centrifuged and washed twice, then passed to MB-10 and MBE-6 (0.2% yeast extract). Results are presented in Table 26.

TABLE 26

GROWTH RESULTS OF SERIES 2B INOCULA FOLLOWING PASSAGE AFTER 48 HOURS OF INCUBATION

Original Source of Inoculum	Culture Media	
	MB-10 (Without Yeast Extract)	MBE-6 (0.2% Yeast Extract)
HRC - 1	-	4
2	-	4
3	-	4
4	-	4
LE - 2	-	4
6	-	4

(Numbers refer to quantitative estimates of growth)

TABLE 27

GROWTH RESULTS OF SERIES 2B INOCULA FOLLOWING PASSAGE AFTER 7 DAYS OF INCUBATION

Original Source of Inoculum	Culture Media	
	MB-10 (Without Yeast Extract)	MBE-7 (0.1% Yeast Extract)
HRC - 1	-	4
2	-	4
3	-	4
4	-	4
LE - 2	-	4
6	-	4

(Numbers refer to quantitative estimates of growth)

TABLE 28

GROWTH RESULTS OBTAINED IN PART B OF SERIES 2B (PROCESSING OF LE AND HRC-E SOIL)

Original Source of Inoculum	Broth Media		Agar Media	
	MB-10 (Without Yeast Extract)	MBE-5 (0.4% Yeast Extract)	M-11 (Morphine Agar)	EM-1 (Enriched Morphine Agar)
HRC - 1	-	4	2	3
2	-	4	1	4
3	-	4	1	4
4	-	4	2	4
LE - 1	-	4	2	4
2	-	4	2	3
3	-	4	2	4
4	-	4	2	4
5	-	4	2	3
6	-	4	2	3
7	-	4	2	4
8	-	4	2	4

(Numbers refer to quantitative estimates of growth)

TABLE 29

AGAR TO AGAR AND AGAR TO BROTH TRANSFER OF POOLED GROWTH FROM MORPHINE AGAR - PARTS B OF SERIED 2B (12 DAY GROWTH READINGS)

Culture Media	Inoculum (Pooled Growth from M-11 Plates)
Broth - MB-10	No growth
MBE-6	Heavy growth (maximum turbidity)
Agar - M-11	Sparse growth of 3 organism types
EM-1	Luxuriant growth of predominantly 3 organism types

TABLE 30

pH DETERMINATION OF SOIL SAMPLES AND OF FLASK CULTURES

Test Methods:

Distilled water was adjusted to pH 7.0 with 1N. NaOH. Slurries of the individual soil samples were made and the pH of each measured on a Beckman Expandomatic pH Meter. Supernatants from current broth cultures were also collected for pH determinations.

Test Results:

SOIL	pH	FLASK CULTURES	pH
LE Soil #1	7.7	LE - 1	7.4
2	8.2	2	7.35
3	8.7	4	7.4
4	8.2	6	7.8
6	8.2	8	7.6
8	8.7	HRC - 1	7.7
HRC Soil #1	8.5	2	7.4
2	8.2	3	7.4
3	8.5	4	7.65
4	7.8		
Afyon Soil	8.2		
Bayat Soil	8.4		

TABLE 31

GROWTH RESULTS OF INITIAL PROCESSING OF HRC-B SOIL

(CULTURE MEDIA ADJUSTED TO pH 8.2 AND pH 7.1)

	Broth Media		
HRC-B Soil	MB-11 (Morphine Broth pH 8.2)	MB-12 (Morphine Broth pH 7.1)	MBE-10 (Enriched Morphine pH 8.2)
Afyon	No growth	No growth	Heavy growth
Bayat	No growth	No growth	Heavy growth

TABLE 32

GROWTH RESULTS AFTER THREE PASSAGES OF ORGANISMS FROM HRC-B SOIL

Original Source of Inoculum	Broth Media		
	MB-11 (pH 8.2)	MB-12 (pH 7.1)	MBE-9 (pH 8.2)
Afyon	2	1	4
Bayat	3	1	4

