INDIVIDUALIZATION OF BLOODSTAINS

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ABSTRACT

The successful scientific evaluation of bloodstain evidence has the potential of identifying a suspect as being associated with a particular crime. Thus as associative evidence, it is potentially the most valuable since it can be used not only to differentiate individuals but also to calculate the uniqueness of that individual in a population. There are many problem areas in the successful scientific evaluation of bloodstain evidence which can only be conquered by extensive research into the problems. Also there are many techniques not commonly used in bloodstain analysis which appear applicable.

This research has been directed toward these problem areas and to expanding the criminalists' knowledge and capabilities to enhance the usefulness of bloodstain evidence in the court system.

First, population frequencies for the isoenzymes esterase-D and carbonic anhydrase-C were determined. These statistics are for the Pittsburgh area and may be incorporated into a national survey being conducted as Project 7910 of The Aerospace Corporation Contract on the Development Contract for LEAA.

Second, Gm typing statistics were initiated although sufficient quantity and quality of antisera in this country greatly limited our efforts. To surmount this problem we have contacted and encouraged several potential sources in the United States. Also, we have successfully completed a pilot study to eliminate a time consuming step requiring fresh indicator cells and one of the difficult to locate antisera. This reagent is specific for one Gm type and the method is potentially applicable to all types.

Third, additional data is being collected using our sex determination technique, developed under our previous grant (LEAA Grant No. 75NI-99-011), to further demonstrate the validity of such testing.

Fourth, a technique for the determination of drugs in a bloodstain has been developed and the work presented to the Criminalistics Section of the American Association of Forensic Scientists in San Diego, February, 1977. This technique for dilantin also works with digoxin and morphine.

Fifth, an improved technique for the isolation and typing of haptoglobins from bloodstains has been developed, was presented at the Joint Meeting of the Northeast and Mid Atlantic Forensic Science Associations and is to be published in Forensic Serology News.

Sixth, we have continued studying the MN system and have submitted a report of our present findings as related to bloodstain testing to the Journal of Forensic Science.

Seventh, several crime laboratory personnel have visited our laboratory for informal training in several areas of developing forensic techniques. Notably:

Name	Location	Procedure
Ralph Plankenhorn	Greensburg, Pa.	Gm, CAII, etc.
James Zotter	Houston, Texas	Gm
Fran Gdowski	Trenton, N. J.	Gm, Hp, CaII, etc.
Shirley Phillips	Baton Rouge, La.	RIA

Additionally we keep our contacts with the Pittsburgh and Allegheny County Crime Laboratory and teach courses in the Forensic Science Masters program taught between the Crime Lab and the University of Pittsburgh.

Finally and quite importantly, we have provided expert technical assistance and witness on several problem cases from various parts of the country; Pennsylvania, New Jersey, New York, Texas, and Kentucky.

PREFACE

This study has been concerned with a continued effort toward individualization of forensically important physiological fluids. It has been sponsored by the Law Enforcement Assistant Administration. It aims at the development of new and necessary study of existing blood individualizing techniques which will increase the knowledge and capabilities of the forensic serologist and the utility of bloodstain evidence in the Criminal Justice System of the United States.

The results of this study will provide the forensic serologist with newly adapted and/or improved techniques to possibly determine information concerning the drug therapy and racial and sexual origin of bloodstain evidence as well as other individualizing factors. The statistical information compiled will have immediate application in the courts of Western Pennsylvania and can also be used as part of a national data base.

This report summarizes the important and practical results which were obtained, as well as presenting detailed methodology for the practicing forensic scientist.

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SUMMARY

Criminalistics is committed to establishing the truth or falsity of alleged facts, especially the guilt or innocence of the accused. In this regard, criminalistics is truly an integral part of the criminal justice system. Without the scientific evaluation of physical evidence, few convictions could be obtained during the course of litigation.

Blood evidence is potentially the most discriminatory type of associative evidence in terms of relating to a specific suspect or victim except fingerprints. Advances in blood individualizing capabilities, therefore, will allow the criminalist to select from a variety of specific systems, those best suited to the requirements of each case.

Although blood frequently appears as clue material in a wide variety of crimes, it presently falls short of its potential to provide better associative evidence for several reasons: 1) Reliable population frequencies for some blood factors have not been established, especially in the U.S. 2) Methodology for other factors has not been developed for dried blood; 3) The forensic applicability of other potentially rewarding systems has not been investigated; and 4) Most crime laboratories cannot afford personnel time for research projects, especially complicated blood-profile studies, nor can they afford costly equipment solely for research purposes.

That blood will continue to be an important aspect of associative evidence examination was underscored by the crime laboratory directors when, at their symposium sponsored by the Federal Bureau of Investigation in Quantico, Va., December 1973, they, as a group, gave top priority to bloodstain evidence and decreed that this area should be further investigated.

The National Institute of Law Enforcement and Criminal Justice, Law Enforcement Assistance Administration, also recognizes the problem of bloodstain analysis and its value as associative evidence. It has contracted with the Aerospace Corporation to evaluate the blood problem. The findings were published as "Survey and Assessment of Blood and Bloodstain Analysis Program", April, 1974. Their conclusions indicate that blood is potentially one of the most valuable types of associative evidence but that its utilization in the crime lab is limited because of inadequate personnel training, the lack of statistical data for several forensically important systems, and the availability of reliable methods. They also conclude that other systems, not currently in forensic use, have potential discriminatory power greater than that of many systems currently in use.

The discriminatory power of an analytical system is a measure of the ability of that technique to "individualize" the specimen in question. The term "individualization" means a total distinction from all other possibilities within the same class or category. To date, forensic serology has been concerned mainly with the identification and typing of inherited biochemical factors found in blood. These include red cell components such as antigens found on the red cell, e.g., ABO, MN and Rh, and isoenzymes found within the red cell, e.g., phosphoglucomutase (PGM), erythrocyte acid phosphatase (EAP), and adenylate kinase (AK), and certain serum components e.g., haptoglobin, Gc, and Gm and Inv(Km) antigens. The data obtained from such analyses permits the analyst to make certain calculations regarding the frequency that a particular blood specimen can be found in the general population, i.e., its uniqueness.

This study was designed to further investigate the individualization of bloodstain evidence and was divided into several parts. Data collection is comprised of three parts.

First, under the LEAA Grant No. 76NI-99-0099, it was proposed to use the method developed by this research group under LEAA Grant No. 75NI-99-0011 to start a much needed data base for Gm types. Even though this effort has been greatly thwarted by a lack of reliable antisera, over 200 samples have been analyzed for Gm types 1,2,3(4), 12(5), 13(10). Dr. Arthur G. Steinberg, a leading American Gm expert, has been contacted to provide us with proper standards and is aiding in our active search for reliable antisera. Additionally, minimizing the problems of time and effort required and the difficulty of reliable test readings of the present assay are the objectives of an improved technique which we have developed.

Second, the use of isoenzyme systems for which the different polymorphic forms are under genetic control are of two types. Those for which the polymorphic system is applicable to the population as a whole regardless of ethnic background and those unique in that the polymorphisms can distinguish between ethnic races.

Approximately 700 samples have been typed for each of two systems, esterase-D a general population system and carbonic anhydrase-C(CAII) an ethnic race distinguishing system. Esterase-D showed variants for approximately 20% of the general population, whereas approximately 18% of Blacks exhibit variants for carbonic anhydrase-C(CAII).

With these methods for typing from dried blood by two more isoenzyme systems of great potential for individualization, the capabilities

of the criminalist are purposefully expanded. The data is applicable to Western Pennsylvania immediately and to future national needs.

Third, in this study the radioimmunoassay method of determining the sexual origin of a bloodstain was to be used to process an additional 500 serum samples for a total of 2000, to analyze the same stains of two different ages for differences in steroid ratios, to analyze samples from endocrine abnormal patient as a possible explanation of the 5-8% error associated with this sexing procedure and to collect samples from crime laboratories as a blind test of the procedure.

The samples have been collected and processed through the two column chromatography steps and are awaiting newly ordered reagents for completion of the radioimmunoassay.

The division concentrating on new and/or improved techniques encompasses Gm, haptoglobins and RIA of drugs from bloodstains.

First, the testing for Gm factors, which have great potential forensically because of their location on the abundant and stable IgG class of immunoglobulins, needs an improved test procedure for use in dried bloodstains. The technique of absorption inhibition is reliable in wet blood, but falls short in dried blood by the nature of the inhibition test where the presence of the Gm antigen being detected is indicated by no agglutination of appropriately sensitized cells, i.e., a negative result.

It has been demonstrated that anti-Gm, can be covalently bound to acrylamide beads, that this bound antibody will selectively bind the Gm containing IgG molecule which can then be positively located by commerical rabbit anti-human IgG sera which is fluorescently labelled. A simple fluorometer can show the presence of a Gm antigen and even be used to quantitate it. This procedure accomplishes several ends: 1) It gives a positive indication for a positive result which is quite desirable in forensic work, 2) Difficult to obtain anti D-Gm specific sera for sensitizing

0^+ red blood cells, the fresh 0^+ cells and an inherent variability of different cell sources is eliminated and 3) The system is potentially adaptable to all Gm and Inv(Km) typing.

Second, a pilot project was initiated to study the quantitative extraction of haptoglobins in older stains. Variable extraction conditions of high and low pH with and without a variety of detergents at several concentrations were employed with the haptoglobins quantitated by rocket immunoelectrophoresis.

The results of this study show that the isolation and detectability of haptoglobins and presumably other genetic markers as well are dependent upon the extraction conditions, the time of extraction and the age of the stain. Thus, in older stains the ability to successfully analyze genetic markers may be due, in part, to the ability to isolate the substance from the stain.

Third, it would be advantageous to identify substances in the blood which would serve to differentiate bloodstained evidence and which are not subject to the same degradative influences as the genetic markers, because the substances are non-genetic in humans. Such substances could be prescription or illicit drugs from a victim or suspect. Such knowledge would be particularly useful where prior information indicated one of these individuals was using a drug and the technique could also be used for general screening.

To obtain this identification a commercially available radioimmunoassay (RIA) was adapted to determine levels of diphenylhydantoin (DPH) in bloodstains, saliva and semen. Methods for extraction of DPH from stains and the size of stain required were investigated. Although these tests were conducted with DPH, a drug used in epilepsy therapy, because of the

availability of a known DPH treated patient, the potential exists for the detection of many other drugs, by this same general method, that would be of interest to those involved in the investigation of crime. The technique has currently been expanded to cover digoxin and morphine.

A third division of this study has concentrated on the problems of mistyping of the MN system in bloodstains. The recently released results of a nationwide proficiency test of crime laboratories show an error as high as 40% is obtained in MN typing as compared to 1.6% for ABO. These results suggest that the fault is not necessarily in the ability of crime laboratory personnel to use these techniques, but quite possibly with the system, reagents, or the application of these particular techniques to the MN system.

From various tests aimed at the cross-reactivity problem and testing of several commercial, as well as in-house manufactured antisera, it has been concluded that this is a problem in a very useful system, that has an answer given sufficient study, but that it should be discontinued in dried stains until existing testing procedures and antisera production are changed or modified to allow consistently accurate analysis.

A fourth division of our work has involved the direct application and distribution of our research gained knowledge in addition to normal publishing of results and presentation of papers. During the past year ten cases from various parts of the country, in which the evidence was either old or beyond the capability of the local crime laboratory, were analyzed in our laboratory. In some of the cases Dr. Shaler then provided expert testimony. In other cases crime laboratory personnel from the originating jurisdiction accompanied the evidence to our laboratory and were themselves instructed and aided in particular analyses. This appears to be a good method of introducing new techniques for court

acceptance and at the same time providing some degree of training. Also, we have retained our contact with the Pittsburgh and Allegheny County Crime Laboratory and teach selected courses in the University of Pittsburgh Forensic Science Masters degree program coordinated with the Crime Laboratory. This spring a newly revised problems course will introduce newly developed and potentially problem solving methodologies to these fourteen students.

CHAPTER I. INTRODUCTION AND BACKGROUND

"Individualization" implies total distinction from all other possibilities within the same class or species. Thus certain criteria must be met:

1. Methodology for the unequivocal identification of a suspect material as to class (blood, seminal fluid) and to species (human, dog, etc.).
2. Methodology to sub-classify the material to approach individuality.
3. Available statistical data to allow a decision to be made concerning the uniqueness of the material.

This research has been designed to:

I. Investigate the population frequencies for the Western Pennsylvania-Pittsburgh area for two additional potentially rewarding isoenzyme systems and the serum immunoglobulin antigenic markers of Gm (all values to be included in a national survey now being conducted.

II. The collection of additional data to broaden the sample base of the sexing technique developed by this laboratory under the previous LEAA Grant No. 75NI-99-0011.

III. The development of new and/or improved methodology for determination of Gm's, MN, haptoglobins and drugs from bloodstains.

I. Population Frequencies for Esterase-D, Carbonic Anhydrase II and Several Gm Factors.

Of the more than 300 antigenic systems theoretically available relatively few (ABO, MN and Rh) are routinely identified by criminalists. As a result of extensive, blood bank typing throughout this country extensive local population frequencies are available for ABO and Rh systems[1].

In addition to these antigenic systems, there are numerous polymorphic protein systems (isoenzymes, albumin, Gc, transferrin, hemoglobins, haptoglobins, etc.)[2] which could be utilized. These determinations are all based

on electrophoretic separations and laboratories with electrophoretic capabilities are becoming increasingly aware of the potential of such methods for individualization of bloodstain evidence.

Courts are especially interested in having frequency values of these systems for the population in which the crime occurred to allow a proper calculation of uniqueness. Prior to our previous grant (LEAA 75NI-99-0011) the only readily available statistics were derived from those of the United Kingdom. That these frequencies do change from one population to another has been illustrated for the Chinese and Korean populations for the immunoglobulin allotypes[3], for phosphoglucomutase in the German population[4] as well as for the ABO system over Europe, Asia and Africa[5]. Following our lead in obtaining statistics for the isoenzymes phosphoglucomutase (PGM), erythrocyte acid phosphatase (EAP) and the serum protein haptoglobin (Hp) several other grantees will now compile statistics for other regions of the country.

With the increased electrophoretic capabilities and knowledge which will follow such a national statistical survey, it is only proper to add additional polymorphic systems and their statistical probabilities which show good forensic potential and utilize the same equipment and general knowledge.

The statistics for the isoenzymes Esterase-D, a general population variant[6], and Carbonic Anhydrase II, an ethnic variant[7], introduced in this study should rapidly become useful to the practicing criminalist.

The Gm and Inv(Km) systems are potentially very useful forensically. These are polymorphic regions of serum proteins which are associated with antibody molecules[8]. Gm groups are polymorphic antigenic determinants carried on the gamma chain (heavy chain) of gamma globulins (IgG) while the Inv(Km) groups are found on the kappa chains (light chains) of IgG, IgM and IgA immunoglobulin molecules[9]. Presently there are approxi-

mately 18 well-defined Gm and 3 Inv(Km) antigenic determinants which have been used in population studies and which reveal that each person inherits a combination of Gm and Inv(Km) types based on his genotype[10,11,12]. Like the isoenzymes, anthropological "races" may retain a certain combination of Gm factors. Thus their value does not lie solely in discriminating among individuals, but also in possibly estimating the racial origin of a bloodstain.

The forensic applicability of Gm and Inv(Km) has been established[13]. The stability of the Inv(Km) factor was more than adequately demonstrated by its identification in a 22 year old bloodstain[14]. Through our previous funding (LEAA Grant No. 75NI-99-0011), Dr. Shaler adapted methodology to allow Gm typing in blood, semen and vaginal stains[15]. The Gm factors are inherited as certain haplotypes with differing gene frequencies in different populations as demonstrated by studies in Hungary[16], China[17] and Scotland[18]. Again as with the other antigenic and polymorphic protein determinants these frequencies are needed to allow calculation of uniqueness.

II. Sexing of Bloodstains

A radioimmunoassay (RIA) technique of quantitating testosterone and 17-β-estradiol to determine the sexual origin of a bloodstain was developed by this laboratory under previous funding, LEAA Grant No. 75NI-99-0011. The basis for such a test was that it had been known for some time that major sex characteristics are to some extent dependent upon plasma concentrations of selected androgenic (testosterone) and estrogenic (17-β-estradiol) steroid hormones. The use of testosterone levels in the pregnant female[19] and in amniotic fluid to determine fetal sex has been demonstrated. The advantages of the RIA over cytological techniques of searching for the chromosome in lymphocytes[20] to show male origin are an

optimal stain (a crust of blood) is not required, the RIA technique does not require the extensive experience that karotyping does, RIA is a useful technique in other areas, such as determining drugs in bloodstains, a negative result by the cytological technique may mean that the Y chromosome was present but not found and stains eight months old have been successfully typed by the RIA technique as to sexual origin.

III. The Gm system, as mentioned earlier with 18 well-defined factors, has great forensic potential, but it has several problems of technique and required antisera presently hindering its widespread use. Each of the factors tested for requires two special antisera of human origin and fresh type O Rh positive cells for the present technique of hemagglutination inhibition[10,21]. A proper dilution of unknown serum is incubated with a proper dilution of anti-Gm serum specific for the the factor sought. If the factor is present it binds to the anti-Gm and inhibits it from causing agglutination when combined with a proper dilution of O^+ red blood cells sensitized with anti-D antisera possessing the same Gm factor. The result is no agglutination if the factor were present or agglutination if it were absent. This type of test works quite nicely on serum, if one has all of the necessary reagents. But good quality reagents, two for each factor, are not at all readily available in this country except for a few research laboratories where their sources are quite guarded and even they are lacking some of the reagents. Also the necessity of coating fresh O^+ red blood cells, obtaining proper sample sizes from bloodstains, obtaining a negative test for a positive result and vice versa require a great deal of effort and controls to obtain reliable results, if the antisera were available.

In this study we had proposed to develop a reagent by coating anti-Gm sera for a particular factor onto latex particles and thus obtain a

positive agglutination of latex particles when the factor was present and eliminate the O^+ cells and the anti-D-Gm sera for each factor. This would make a much simpler test, quite useful for wet serum testing, but still depends on agglutination which is dependent on proper ratio of reagent to sample.

It was decided to devise a means of testing which could eliminate a difficult to obtain reagent and the need for agglutination by covalently binding anti-Gm IgM immunoglobulins to inert beads which would bind the IgG containing factor from a sample, if present, and then qualitatively and quantitatively determine its presence with a fluorescein or radioactively labelled commercially available anti-human IgG serum.

The determination of haptoglobin (Hp) phenotypes in dried blood is another potentially very useful method for differentiating individuals. As part of LEAA Grant No. 75NI-99-0011 this laboratory determined the phenotypic frequencies for Hp. A method of typing Hp's in dried blood stains using density gradient polyacrylamide gel electrophoresis was demonstrated to the Southern Forensic Association in Tallahassee, Florida, 1976. A problem which still remained was a very critical one, that of proper extraction of the haptoglobins from older dried stains. This problem has been studied and a copy of the paper submitted to Forensic Serology News is included as Appendix A.

Although there are sufficient blood factors genetically determined to individualize a bloodstain were analyses for enough of them performed, these factors appear to have variable persistence and some are probably not as useful in older stains. Thus a technique for detection of drugs in bloodstains was developed and presented to the American Association of Forensic Science in San Diego, California, February, 1977. A copy of the paper submitted to the Journal of Forensic Science is included as Appendix B.

The MN system of red blood cell antigens is a system with which the forensic serologist is having problems as demonstrated by the recently released proficiency testing data. The study by this laboratory of the system and sources of these errors is included as Appendix C which has been submitted to the Journal of Forensic Science.

This laboratory's application of new techniques, particularly Gm typing on old evidence as well as proper utilization of existing techniques, has aided the cause of justice in several parts of this country and provided a measure of training for the criminalists of the originating jurisdictions. A detailed discussion of several cases, some of which have not yet come to trial, is included as Appendix D.

CHAPTER II. EXPERIMENTAL

I. Population Frequencies for Polymorphic Systems of Forensic Interest

A. Esterase-D (EsD)

Samples were obtained from both the Pittsburgh Central Blood Bank and the Allegheny County Jail by venepuncture collection in EDTA-anti-coagulant vacutainer tubes. For each sample 2 drops of red blood cells were placed in individual spot plate wells and an equal amount of 0.05M dithiothreitol added to lyse the cells and reduce disulfide bridges to yield active enzyme. Strips of cotton sheeting 2 x 5mm were soaked in each well for 10 minutes before being moved up the side of the well to allow the excess to run off just prior to insertion into 12% starch gel.

Different buffers are used for the gel and bridge as in the method of Parkin and Adams[22]. The gel buffer was a solution of 6.75mM Tris, 1.8mM citric acid, 2.2mM boric acid and 0.2 mM lithium hydroxide with a final pH of 7.2 at 4°. Any adjustments necessary to raise or lower the pH to 7.2 were made with lithium hydroxide and citric acid respectively. The bridge buffer was a solution of 440mM boric acid and 40mM lithium hydroxide, pH 7.2. To aid in dissolving the boric acid the buffer was heated to 40° and then lowered to 4° to adjust the pH to 7.2. The buffers were stored at 4° and discarded at any sign of cloudiness or precipitation.

The 12% starch gel[23,24] was made by dissolving 10.3g of Sigma starch-hydrolyzed for electrophoresis, S-4501, in 100ml of gel buffer in a 500ml flask. The solution was heated over a gas flame, swirling continuously until it began to boil. It was degassed by vacuum aspiration and poured onto a 20 x 20 cm glass plate with 2mm thick borders. The plate was tilted for even distribution of the hot solution over the

entire surface and then allowed to stand level for one hour at room temperature followed by half an hour on the cooling platen at 4°.

Slits, 6mm wide and 5mm apart were cut into the gel 7cm from the end to be the cathode with a cut razor blade. The sample strips were inserted into the slits. The plate was set back on the cooling platen in the electrophoresis tank and connected to the buffer tanks by one thickness of Whatman #3MM chromatography paper soaked in bridge buffer. The plate was run at 12 volts/cm, constant voltage, for eight hours.

The isoenzyme patterns are developed by reaction with a 4-methylumbelliferylacetate (4MUBA)(Sigma) filter paper overlay. Ten milligrams 4MUBA was dissolved in 2ml of acetone and then diluted to 10ml with reaction buffer composed of 0.05M sodium acetate-acetic acid, pH 6.9. This solution, made up just before the completion of the electrophoretic run, was poured onto a piece of filter paper, carefully laid over the gel to prevent trapping any air bubbles. The plate was incubated at 37° for ten minutes after which the paper was removed and the patterns read under long-wave ultraviolet light.