(Numbers refer to quantitative estimates of growth)

TABLE 33

GROWTH RESULTS AFTER PASSAGE OF ORGANISMS FROM MORPHINE BROTH AND ENRICHED MORPHINE BROTH

(HRC-B SOIL ORGANISMS)

Inoculum		Broth Media	
Original Soil	From Growth Medium	MB-11 (Non-Enriched)	MBE-10 (Enriched)
Afyon	Enriched	1	4
Afyon	Non-Enriched	2	4
Bayat	Enriched	-	4
Bayat	Non-Enriched	-	4

(Numbers refer to quantitative estimates of growth)

TABLE 34

GROWTH RESULTS FROM PROCESSING OF MORPHINE-FED SOIL

Inocula		Broth Media		
Culture Medium Source	Soil and Site	MB-11 (Morphine Broth pH 8.2)	MB-12 (Morphine Broth pH 7.1)	MBE-8 (Enriched Morphine Broth pH 8.2)
MB-11	LE-1	1	1	4
	LE-2	-	-	4
	LE-3	1	1	4
	HRC-1	-	-	4
	HRC-2	-	-	4
	HRC-3	2	-	4
MBE-8	LE-1	2	2	4
	LE-2	2	1	4
	LE-3	2	1	4
	HRC-1	-	-	4
	HRC-2	1	1	4
	HRC-3	1	-	4

(Numbers refer to quantitative estimates of growth)

APPENDIX

Pages 61-71

A NEW SENSITIVE ENZYMATIC FLUOROMETRIC ASSAY FOR MORPHINE

DAVID N. KRAMER, PH.D. ____ WILLIAM SWAIN

Kupferberg et al. (1) reported a sensitive fluorometric assay for morphine in plasma and brain based on the oxidation of morphine to pseudomorphine by the use of potassium ferricyanide in weakly alkaline medium. Figure #1.

The method required extraction of the tissue at ph9.0 (bicarbonate buffer) with 10% butanol in $CHCl_3$ and re-extraction with 0.01N HCl. The sensitivity achieved was 0.1 µg-10 µg/ml of solution measured.

Takamori (2) by reducing the volumes of the reaction mixture, obtained more efficient oxidation of morphine at low concentrations, and was able to assay quantities of 10 nanograms in a reaction volume of 42 microliters. Takamori also added a protein precipitation step prior to extraction from plasma. In samples low in protein content, however, the protein removal was not found to be beneficial.

Blackmore et al. (3) reported an automated fluorometric method based on the chemical oxidation of morphine to the fluorescent pseudomorphine.

The disadvantages of the ferricyanide oxidation method are:

1. excitation wave length at 254 mµ results at a lower fluorescence efficiency as compared to λ ex=320 n m (which has twice the fluorescence efficiency).

2. the concentration range is limited to 0.1 µg - 10.0 µg as reported by Blackmore et al. (3) requiring 10 nanograms -- 100 nanograms per sample volume assay.

3. since ferricyanide has considerable absorption,it affects the calibration curves and the method requires preparation of appropriate calibration curves.

Guilbault and Kramer (4) reported that α-naphthols may be oxidatively coupled by use of peroxide and peroxidase. The reaction was found to proceed smoothly and rapidly and was used as an assay method for microgram quantities of peroxide as well as an assay for peroxidase.

In conjunction with a study of the metabolism by microorganisms of morphine, it was necessary to assay residual morphine in bacteriological media and the peroxidatic assay of morphine was investigated. The oxidative coupling is given in Fig. #2.

EXPERIMENTAL

Reagents

Peroxidase. A stock, 400 units/ml. solution of horseradish peroxidase (California Biochem. Co., Los Angeles, Calif., specific activity 400 purpurogallin units per mg.) was prepared by dissolving 100 mg. of enzyme in 100 ml. of distilled water.