B. Carbonic Anhydrase-C (CA II)

Because polymorphic variants of carbonic anhydrase-C (CA II) are almost exclusively associated with Blacks, blood samples were collected from American Blacks of the Pittsburgh area, largely from the Allegheny County Jail, by venepuncture into EDTA-anticoagulated vacutainer tubes. The samples were stored at 4° and then frozen at -20° to lyse the red blood cells which contain the CA II. Lysed whole blood was absorbed onto Whatman #3 filter paper strips (2 x 5 mm) and inserted into a 12% starch gel.

Gel and tank buffers are essentially those of Hopkinson, et al[25]. A stock buffer is 0.9M Tris, 0.5M boric acid, 0.02M EDTA pH 8.6 at 4°.

The tank and bridge buffer is a 1:14 dilution and the gel buffer is a 1:30 dilution. The starch gel is made of 10.3g sigma starch dissolved in 100ml of gel buffer, heated to boiling, degassed and poured onto a 2mm x 20 x 20cm plate which is allowed to cool for 1 hr prior to insertion of the sample stips. Electrophoresis is carried out at 4° at 100 volts for 16 hours or 400 volts for 4 hours.

Visulaization of CA II isoenzymes was carried out by dissolving 10mg fluorescein diacetate in one ml of acetone, diluting it to 10ml with 0.1M sodium phosphate buffer pH 6.5 and pouring this over the filter paper covered gel from which the sample inserts had been removed. After 35 minutes of incubation at 37°, the phenotypes were observed under long-wave ultraviolet light.

C. Procedural Analysis of Serum for the Presence of Gm Factors[15].

1. Titration of Gm antisera. Appropriate antisera were serially diluted (1:2, 1:4, 1:8, 1:16, 1:32, etc.) to 1:512. One drop of each dilution was incubated with one drop of a 2% suspension of sensitized red cells (see below) for 30 minutes at room temperature. The suspension was centrifuged at 1000 xg for 30 seconds and the agglutination was read macroscopically. The working dilution was the next to the last one which gave a 4+agglutination.

2. Sensitization of red cells. One drop of packed saline washed 4 times, O^+ red blood cells were incubated with three drops of incomplete anti-D serum containing the appropriate Gm factor. The cells and antisera were incubated for 1 hour at 37°C, centrifuged, washed 3 times in saline and resuspended to yield a cell suspension of approximately 2%.

3. Identification of Gm antigens in serum. Serum to be tested for the presence of Gm antigens was diluted 1:10 with saline. One drop of

the diluted serum was mixed with one drop of appropriately diluted antiserum and incubated for 30 minutes at room temperature. Then one drop of sensitized red cells were added and incubated for 30 minutes at 4°C. The suspension was centrifuged for 30 seconds at 1000 xg and agglutination read macroscopically. Because this is a hemagglutination inhibition test a positive agglutination means that the Gm factor was not present in the serum tested, whereas a negative agglutination means that the Gm factor was present.

II. Sexing of Bloodstains

The determination of the sexual origin of a bloodstain is carried out by a lengthy procedure developed in this laboratory as part of LEAA Grant No. 75NI-99-0011, the final report of which contains a complete detailed procedure[15]. This method was presented to the American Association of Forensic Science, Washington, D.C., February, 1976, and the above mentioned final report is to be published.

A. The procedure involves the following steps:

1. Solubilization of the bloodstain with phosphate buffered saline.
2. Extraction of the steroids by XAD-2 Amberlite resin.
3. Separation of the steroids Progesterone, Teststerone and Estradiol by LH-20 Sephadex Chromatography.
4. Radioimmunoassay quantitation of the separated steroids.
5. Calculating the ratio of Testosterone to Estradiol which allows a greater than 90% correct determination of the sexual origin of the stain.

B. In this study 500 serum samples with 200 bloodstains 1 to 3 days old and 200 corresponding bloodstains 3 to 4 months old were processed.

Additionally, pathological samples obtained from Dr. H. Nakkin of Montefiore Hospital and Dr. L. Loreaux of N.I.H. are being examined.

III. Improved Methodology and Study of Gm's, MN, Haptoglobins and Drugs from Bloodstains

A. Development of an Imporved Reagent to Detect Gm Factors

1. Isolation of Anti-Gm_1.

The anti-Gm_1 immunoglobulin largely IgM of sera obtained from humans (Plasma Blood Alliance, Knoxville, Tenn.) was isolated by a general procedure of Cambier and Butler[26]. Clarified whole whole serum is dialyzed against 0.01M pH 5.4 phosphate buffer. The euglobulin precipitate is washed two time in the same buffer and resolubilized to 20% of the original volume in 0.01M sodium acetate, pH 5.4 that is 0.15M in NaCl. The solution is clarified by centrifuging at 3000 xg for 15 minutes before being applied to a Sephadex G-200 column equilibrated in 0.1M phosphate, 1.0M NaCl and 0.02% NaN_3, pH 7.2. The IgM-anti-Gm elutes in the void volume as calibrated by blue dextran.

This procedure gives anti-Gm_1 free of any IgG contamination. It is necessary to eliminate IgG of the anti-sera to allow determination of IgG molecules containing specific Gm factors of the sample by using labelled rabbit anti-human IgG antisera.

2. Activation of Polyacrylamide Beads

The idea behind this reagent was to replace O^+ red blood cells with polyacrylamide beads and not have to depend on the hemagglutination-inhibition negative result for a positive determination. In order to attach the anti-Gm_1 to polyacrylamide beads the beads are activated by reaction of the amide side groups with a 10 times excess of pentanedial, glutaraldehyde, according to Ternynck and Avrameas[27].

a. Four grams polyacrylamide beads (P-300, Bio-Rad) were swollen in distilled water for 24 hrs at room temperature. The swollen beads are washed in distilled water 10 times.

b. To 100ml of swollen gel add 500ml of 6% glutaraldehyde in 0.1M phosphate, pH 7 buffer. Adjust the pH to 7.0 and incubate at 37°C overnight.

c. Wash the activated beads 15 times with distilled water and store at 4°C.

3. Attachment of Anti-Gm_1 to Activated Polyacrylamide Beads[27]

a. Mix 10ml of purified anti-Gm_1, 15-30mg protein, 0.1M phosphate, 0.15M NaCl, pH 7.4 (PBS) with 10ml of activated beads, 3000 x g, 4° and incubate at room temperature for 48 hr.

b. Collect the derivatized beads by centrifugation, saving the supernatant, and wash the beads with phosphate buffered saline, pH 7.4 until A^{280} is less than 0.05.

c. Block any remaining activated positions by incubating the beads with 0.1M lysine or Tris at pH 7.4, room temperature for 18 hr.

d. Wash with (PBS) 10 times followed by 2 washes with 50ml 4°C 0.2M glycine-HCl pH 2.8 and one time with 0.2M K_2HPO_4.

e. Then wash with (PBS) until A^{280} equals zero and store at 4°C with 0.01% NaN_3.

4. Testing for Gm_1 by manufactured reagent.

a. One ml of suspended beads (above prepared beads as 50ml PBS suspension) was added to each of three 12 x 75 borasilicate culture tubes.

b. To tube number one was added 10µl of saline, 10µl of Gm_1 negative serum to tube number 2 and 10µl of Gm_1 positive serum to tube number 3.

c. The tubes were mixed and allowed to incubate for one hour at room temperature to allow any Gm_1 positive IgG to bind to the anti-Gm_1 derivatized beads.

d. The beads were washed 2 times with PBS and then 50µl of fluorescein labelled rabbit anti-human IgG (Bio-Rad) added to each tube mixed and allowed to incubate at 37°C for 1 hr.

e. The beads are then washed 4 times with PBS by resuspending, precipitating at 1000 x g for 2 minutes, and decanting to remove unreacted fluorescein labelled antibody.

f. The fluorescence and therefore indirectly the bound IgG containing Gm_1 are measured in a fluorometer by exitation at 485nm with emission measured at 525nm.

B. The methods for MN study are included in Appendix C.

C. The methods for haptoglobin extraction are included in Appendix A.

D. The methods of radioimmunoassay determination of dilantin in blood-stains are included in Appendix B.

CHAPTER III. RESULTS AND DISCUSSION

I. Population Frequencies for Esterase-D, Carbonic Anhydrase II and Gm.

The statistical results of this study are presented in Tables 1, 2 and 3 for esterase-D(EsD), carbonic anhydraseII (CAII) and some Gm factors respectively. For esterase-D and carbonic anhydrase II these results, plus the experimental section for each of these isoenzyme systems, provide for forensic serologists a practical approach to the determination of two more genetic markers in bloodstains. As well as providing reliable methods for identifying these polymorphic systems, these data can be used for calculating the uniqueness of a particular blood sample in the population.

The samples for these studies were obtained from both the Pittsburgh Central Blood Bank and the Allegheny County Jail. The race, age and sex of the donor and the drawing data were recorded for each sample. Care was taken to insure that no duplicate samples were taken. The jail samples were the main source of the Black donors. Samples were taken from all persons admitted to the jail whether they were released or held.

A. Population Frequencies of Esterase-D Isoenzymes.

Table 1 shows the results obtained for the esterase-D system. Esterase-D is considered a general population variant and these values for Blacks and Caucasians demonstrate this consideration with nearly equal percentages for both classes. Although no 2-2 types were seen for Blacks, with the presence of 2-1 types the 2-2 phenotype is possible and was probably not seen because of the small number (152) of Blacks analyzed.

B. Population Frequencies of Carbonic Anhydrase II Isoenzymes.

Table 1. Esterase-D

	N		Phenotypes 1-1	2-1	2-2	Phenotypic Frequency 1-1	2-1	2-2	Gene Frequency EoD_1	EsD_2	X^2	Probability
	697	Observed Number	540	149	8	0.775	0.214	0.011	0.882	0.118	0.415	0.53
545 Caucasians + 152 Blacks		Expected proportion	0.778	0.208	0.014							
		Expected Number	542	145	9.7							
		$\frac{(O-E)^2}{E}$	0.007	0.110	0.298							
Caucasians	545	Observed Number	429	108	8	0.787	0.198	0.015				
Blacks	152	Observed Number	116	36	0	0.763	0.237	-				

Table 2. Carbonic Anhydrase II

	N		Phenotypes 1-1	2-1	2-2	Phenotypic Frequency 1-1	2-1	2-2	Gene Frequency $CAII_1$	$CAII_2$	X^2	Probability
American Blacks	646	Observed Number	526	114	6	0.814	0.176	0.009	0.902	0.098	0.0076	0.93
		Expected Proportion	0.814	0.177	0.010							
		Expected Number	525.6	114.3	6.2							
		$\frac{(O-E)^2}{E}$	0.0003	0.0008	0.0065							

Table 2 shows the statistics for the distribution of carbonic anhydrase II isoenzymes in Blacks. The variants of carbonic anhydrase II are apparently found only in Blacks[25] and so only Black donors were used in this statistical survey. With the 2 band of CA II showing up over 18% of the time, this system combined with peptidase-A and hemoglobin variants could make a powerful electrophoretic combination of determining blood originating from a Black.

C. Population Frequency of Gm Factors

The results of approximately 250 samples assayed for Gm factors 1,2,3(4) and 5(12) are shown in Table 3. The same samples were not assayed for the same factors because we didn't have all the reagents at the same time. Data was collected for Gm 13(10) also, but it seemed to be in gross disagreement with the literature and Dr. Steinberg, noted geneticist and Gm specialist, informed us that the Behring anti-Gm_{10} sera used are not of good quality and should not be used.[11] We agree and therefore have not reported these results. All of these sample sizes are too small to consider the frequencies as being those of the Pittsburgh area and further large scale testing should be delayed until a proper array of reagents is available. Our inquiries have prompted several serological firms to start screening for donors of anti-Gm's and anti D-Gm containing antisera so the reagents for the hemagglutination inhibition testing will become more readily available.