Hydrogen Peroxide

A 4.3x10-5 solution was prepared by diluting a 30% stock solution of hydrogen peroxide (Merck & Co., Rahway, N.J.) in distilled water.

Buffer

0.5M Tris, pH 8.5 was prepared in the standard way. An 0.5M glycine-NaOH buffer pH9.0 was employed to alkalinize solutions prior to extraction.

Morphine Sulfate

A solution containing 2.66 micrograms/ml. was prepared in distilled water (a concentration of 1.0 µg/ml. of free base). The stock solution was diluted with distilled water, as necessary. Appropriate concentrations of morphine were added to bacteriological media containing standard Trypticase soy broth and nutrient broths.

Extraction Solvent

An extraction solvent was prepared containing 10% butanol in $CHCl_3$.

Procedure

Preparation of standard curve: One-half ml. volumes of spiked bacteriological media was diluted to one ml. with glycine buffer and extracted once with 10 ml. of 10% n-butanol in chloroform. After phase separation, the upper layer was removed

by aspiration and 9 ml. of the extract was transferred to a 12 ml. centrifuge tube and 1.2 ml. of 0.01 HCl was added. One 1 ml. of the aqueous extract was evaporated to dryness in vacuo and 0.8 ml. of this buffer, and 0.1 ml. standard peroxidase solution and 0.1 ml. peroxide solution were added. After 15 minutes, the volume was brought to a total of 3 ml. and assayed spectrofluorometrically.

Suitable blanks are run concurrently, λ ex 320, λ em-436 nm. Fig. #3 gives the calibration curve obtained by this procedure.

RESULTS AND DISCUSSION

A calibration curve (Slide #3) was obtained by spiking bacteriological media with known concentrations of morphine. The fluorescence was found to be linearly related to morphine concentration in the 0.1-10 µg/ml. range. While the indications are that the sensitivity could be extended to significantly lower concentrations by decreasing the peroxidase concentrations used since the blank fluorescence was attributed to the peroxidase.

Table I summarizes results of analysis of samples of unknown bacteriological media in the presence and absence of microbial growth.

Fig. 4 represents a study of the stability of the pseudomorphine in the presence of peroxide and peroxidase. The fluorescent product concentration appears rapidly within the first five minutes, reaching a maximum in 10 minutes, and remains constant for at least 100 minutes.

With respect to the oxidation of morphine, either by alkaline ferricyanide or peroxide/peroxidase, it is assumed that by analogy to the oxidation of β-naphthols the structure of pseudomorphine is shown in Slide #1. It is not known whether the allylic hydroxyl or the allylic double bond are affected.

The advantages of the peroxide-peroxidase method over the alkaline ferricyanide procedure would appear to be in that the enzymatic oxidative coupling is affected by less drastic conditions, decreasing the probability of side reactions. The solutions

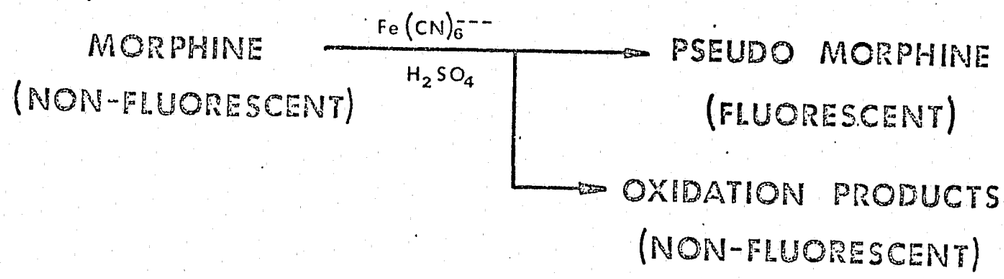
are colorless and are amenable to spot-type tests for use in routine screening. Studies are in progress to determine the utility of the method for urine and plasma analyses. A scheme is presented for routine assay of morphine in biological fluids. (Fig. #5)

TABLE I Recovery of Morphine from Bacteriological Media

	Morphine mg/ml Expected	Morphine mg/ml Found
1.	0.76	0.75
2.		0.70
3.		0.75
4.		0.80
5.		0.85
6.		0.90
7.		0.80
8.		0.85
9.		0.85
10.		0.98

FIGURE #1

CHEMICAL ASSAY OF MORPHINE



BIOCHEMICAL ASSAY OF MORPHINE

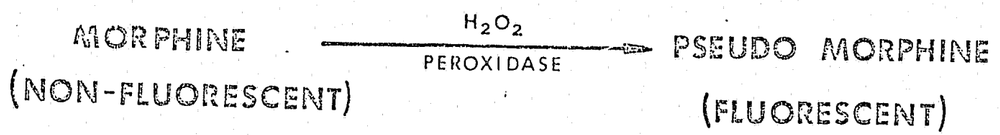
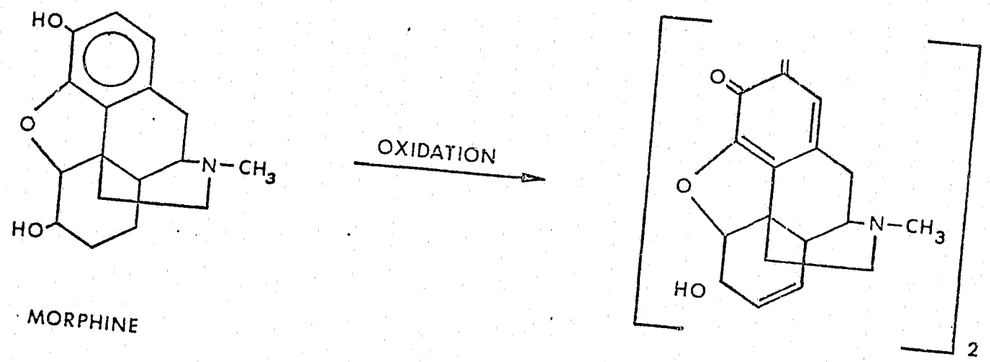