Of the data in Table 3, that pertaining to the percentage of Gm_1 and Gm_2 of Black donors is of interest. From African population surveys reported in the literature[10,11,12,28] Gm_1 is present in the haplotypes almost 100% of the time and Gm_2 is absent almost 100% of the time.

Table 3. Gm Frequencies

	Gm 1	Gm 2	Gm 3(4)	Gm 5(12)
White				
Number Analyzed	86	75	25	21
Number Positive	36	22	20	15
Percent Positive	42	29	80	71
Black				
Number Analyzed	170	188	2	72
Number Positive	148	60	2	70
Percent Positive	87	32	100	97

Certain haplotypes of Caucasians lack Gm_1 and others possess Gm_2, thus one can see the white ancestry of many American Blacks who are either Gm_1 negative or Gm_2 positive. The Gm_2 case is easily obtained with one white ancestor who was Gm_2 positive, although the percentage in this small sample seems large. The Gm_1 negative would require two white ancestors who were Gm_1 negative. This is difficult but far from impossible for Gm_1 negative constitutes 70% of the Caucasian haplotypes. American Blacks are of 30% white ancestry[11] and a number of these Gm_1 negative could occur in the population of a northern city. Of five personally known Blacks, along with seven whites whose serum was typed graciously by Dr. Steinberg to give us standards for Gm 1,2,3,5,6,11,13,14,17,21 and 26, one Black girl gave a typical Caucasian Gm typing indicating two white ancestors. Dr. Steinberg states that he would expect a typically Caucasian Gm genotype in approximately 9% of American Blacks. I find it easily imaginable to find pockets of population where this percentage would be exceeded and feel that only a larger study for each major city or region could allow a proper estimation.

II. Sexing of Bloodstains

All of the serum, bloodstains and pathological samples collected were processed through the Amberlite XAD-2 and Sephadex LH-20 columns to extract and separate the steroids and held to efficiently determine the quantities by radioimmunoassay after all of the column chromatography. When the standard curves for the radioimmunoassay were prepared it was realized that we were experiencing difficulties with our reagents and that we should order new ones. Because of the transfer from Dr. Shaler to Dr. Mortimer of the research directorship, a new application was

required by the Health Physicists before new radioactive material could be ordered. The application has now been approved and new reagents ordered so these results will be forwarded when the analysis is complete in approximately 6 weeks.

III. Development of Improved Methodology for Gm, Hp, MN and Drugs

A. Determination of Gm_1 by Anti-Gm_1 Bound to an Acrylamide Bead.

Assay of a saline blank, serum negative for Gm_1 and serum positive for Gm_1 gave the following fluorometer readings:

Sample	Intensity of Emission at 525 nm
Saline Blank	0.25
Gm_1 negative control serum (Mortimer)	0.31
Gm_1 positive control serum (white)	0.62

It can readily be seen that the Gm_1 positive sample yields a reading significantly greater than those for the saline blank and negative control. It is therefore possible to determine Gm_1 by this procedure and the procedure is applicable to all of the Gm types. This procedure of reacting a Gm containing substance with anti-Gm attached to a polyacrylamide bead and then using fluorescein labelled rabbit anti-human IgG to quantitate the attached immunoglobin accomplishes the several goals behind its development: 1) Elimination of the sensitization of fresh 0^+ red blood cells, 2) Obtaining a positive result for a specific Gm positive sample, 3) Elimination of reading of agglutination reactions and 4) Elimination of the requirement of proper dilutions of

each serum and red blood cell preparation. Additionally, I believe that this type of reagent holds potential for a combined reagent to assay for several Gm factors at once from the same sample. This latter feature would fit nicely into a goal towards which this laboratory is working, part of which is in our new proposal under the heading of Maximum Utilization of Bloodstain Evidence.

The results with this new reagent, although quite encouraging, at the same time point to areas which need improvement for a reliable procedure. The most obvious is that the blank and negative control did not give approximately zero for emmission at 525 nm. This problem is largely related to the type and amount of beads used which allowed for nonspecific entrapment of fluorescein labelled anti-human IgG and would require many additional washes to remove. A different porosity bead and density of anti-Gm is to be used on the next preparation of bead reagent. This reagent will then be checked for sensitivity, specificity, ease of handling and storage stability. Generally enzymes and other proteins are more stable when in a solid state form. Bio-Rad, who sells reagents for determining IgG, IgM and IgA concentrations in serum, ships and stores their bead bound antibodies as lyophilized powders.

The quantitative properties of this type of reagent, potentially applicable to all Gm typing, would allow study to optimize the extraction of Gm containing IgG molecules from dried stains. Several of these reagents where the anti-Gm's are attached to separate plastic tubings or screens could be combined to produce a system capable of assaying for several Gm factors from the same sample.

The forensic potential of the Gm system for individualization fully qualifies it for development as a forensic test. The difficulties of Gm

testing require that a method to eliminate inconsistency and incompetence be developed before it is a commonly used crime laboratory test. The method being developed in this laboratory should provide this measure of consistency and competence.

B. Haptoglobin Extraction

It has been the contention of this laboratory that the problems of assaying genetic factors in dried bloodstains are equally interdependently related to the persistence of the factors under various storage conditions and the ability of the forensic serologist to extract the factors in an analyzable form. Study of this interplay is a major feature of our new proposal.

As a pilot project the extraction of haptoglobins from older stains has been conducted. A paper covering the extraction study has been submitted to Forensic Serology News and is included as Appendix A. Essentially the results are that to quantitatively extract haptoglobin from a four month old stain one should use 0.1% Triton X-100 at pH 8.4 for 24 hr. Additionally, we have been experimenting with subtyping of haptoglobins as to whether they contain fast or slow sub groups of the α-1 chains.[29,30] This could greatly increase the genetic information available from haptoglobins giving 6 instead of 3 possible phenotypes. I believe that any national survey for frequency of haptoglobins should be conducted to look for the frequency of the subtypes and not just the usual 1-1, 2-1 and 2-2.

C. Detection of Drugs in Bloodstains

The determination of non-genetic factors such as drugs in bloodstains could be very useful in older stains, where persistence of genetic factors

could be a problem, or when victims' or suspects' medication or drug habits are known. To take advantage of this situation a technique was developed for dilantin which is applicable to other drugs. This method was presented to the American Association of Forensic Science, San Diego, 1977. A copy of the paper submitted to the Journal of Forensic Science is included as Appendix B.

This technique was developed for dilantin, a drug of epilepsy therapy, however, we have promising preliminary experiments with radioimmunoassays to determine therapeutic levels of digoxin and morphine in 100 µl bloodstains. These assays are being studied to improve sensitivity before formal presentation.

D. Problems of the MN System in Bloodstains

The study by this laboratory of the MN system is included as Appendix C which has been submitted to the Journal of Forensic Science.

E. Cases Analyzed as Assistance to Several Crime Laboratories

This laboratory's application of techniques, developed over the past two years on old evidence has aided the cause of justice in several parts of the country and provided a measure of training for the criminalists of the originating jurisdictions. A summary of several cases, some of which have not yet come to trial, is included as Appendix D.

CHAPTER IV. CONCLUSIONS

It is concluded that the results of the esterase-D and carbonic anhydrase II phenotypic frequency survey along with the haptoglobin extraction and the increased knowledge of the MN system can be put into immediate use in the Criminal Justice System in the United States. This research has provided the criminalist and forensic serologist with additional capabilities to individualize a bloodstain.

The radioimmunoassay techniques for drugs (dilantin, digoxin and morphine) and sex identification from a bloodstain have progressed such that they are quite informative when fully implemented.

The solid-phase Gm testing procedure deveoped for Gm_1 demonstrates the feasibility of expanding to the other Gm factors and developing a simple sure procedure to add this genetic system to those routinely analyzed by forensic serologists.

The utility of methods developed in this research has been shown in actual difficult to solve criminal cases.

Thus, this research will upgrade as well as expand the technological capabilities of the forensic scientist and show promise of future expansion as it also increases the usefulness of bloodstain evidence as an aid in the criminal justice system.

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Appendix A

The Isolation of Haptoglobins from Bloodstains

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The determination of haptoglobin phenotypes in dried blood is potentially a very useful method for differentiating individuals.[1] Although there have been many descriptions of moderately successful methods of phenotyping haptoblobins in stains[2-4] little has been published regarding their extractability. It has been our experience that as proteins dry and become older, they become increasingly difficult to put into solution. Thus, the normal convention of moistening bloodstains in an appropriate electrophoretic tank buffer for 15 minutes to two hours before insertion into gels was questioned and investigated. A pilot project was initiated to study the quantitative extraction of the haptoglobins in older stains.

A Rocket immunoelectrophoreic technique[5] in which aqarose (1.0%) containing 1% anti-human haptoglobin sera was used to study the extraction of haptoglobins from dried bloodstains.

The project was divided into two phases. The first was the study of the extractability of haptoglobins from a 1 month old stain. The second studied the ability to immunologically detect haptoglobins in older stains. Various conditions were employed for these studies. Three detergents (0.1%), triton X-100, tween-20 and sodium dodecylsulfate (SDS), and buffer were studied for their ability to extract the Hp at different incubation times and at different pH's.

Table I indicates that for all conditions studied with all the extraction media employed, extraction is incomplete even after six hours of incubation. The increase in the amount of haptoglobin extracted from 6 - 24 hours is dramatic - ranging from 24 - 133%. The pH of the extracting medium is also important. It can be seen that the haptoglobins are very inefficiently

extracted at low pH for all the conditions studied. The most noticeable is the ineffectiveness of SDS at low pH. This effect is not surprising since it is an anionic detergent which at low pH would not be expected to maintain its charge. Interestingly enough, hemoglobin is not extracted at all at low pH by SDS. An examination of the results show that the non-ionic detergents triton X-100 and tween-20 are the best solvators of the haptoglobins. Since triton X-100 appears to at least as good, if not better than, the other extracting media, it was the detergent used to determine the time required to obtain complete extraction of the haptoglobins. Table II shows that for all conditions, low pH with detergent and high pH with and without detergent, essentially complete extraction is obtained within 24 hours. Thus, extraction beyond 24 hours is not necessary.

The persistence of immunologically identifiable haptoglobin in aged stains was also studied. Table II shows quite clearly that the ability to extract haptoglobins from very old stains at low pH is greatly diminished. Haptoglobins were detected, however, in these same stains after extraction at high pH with and without detergent present. The solubility of the proteins at low and high pH is dramatically illustrated. These results, it must be remembered, only indicate immunological persistence in old stains and should not be equated with the ability to successfully phenotype. One interesting aspect of the study is the detectability at low pH of haptoglobin in two different stains of comparable age, 124 and 113 days respectively. In the 124 day old stain haptoglobins could not be detected while in the 113 day old stain they could. This differential ability to detect significant amounts of haptoglobins in stains has been observed in other samples and even in relatively fresh stains. Apparently the haptoglobins in certain stains degrade at a faster rate than in others, or were never present at all.