FIGURE #2

OXIDATION OF MORPHINE TO PSEUDO MORPHINE



MORPHINE

PSEUDO MORPHINE
2,2'-bi MORPHINE

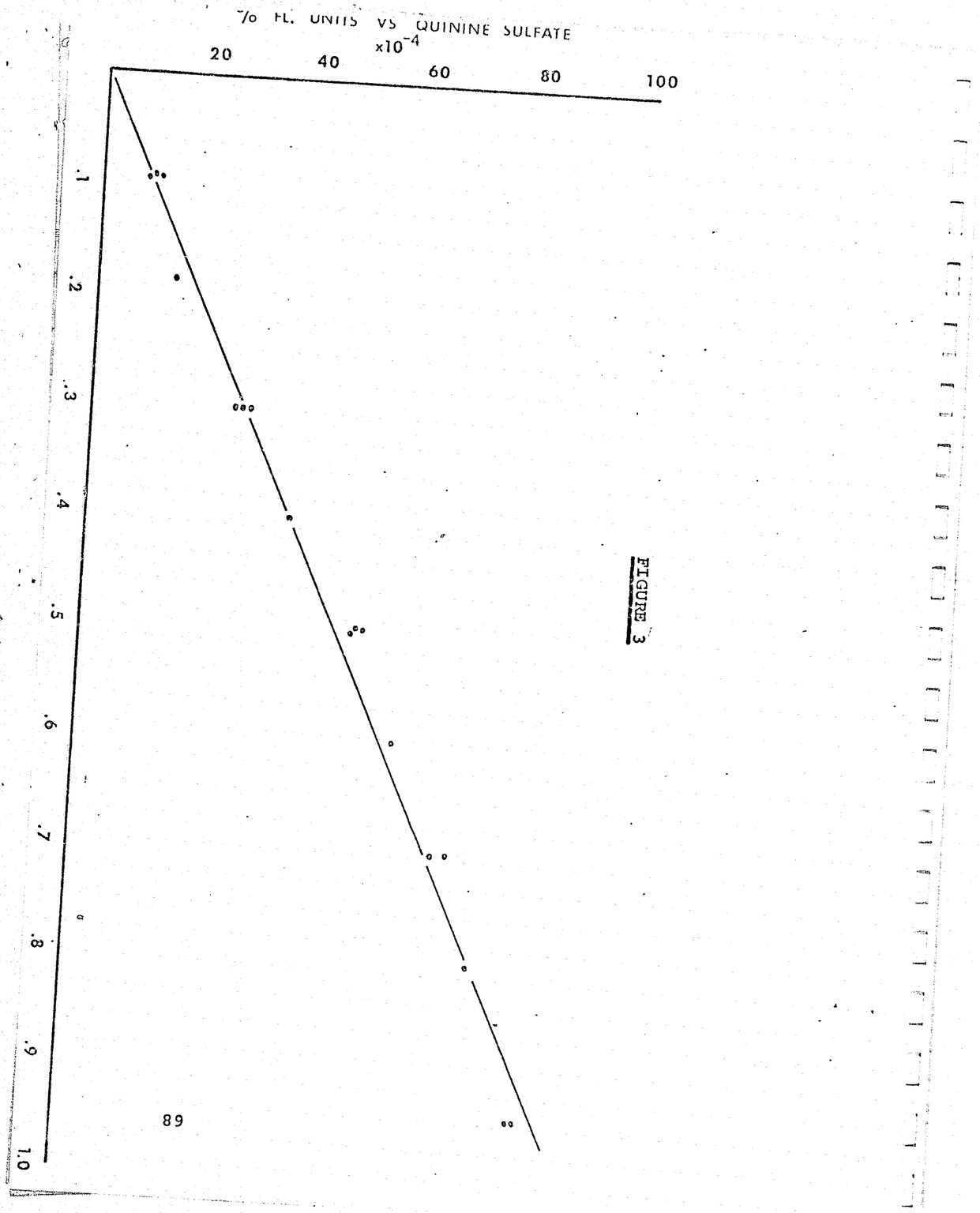


FIGURE 3

FIGURE 4 - STABILITY OF PSEUDOMORPHINE UNDER CONDITIONS OF TAKEMORI