The results of this study show that the isolation and detectability of haptoglobins and presumably other genetic markers as well are dependent upon the extraction conditions, the time of extraction and the age of the stain. Thus, in older stains the ability to successfully analyze genetic markers may be due, in part, to the ability to isolate the substance from the stain. This same situation has been observed in the extraction of drugs from bloodstains.[6]

Acknowledgments

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Table I

Haptoglobin Extractions From Dried Bloodstains

Extraction Medium	pH	Rocket height (mm) 6 hrs.	24 hrs.	% Increase
Buffer	4.6	10.5	13	24
+ TRX 100(0.1%)	4.6	9.5	13.5	42
+ SDS (0.1%)	4.6	3.0	7.5	133
+ TW 20 (0.1%)	4.6	8.0	9.7	21
Buffer	8.4	11.0	15.0	36
+TRX 100 (0.1%)	8.4	11.5	19.0	65
+SDS (0.1%)	8.4	9.0	15.5	72
+TW 20 (0.1%)	8.4	10.0	18.5	85

Table II

Extraction Medium Triton X-100(0.1%)	pH	Hours Extracted	Immunological Stain 202	Immunological Stain 166	Immunological Age 124	Rocket height (mm) (Days) 113	70	44
+	4.6	24	-	-	-	12	15	12.5
+	4.6	48	-	-	-	11	15	15
+	4.6	72	-	-	-	12	16	13
+	8.4	24	12	13	9	14	16	12
+	8.4	48	12	13	10	14	17	12
+	8.4	72	12	13	10	15	17	13
-	8.4	24	13	-	10	15	16.5	13
-	8.4	48	14	13	10	15	17	13
-	8.4	72	14	13	10	16	17	13

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Appendix B

1) Title of Paper:

Detection of Drugs in a Bloodstain. I. Diphenylhydantoin (DPH).

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3) This work was presented to the Criminalistics Section of the AAFS in San Diego, California, February, 1977.

Detection of Drugs in a Bloodstain. 1. Diphenylhydantoin (DPH).

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INTRODUCTION

The blood of individuals differs in many ways. Forensic scientists have taken advantage of the fact that there are identifiable genetic differences which have the added reward of permitting calculations to be made regarding the uniqueness of a particular blood specimen in the general population.

Although these genetic markers are of great utility in forensic serological analyses they have, with few exceptions, a limited persistence in the dried state. Thus, although very old stains can be typed for the ABO and Gm systems, the other genetic markers i.e. red cell antigens and polymorphic protein and enzymatic systems are no longer detectable sometimes even after a few weeks.

Attempts have been made to utilize non-genetic factors of the blood to arrive at other parameters which could be used to differentiate bloodstain samples. Sweet et. al.[1] have attempted immunoelectrophoretic protein separations to arrive at an individualization index of the unknown stain. Whitehead et. al.[2] have described a technique which permits the detection of allergen associated antibodies in bloodstains. In both of the above studies the blood factors being detected are proteins of endogeneous origin and thus are subjected to the same degradative influences as the genetic markers.

It would be advantageous to identify substances in blood which would serve to differentiate bloodstained evidence and also not be

subjected to the same degradative influences as the genetic markers. Shaler et. al.[3] and McWright and Brown[4] have utilized hormonal steriod concentrations to arrive at estimations concerning the sexual origin of the bloodstain as presented at the 1976 annual meeting of the American Academy of Forensic Sciences. This report is concerned with identification of the drug diphenylhydantion in bloodstains, saliva and semen using a radioimmunoassay procedure. This communication illustrates a technique which has great potential utility in forensic science not only as a tool for differentiating and individualizing bloodstains, but also as an investigative tool for police agencies.

EXPERIMENTAL

1. STANDARD CURVE

A standard concentration curve was prepared by assaying known amounts of unlabelled Diphenylhydantoin (DPH) equal to 0, 50, 100 250, 500, and 1,000 pg (10^{-12} grams) in duplicate by the RIA method. This was accomplished by dispensing 0, 5, 10, 25, 50, and 100 µl respectively of a 10 ng/ml DPH standard solution to 10 x 75 mm round bottom glass culture tubes. Next, bovine serum albumin (BSA) buffer, pH 7.4 (0.06% BSA, 0.01 M phosphate-saline) was added to a final volume of 1.0 ml followed by 50 µl ^{3}H-DPH equivalent to approximately 7000 cpm/tube. The standards were then vortexed to disperse the ^{3}H-DPH in solution. DPH-antibody solution (50 µl) commercially prepared from rabbit serum was transferred into each of the above tubes, vortexed, and incubated at 4°C for 30 minutes. Dextran-coated charcoal suspension, (500 µl), (0.625% Charcoal, 0.0625% dextran-T 70, 0.01 M phosphate-saline buffer, pH 7.4) was then added. The suspension was vortexed, incubated at 4°C for 10 min. and precipitated by centrifugation at 2000 x G for seven minutes. The supernatants were decanted into glass scintillation vials containing

10 ml of a suitable scintillator and counted in a Beckman LS-100C liquid scintillation counter for 5 minutes per sample. In addition to the above standards, "total" samples were run containing only 1.0 ml BSA buffer, 50 µl ^{3}H-DPH as well as "blank" samples containing 1.0 ml buffer, 50 µl ^{3}H-DPH, and 500 µl charcoal suspension.

Data obtained was plotted on two different types of graphs. The first graph, described by Wien Laboratories[5], is simply a ratio of the "zero" standard cpm to each standard cpm vs. picograms DPH added per standard. The logit method[6] shown in the second example represents a mathematical manipulation of the same numbers where the logit of the efficiency-corrected CPM is plotted vs. the log of the dose. Results quoted in this report were extrapolated from the second (logit) standard curve. Experimental error of this RIA for DPH is ± 9%.

2. SAMPLE PREPARATION

a. Dried Bloodstains:

Whole blood drawn by venepuncture into EDTA-anticoagulated tubes was measured using a 10 µl pipeting device (Sigma, St. Louis) and placed on white, unbleached cotton sheet cloth to dry. Storage was at room temperature in an open space for the designated aging times. At the time of assay the dried blood stains were cut with scissors into approximately 1 mm squares, placed in 10 x 75 mm round bottom glass culture tubes, and eluted with 2.0 ml of an appropriate solvent for the described time intervals. For DPH determination by RIA, 10 µl aliquots of the eluant were placed in BSA buffer to a final volume of 1.0 ml, etc. as per the method described above under "standard curve". This dilution produced an equivalent of 0.05 µl of the original 10 µl bloodstain.

b. Saliva and Semen:

Neat saliva and semen samples were collected and stored at 4°C in glass culture tubes. Dilution for RIA was as follows: 10 µl neat sample was added to 100 µl physiological saline solution. Aliquots, (10 µl), of this dilution were tested by adding BSA buffer to a final volume of 1.0 ml, etc. as per the method reported under "standard curve". This was the equivalent of 0.91 µl of the original neat sample.

c. Saliva Stain:

Saliva stains on a cigarette filter were created by having an individual on DPH therapy hold a cigarette in his mouth as if he were smoking. The filter and filter paper were dissected from the body of the cigarette. The paper was then cut into approximately 1 mm squares and eluted with 400 µl 0.1% SDS-saline for 24 hours at room temperature in a parafilm-covered round bottom disposable glass culture tube. This amount of solvent was sufficient to cover the sample. Similarly, 800 µl of the same solution was used to elute the filter under identical conditions. 100 µl aliquots of the eluates were tested by adding BSA-buffer to a total volume of 1.0 ml and proceeding with the RIA method represented under section No. 1 above.

d. Whole Blood:

10 µl whole blood obtained by venepuncture into EDTA-anticoagulated tubes was diluted with 5.0 ml saline. The sample (10 µl) was analyzed for the presence of DPH by adding 1.0 ml BSA-buffer and progressing to the RIA described under section No. 1 above.

e. Plasma:

Whole blood was centrifuged for 3 minutes at 1000 x G to separate plasma from the red blood cells. The plasma (10 µl) was dispensed into

5.0 ml saline and 10 µl of this dilution was tested by adding 1.0 ml BSA-buffer and continuing with RIA as described above.

f. Red Blood Cells (RBC):

Red blood cells, (10 µl), obtained by centrifugation of whole blood were transferred to 1.0 ml saline and resuspended by gently inverting the covered glass disposable culture tube. The cells were then re-collected by centrifugation at 1000 x G for 3 minutes. The supernatant was discarded, and the cells were resuspended in 1.0 ml saline. Five such washes were utilized before the RBC precipitate was taken up in 1.0 ml BSA-buffer and tested by RIA procedure stated under "standard curve".

3. ELUTION BY DETERGENTS:

DPH, (10 µl), and control dried bloodstains were prepared as described above. A non-ionic detergent, Triton X-100, $CH_3C(CH_3)_2CH_2C(CH_3)_2C_6H_4$-O-$[CH_2-CH_2-O]_n^H$, and an ionic detergent, sodium iodecyl sulfate, $CH_3(CH_2)_{11}O-SO_3^-Na^+$ (SDS), were examined for their efficiency at extracting DPH from dried bloodstains. Detergent solutions (0.1 and 1.0%) of each were prepared in physiological saline. Each 10 µl bloodstain was cut into approximately 1 mm squares and eluted with 2.0 ml of these solvents in glass round bottom disposable culture tubes for 60 minutes at 23°C. Each sample was vortexed for 5 seconds at the start and finish of incubation. 10 µl aliquots of eluant were transferred for RIA as described above.

4. MATERIALS

Known DPH blood, saliva, and semen were the generous gift of Mr. Herb Schuetzman. Control samples for comparison were obtained from various sources including the Central Blood Bank of Pittsburgh. A "DPH

Test Set" was purchased from Wien Laboratories, Inc., Succasunna, NJ 07876. Triton X-100 was purchased from Rahm and Haas, Philadelphia, Pa., and SDS from Fisher Scientific, Pittsburgh, Pa.

RESULTS:

The standard curves, Figures 1 and 2, illustrate the extreme sensitivity of the RIA procedure for DPH determinations. It is possible to detect DPH to the 50 pg level. Figure 3 illustrates the concentrations of DPH found in whole blood, blood plasma and washed red blood cells. The data shown, as expected, indicates that blood plasma, has larger concentrations of DPH than whole blood, 12.5 µgm/ml ±1.1 µgm/ml versus 5.5 µgm/ml ±0.5 respectively. The whole blood samples were run to give levels which would be expected from bloodstains made from that same volume of blood. Figure 3 also shows that red blood cells contain no readily detectable levels of DPH thus the difference in concentration between whole blood and blood plasma represents the dilution factor of the red blood cells (blood particulates) in the particular blood sample.

Figure 3 also shows the amount of DPH found in saliva and seminal fluid. The amounts present in each, although lower than blood plasma (0.7 ±0.1 µgm/ml versus 12.5 ±1.1 µgm/ml respectively) are not significantly different from each other. Although there is significantly less DPH in the physiological fluids, saliva and seminal fluid, the concentration is sufficient to reliably detect DPH in 0.9 µl of neat fluid. Thus the ability to detect the drug in physiological stains other than blood is a viable reality.

One theoretical concern with aged stains might be associated with the extractability of the drug. This problem was investigated and the results are shown in Table I. Blood stains were aged for six months

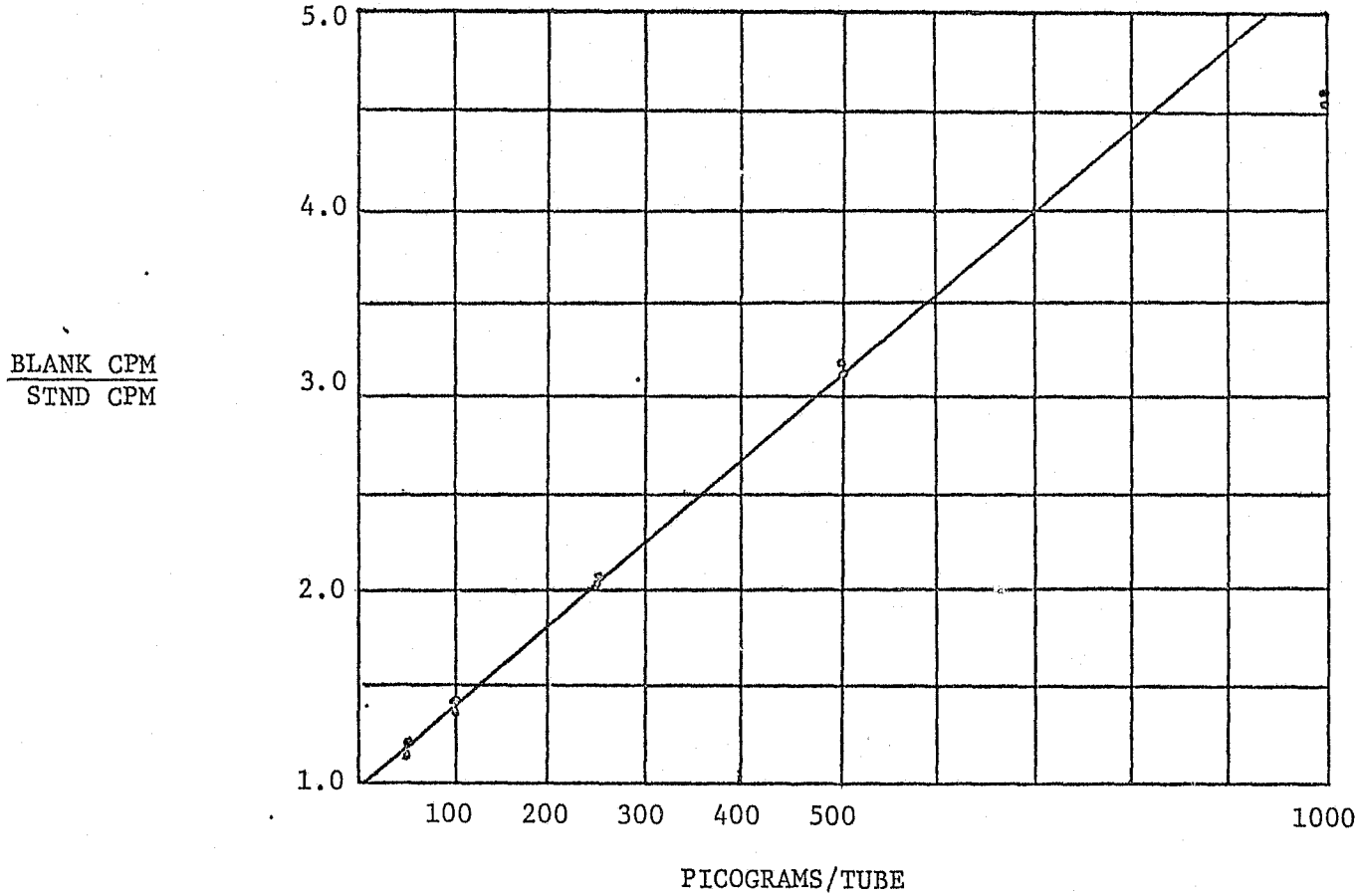

Figure 1. DPH Radioimmunoassay Standard Curve per Wein Laboratories Directions.

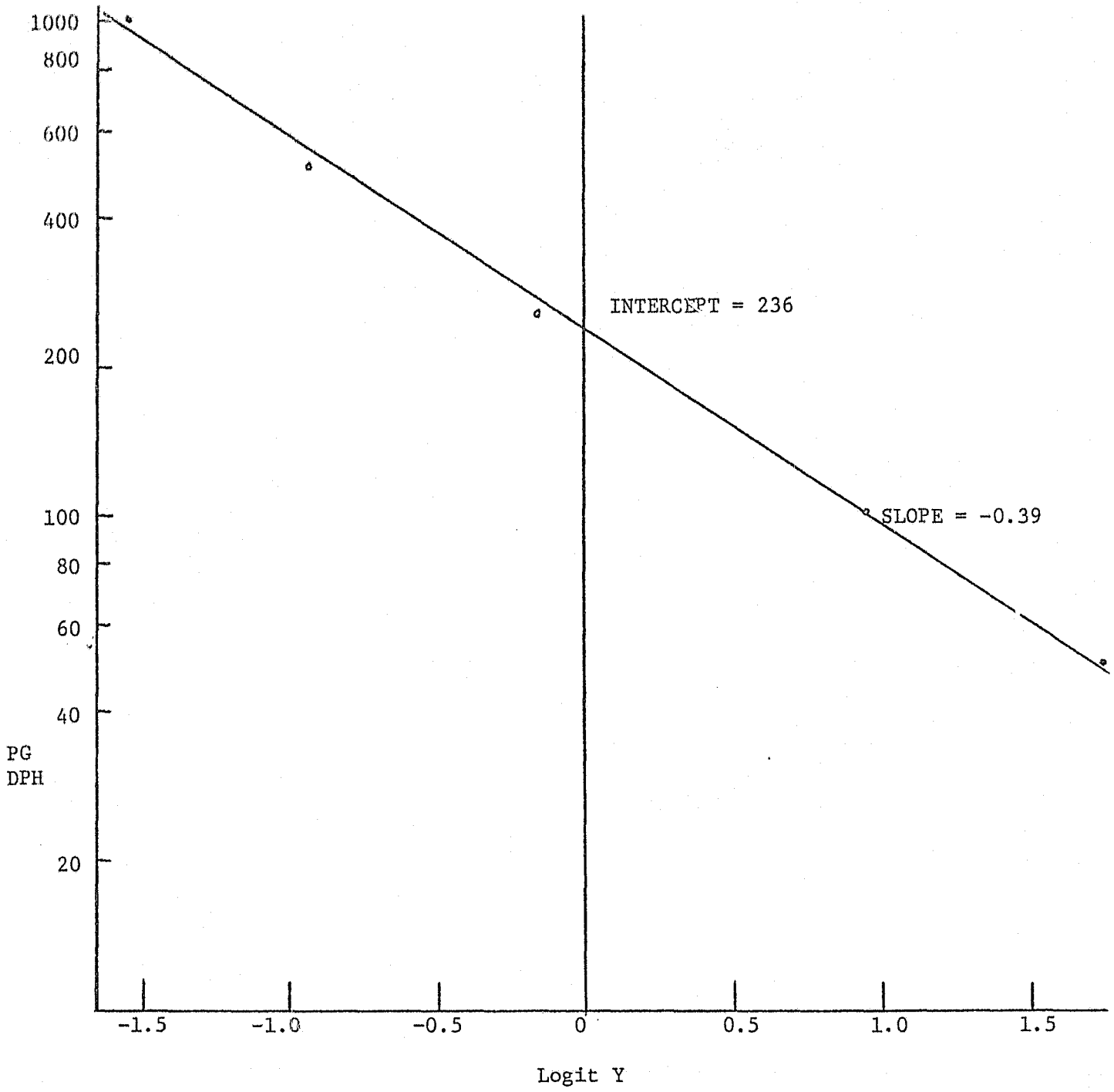

Figure 2. DPH Radioimmunoassay Standard Curve by a Logit Plot.

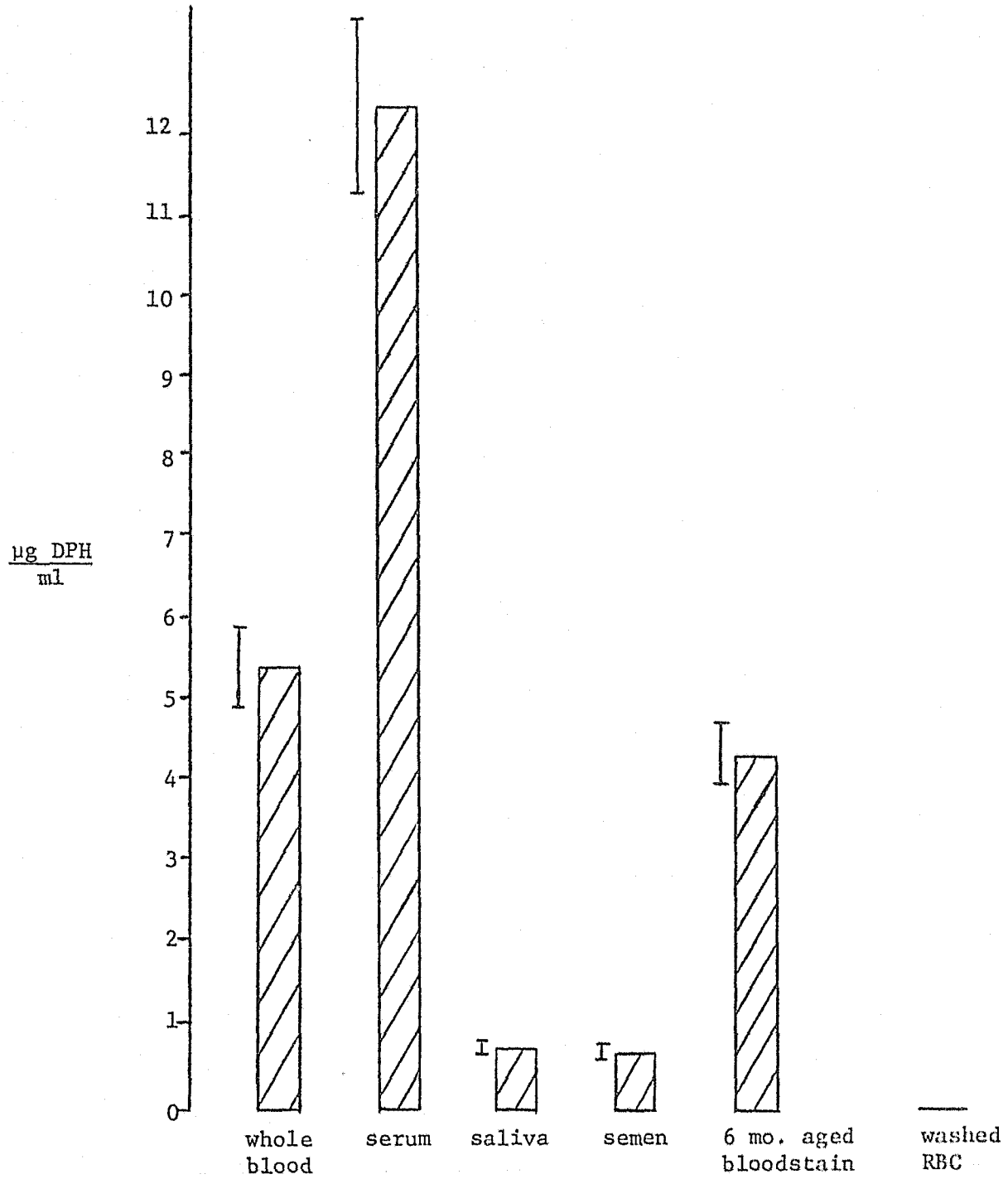

Figure 3. Concentrations of DPH determined in whole blood, serum, saliva, semen and a bloodstain.