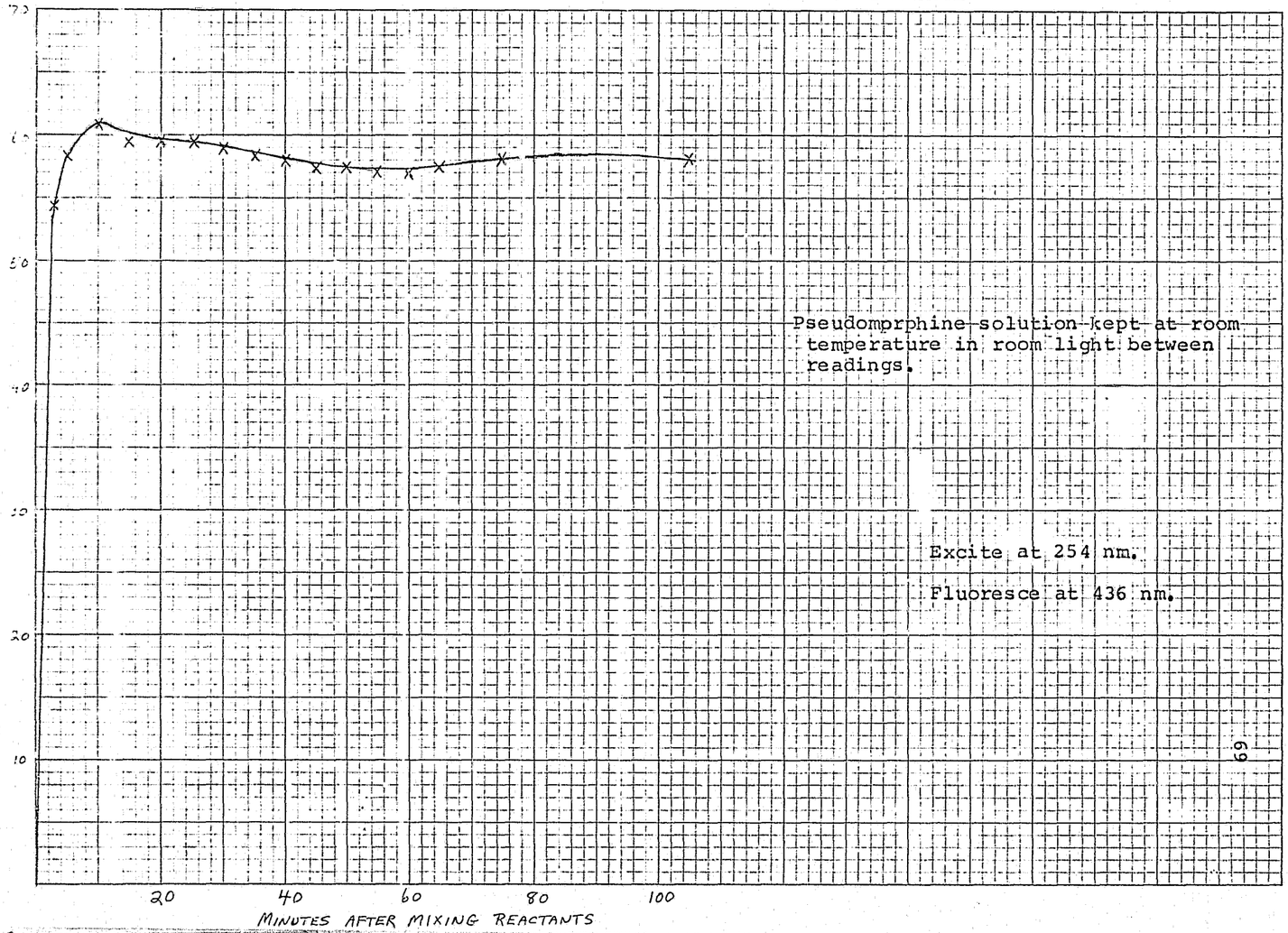
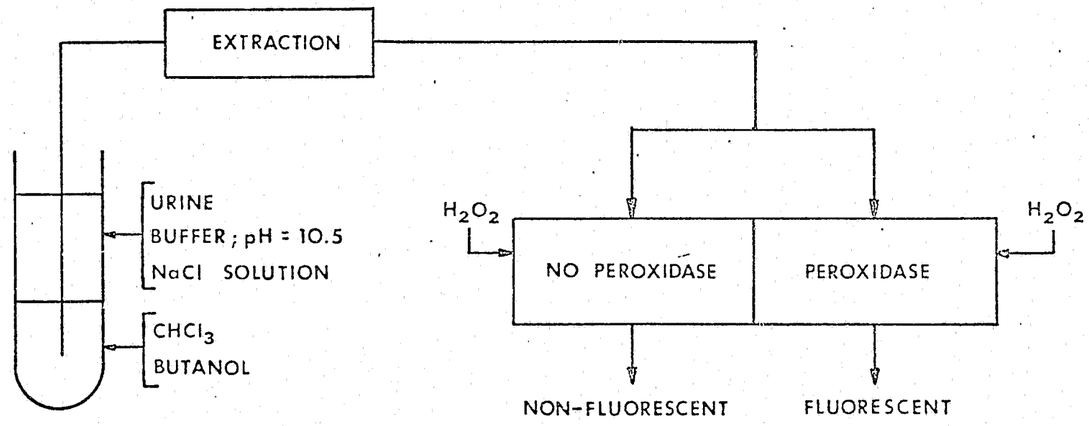


FIGURE #5



FLUORESCENT: + TEST FOR MORPHINE

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