LEVELS OF DPH

Sample	amt. det.	Calculated µgm/ml sample
serum	250pg/0.02µl	12.5 ± 1.1
whole blood	110pg/0.02µl	5.5 ± 0.5
fr.stn.1hr. saline	110pg/0.05µl	2.2 ± 0.2
24hr. saline	190pg/0.05µl	3.8 ± 0.3
1hr.1.0% TritonX-100	200pg/0.05µl	4.0 ± 0.4
1hr.0.1% TritonX-100	230pg/0.05µl	4.6 ± 0.4
1hr.1.0% SDS	260pg/0.05µl	5.2 ± 0.4
1hr.0.1% SDS	290pg/0.05µl	5.8 ± 0.5
Aged stn. - 6 mo. 24hr.saline	220pg/0.05µl	4.4 ± 0.4

Table I. Levels of DPH Extractable from Dried Bloodstains

and the ability to extract DPH was compared to a fresh (less than 1 week old) stain prepared from the same volume of blood. The length of time required to extract the stain is important. For example the data shows that extraction of the drug from a fresh stain for one hour in physiological saline yields 2.2 $\pm$ 0.2 µgm DPH/ml or 40% of the total while extraction for 24 hours yields 3.8 $\pm$ 0.3 µgm DPH/ml or 70% of the total. This represents a 75% increase in the extractability of the drug. Similarly extraction of the six month old stain for 24 hours in physiological saline gave 4.4 $\pm$ 0.4 µgm DPH/ml which is not significantly different than that obtained from the fresh stain incubated for the same length of time. In addition these results indicate an upper limit (70%) for the extractability of the drug from stains incubated in physiological saline whether the stain is relatively fresh or not.

Methodology was investigated which would permit the extraction of the drug from stains with greater efficiency and shorter incubation times. Thus different detergents at varying concentrations were studied. Table I illustrates these results. The stains were extracted with non-ionic triton X-100 and anionic sodium dodecyl sulfate (SDS) detergent at concentrations of 0.1% and 1.0% in physiological saline were for one hour. The triton X-100 extracted 4.6 $\pm$ 0.4 and 4.0 $\pm$ 0.4 µgm DPH/ml at concentrations of 0.1% and 1.0% respectively. This corresponds to approximately 80% of the total drug available. The anionic detergent, SDS, extracted 5.8 $\pm$ 0.5 and 5.2 $\pm$ 0.5 µgm DPH/ml or 100% of the total for 0.1% and 1.0% solutions respectively. Thus the use of detergents, especially the anionic detergent SDS, quantitatively extracts the drug in very short periods of time.

Saliva stains prepared on a cigarette were extracted in SDS detergent solution and analyzed by the RIA procedure for the presence of DPH. The

cigarette was dissected into its filter paper and filter components. The results are interesting since the amount of DPH on the outside filter paper was less than that in the filter, 320 pg versus 2600 pg respectively.

DISCUSSION

The radioimmunoassay technique works on the principle that non-radioactive endogenous antigens (DPH) competes with exogenous radioactive (^{3}H-DPH) for antibody combining sites in a manner proportional to their concentrations.[7] Radioimmunoassay, because of its extreme sensitivity, is rapidly becoming a technique of great utility in forensic science.

The described technique concerning the identification of diphenylhydantoin in bloodstains, per se is not necessarily an end in itself but a model system regarding the potential of non-genetic information available in bloodstain evidence. Similar techniques for other drugs can be worked out which in a very small stain could describe the state-of-health or the habits of an individual. A more pragmatic aspect of this model system may be its value to investigating police officers. Blood left by the perpetrator of a crime could point to his socio-economic background. Also, if the victim of a violent crime was taking drugs, in this case an epileptic on Dilantin therapy, the presence of DPH information found in the bloodstain on the clothing of the suspected perpetrator of the crime would go a long way toward establishing that stain as originating from the victim. Thus, although these tests were conducted with DPH because of the availability of a known DPH treated patient, the potential exists for the detection of many other drugs by this same general method that would be of interest to those involved in the

investigation of burglaries, homicides, motor vechicle violations, assults, and any crime where blood and physiological fluid evidence exists.

Acknowledgements

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MN DETERMINATION IN BLOODSTAINS - SELECTIVE DESTRUCTION OF CROSS-REACTING ACTIVITY

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INTRODUCTION

By definition, serology is the study of anitgen-antibody reactions in vitro. 1/ Forensic serology is, however, an expansion of this study and encompasses not only serology but the essentials of biochemistry, immunohematology, immunology and immunochemistry. The forensic serologist is faced with the dual responsibility of performing analyses within this framework of disciplines and functioning as a criminalist faced with the science of identifications. 2/ It is the serologist's ulitmate goal to link evidence to a source of origin, if possible.

In terms of potential, blood evidence holds the most promise of being a forensically discriminating tool. This genetic broth requires efficient analysis to yield extensive information as to its source. Currently, serological equipment includes not only the simpler microscopes and centrifuges, but high resolution electrophoresis and the scanning electronmicroscope.

Periodically, it becomes imperative to pause and reflect on analytical procedures, especially when development is expansive and needs are great. It is necessary to make value judgments on whether tests are appropriate, informative and ultimately, valid.

An effort to perform such a critique is the proficiency testing. 3/ It is sobering to see that the results are less than encouraging. This

project is a definitive statement that present laboratory difficulties should be surveyed and corrected.

A subject which warrants elucidation in view of this preface is the MN blood group system. Discovered in 1928 by Landsteiner and Levine, 4/ the MN system is potentially valuable in discriminating between individuals. It concerns antigen sites of the same name located on the red cell surface. In frequency of occurrence they distribute among population studies as 30% M, 20% N and 50% MN. Combined with its running mate, the Ss system, it becomes a powerful tool for accomplishing this end. 5/ For forensic purposes, the detectability of this system in dried blood has been demonstrated. 6/ The techniques in dried blood are the same as that used for the ABO system-namely absorption-elution or inhibition. The results of the proficiency testing demonstrate that an error as high as 40% is obtained with MN as compared to 1.6% for ABO. These results suggest that the fault is not necessarily in the ability of crime laboratory personnel to use these techniques but quite possibly with the system itself and/or the application of these particular techniques to the detection of M and N antigens in dried stains.

The MN problem can be considered in its most simplistic terms as being due to two factors. First, paradoxically, it is possible to detect N antigen on M cells. 7-9/ This cross reactivity, as it is referred to, is potentially troublesome because of the possibility of mistyping M stains as MN. Second, the quality of commercially available anti-N sera is not controlled for forensic purposes resulting in the production of antisera

unsuited to bloodstain typing. This report is concerned with the selective destruction of the cross react' / associated with the MN system. The results of this work are applied to detection of MN antigens in bloodstains.

EXPERIMENTAL

MATERIALS AND METHODS

1. MATERIALS

∝ Chymotrypsin (thrice crystallized) Type VI was a product of Sigma Chemical Company. Amicon Miniconcentrator B15 was produced by Amicon Corporation, Lexington, Mas[illegible]husetts. Ortho Diagnostics supplied the Anti-M and -N sera (L[illegible]ot numbers 147 and 148). Acrylamide was produced by Sigma Chemical Company. Cooke Microtiter equipment was supplied through Scientific Products.

2. DIRECT AGGLUTINATION OF KNOWN MX CELLS

Whole blood known to be cross-reacting (Mx,M'N'), was washed 3x with physiologic saline and were made into a 2% saline suspension. This suspension was tested in a Cooke microtiter plate against serially diluted anti-M and N sera. Aliquots (25 ml) of the red cell suspension were added to each test well, the first of which contained neat antisera and in the remaining wells, sera serially diluted to 1:2048. This test stood at room temperature for thirty minutes and was read. The M was reactive to a 1:8 dilution. The N was reactive only as a neat solution.

3. INCUBATION OF WHOLE RED BLOOD CELL WITH CHYMOTRYPSIN

Experiments were conducted on Mx blood cells in order to duplicate the results of other investigators. Fresh 3x washed, packed red cells of the same Mx type were treated with a variety of concentrations of chymotrypsin; 4, 2, 1, 0,5 mg./ml. Each of these concentrations was tested against a varied timetable for optimum incubation conditions; 120, 60, 40, 20, 10 and 5 minutes. The test was designed so the 24 tubes represented

the M-antisera and a second set of 24 for the N-antisera. The tubes were arranged in four rows, each row representative of the four α chymotrypsin concentrations and six tubes deep to accommodate the varied time incubations. Two milliliter quantities of the α chymotrypsin were prepared by dissolving the enzyme in .01 M phosphate saline, ph 7.6.

One Pasteur pipette drop of an appropriate α chymotrypsin concentration was added to each of the four tubes in the row representing a 120 minute incubation. Two Pasteur pipette drops of a 2% cell suspension of the previously prepared cells were added to each of the four tubes. The tubes were finger tapped and placed in 37 C incubator for 60 minutes. At that time the row representing 60 minutes was prepared and both sets incubated for 20 additional minutes. The rows continued to be prepared in this manner until all were completed and 120 minutes total time had elapsed. At this point, the tubes centrifuged (3400 rpm) in a serofuge for 15 seconds. The supernatant enzyme was removed and the cells were washed 3x with physiologic saline, the last wash being decanted.

One drop of anti-M, 1:8 was added to each tube in the first set of 24 and one drop of neat anti-N sera to each tube in the second set. All tubes were finger tapped and stood at room temperature for a time period not exceeding 30 minutes. Readings were taken at that time or as soon as visible agglutination occurred prior to that time. After reading, all the tubes were centrifuged for 30 seconds. The supernatants were tested against indicator cells of the corresponding type, i.e., M cells against the M supernatants. Table I illustrates this procedure.

4. INCUBATION OF BLOODSTAINS WITH CHYMOTRYPSIN

The next series of experiments were run using an absorption elution technique on MX (M'N') bloodstained fibers.

Absorption-elution plates were prepared and run, initially without α chymotrypsin treatment, and later using the four concentrations using the same varied time study. It was noted with the first plates that commercial strength anti-N sera was ineffective in demonstrating the N antigen even when new lot numbers were substituted.

Throughout the experiment only concentrated anit-N sera was used. This was accomplished by concentrating a 5 ml. bottle of anti-N sera, lot # 148 and later lot #147, in a minicon concentrator to the 5X mark. this worked very well and as the test proceeded was found to be even a bit strong. The strength was eventually adjusted to an optimum 2.5X. On all plates, the anti-M sera was used neat. (The diluted 1:8 anti-M sera was of insufficient strength for absorption-elution testing.)

The actual preparation of the plates consisted of affixing pairs of threads with nail enamel to the individual wells of ceramic ring slides. The prepared plates were permitted to dry overnight. The threads selected for the tests were:

(1) Control threads cut from new clean cotton sheeting

(2) Cross-reacting M threads previously determined

(3) M threads from a stain made with M type blood

(4) N threads from a stain made with N type blood

An illustration of a sample plate follows:

The ∝ chymotrypsin was incubated on the threads at 37 C in a moisture chamber using the four concentrations and times ranging from 120, 60, 40, 20, 10, and 5 minutes. The plates were rinsed in ice cold physiologic saline and placed in beakers of fresh cold saline for three periods of 15 minutes each in the freezer compartment of a refrigerator. The plates were blotted dry with paper towels and incubated with antisera for one hour at 4 C. The washing procedure was repeated as before blotting dry at the endpoint. Room temperature saline, one drop/well, was placed on each pair of threads and the plates were incubated at 56 C for 15 seconds in a moisture chamber. At this point, red cells were added to the test in the form of a 1-2% suspension, one drop/well from a Pasteur pipette, and the plates placed on a rotator for a period of 5 minutes. Readings were taken at this time and the plates replaced on the rotator. These readings continued every 5 minutes until maximum agglutination occurred or until a period of one hour has passed.

5. DISC ELECTROPHORESIS OF MEMBRANE COMPONENTS.

Membranes were isolated and their components were separated by detergent polyacrylamide gel electrophroesis according to Fairbanks eal. 26/

RESULTS AND DISCUSSION

It is generally believed that cross-reactivity is due to the presence of N antigen on M cells resulting from the former being a precursor molecule. 8/, 9/. Thus, a variety of M blood cell samples would exhibit various degrees of N reactivity dependent on the amount of N to M conversion which has taken place.

The ability to detect N antigen is directly proportional to the

strength of the anti-sera.

In order to clarify the experiments performed in this report some of the basic biochemistry on MN blood group factors must be presented.

In dried blood analyses the absorption-elution technique, as described by Howard and Martin 22/ or as modified by Kind 28/, is extremely sensitive. That is to say that only a minute quantity of antigen need be present in order to be detected. Thus, if a bloodstain from an M person, tube typed using antisera against whole cells, demonstrates cross-reacting N activity, it may be typed by the absorption-elution technique as MN.

Historically, it has been known that cross-reactivity was related to S activity 5/.

In 1960 Allen 10/ described an individual on whose red cells N antigen could not be detected. This was the first demonstration that all M cells do not contain N antigen. The blood type, MS-s-U-, that Allen described, is rare and was considered an exception to the rule. For nearly 12 years after that announcement, research continued concerning the biochemical nature of these antigens and their structures, and their biosynthesis was moderately well established 9/, 11-19/. During the course of these investigations, knowledge has increased concerning the structure of red cell membranes and has been correlated with the different serological antigens 20-22/. Several laboratories have demonstrated the protein and glycoprotein constituents in red blood cells by detergent disc polyacrylamide gel electrophoresis, Figure #1 shows a diagramatic representation of the results obtained in this laboratory. It can be noted in B that several different proteins are present and in A that few glycoproteins are

present, notably PAS 1, 2 and 3. (The PAS initials indicate the Periodic Acid Schiff reagent used to stain for carbohydrates). For the purpose of the following discussion we will concentrate only on the glycoprotein present in the red cell membrane.

In an attempt to illustrate the rationale behind experiments in this report, more biochemical evidence concerning the nature of M, N, 'N', and S antigenic activities must be explained.

As mentioned M, N, 'N' and S antigenic reside on glycoprotein molecules within the red cell membrane 23-25/. These glycoprotein molecules can be separated by electrophoresis in detergent acrylamide gels (Figure #1). Two of these glycoproteins, PAS-1 and -2 contain M and N antigenic activity while the third, PAS-3, contains cross-reacting N ('N') and S antigenic activity 23-24/.

Although Springer has shown that M and N specificities are related to the number of neuraminic acids on the molecules 9/; a more penetrating analysis of their structures demonstrate that possibly to a greater extent, the antigenic specificities are dependent on the amino acid environment, i.e., the stereochemical environment of the neuramic acid residues 24/.

It is known that treatment of M cells with neuramindase to remove sialic acid (Sialic acid and neuraminic acid are interchangeable terms) molecules will result in the production of non-specific N activity. Also, treatment of M cells with proteolytic enzymes, e.g., trypsin or pronase will achieve the same result.

An important extension of these studies has shown that treatment of M or N cells with the proteolytic enzyme α chymotrypsin has no effect

on M or N antigenic activities. However, α chymotrypsin will selectively destroy 'N' and S antigenic activity, i.e., in essence the PAS-3 band.

Table I illustrates the results obtained with the incubation of whole M 'N' blood cells with α chymotrypsin. These results show that reduction of 'N' activity without concommittant destruction of M antigenicity is possible. These data duplicate the work of others. The extension of these results to bloodstain analysis utilizing the absorption-elution technique poses problems. The concentration of antisera to be used is different. Higher concentrations of antisera, especially to identify N antigen, are required and the incubation times with α chymotrypsin are also different. The ability to extend wet blood biochemistry to dried blood analyses is possible. These extensions are not direct one to one applications, however, and the procedures require significant modification before workable systems are produced.

The selective destruction of 'N' activity with α chymotrypsin has also shown promising results for the identification of the true M and N antigens in bloodstains. Table II illustrates the effect of α chymotrypsin on M, N and 'N' antigens in bloodstains. Using 4 mg/ml chymotrypsin solution and incubating questioned samples for 2 hours at 37 C prior to the cells have been added to the test all positive controls will read +4 and 'N' will only show a +1 to +2 and at 35-40 minutes will only produce a weak +3. Early in the testing it became obvious that the lower concentrations of enzyme and shorter incubation time were ineffectual in removing 'N' activity. The 4 mg/ml concentration of α chymotrypsin incubated for a 2 hour period produced the most dramatic results. Stronger

concentrations of 8 mg/ml and 13 mg/ml incubated for one hour periods showed little influence to inhibit 'N' activity.

To illustrate the speed with which the reaction is visible, at 5 minutes controls are +2 and the test threads negative. At 10 minutes controls are a strong +3 and the test threads still negative. It became obvious that all of the 'N' activity was not destroyed. It appears that prolonged incubation in the α chymotrypsin has a detrimental effect on the ability to determine any of the desired antigenic activities. Thus, conditions were adjusted to accomplish this desired end without introducing either a reduction in sensitivity or the production of non-specific activity.

The results of this preliminary work are not intended to be a new procedural inclusion into crime laboratory routine. Instead, these data are presented to illustrate to forensic serologists the complex nature of serological systems. The interpretation of serological results must be done with a great deal of caution especially until all the facts, serological, immunological and biochemical are known. When there are obvious inconsistencies in results and errors being made during blood grouping then there is wisdom in a cautious and skeptical approach.

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Acknowledgments

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TABLE I

α Chymotrypsin concentrations - mg/ml

	4	2	1	0.5	4	2	1	0.5
Incubation time in minutes								
5	+	+	+	+	-	-	-	-
10	+	+	+	+	-	-	-	-
20	+	+	+	+	-	-	-	-
40	+	+	+	+	-	-	-	-
60	+	+	+	+	-	-	-	-
120	+	+	+	+	-	-	-	-

All tubes tested with anti M 1:8 All tubes tested with neat anti-N

LEGEND TO FIGURE #1

A) Continuous 10% polyacrylamide disc gel stained with Periodic Acid-Schiff and; B) identically run gel stained with Brilliant Coomassie Blue (G).

FIGURE #1

Diagramatic Representation of Protein and Glycoprotein (PAS) bands

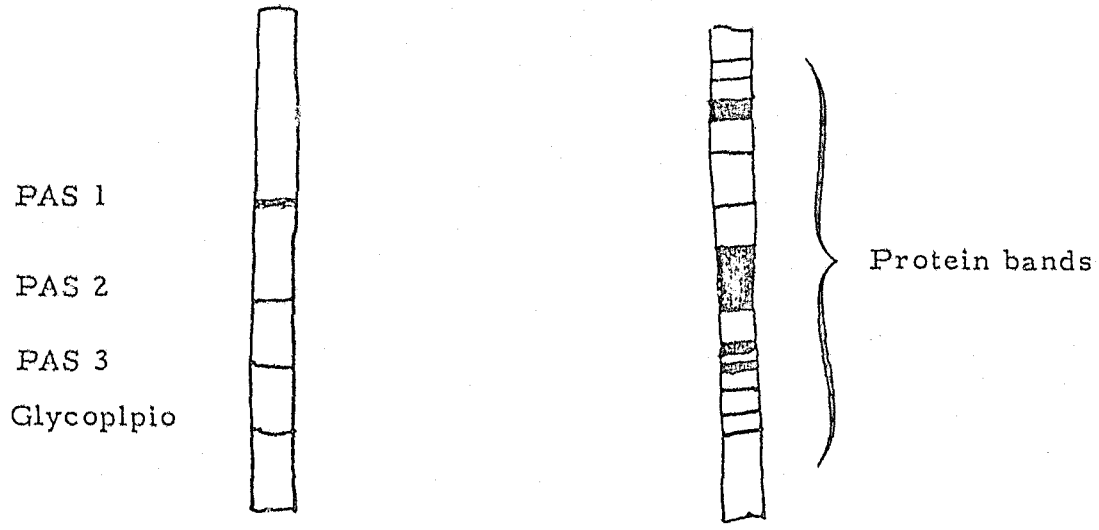

TABLE II

EFFECTS OF α-CHYMOTRYPSIN ON

M, N, 'N' AND ACTIVITY

Bloodstained Threads	Incubation α-Chymotrypsin 4 mg/ml	Incubation Times Minutes						
		5	10	15	22	20	32	37
M	-	+1	+4	+4	+4	+4	+4	+4
N	-	+2	+4	+4	+4	+4	+4	+4
'N'	-	+2	+4	+4	+4	+4	+4	+4
M	+	+2	+3	+3^{S}	+4	+4	+4	+4
N	+	+2	+3^{S}	+3^{S}	+4	+4	+4	+4
'N'	+	-	-	-	+1	+2	+2^{S}	+3^{W}

LEGEND TO TABLE II

The effect of α-chymotrypsin on M, N and 'N' activity in Bloodstains:

1. Incubation of bloodstained fibers was for 2 hours at 37^{o} with -chymotrypsin (4m/ml). The absorption-elution conditons are described in Materials and Methods. The concentration of anti-N sera was 2.5 times concentrated commercially prepared Lot No. 148 Ortho diagnostics:

   S = Strong reaction

   W = Weak reaction

Cases Analyzed as Assistance to Several Crime Laboratories

Appendix D

As a result of LEAA Grant No.'s 75NI-99-0011, 76NI-99-0099 and ensuing related publicity, several agencies have contacted this laboratory in order to have physical evidence, specifically blood evidence, worked in cases of rape, homocide, and homocide-rape. The general details of the cases will be enumerated below:

1. The first case from New York City was a homocide. The blood evidence was contained on a vinyl jacket and was 14 months old at the time it was brought to the laboratory. Previous tests done by the New York City Medical Examiner's Office found the presence of B blood determined by the absorption-inhibition method. The presence of blood was determined by the Takayama test, and the presence of human proteins was determined by immunoprecipitation. The blood evidence in the case was the only important physical evidence. The requesting agency, the Queen's County District Attorney's Office, was interested in identifying blood group factors which would differentiate the victim and suspect. The vinyl jacket belonged to the suspect. Gm testing was done on the jacket and Gm factors 1,2,4,(3) and 12(5) were tested. Differences between the suspect and victim were found in both Gm factors 4(3) and 12(5). The results of the New York City Examiner's Office were also confirmed. The New York City Medical Examiner's Office did not have the capabilities of identifying blood group factors beyond the A, B, O system.

2. The second case was from the Prosecutor's Office in Maysville, Kentucky. At the time the evidence was brought to the office it was nine months old. The evidence to be analyzed was a leather boot on which were two extremely small stains. The evidence was originally analyzed by the Cincinnati crime laboratory and blood group A was found by the absorption elution technique. The presence of blood was identified by the Takayma test and

and presence of human proteins were evident by immunoprecipitation from crusts of blood taken from the boot. The prosecutor's office requested that blood group factors be identified which could distinguish between the victim and suspect. Both the victim and the suspect have group A blood. The blood serum which had soaked into the boot was tested for the presence of Gm factors. Factors 1, 4(3) and 12(5) were tested and differences were found between the victim and the suspect on Gm factor number 12(5).

3. The third case was from the State of New Jersey and was requested by the Prosecutor's office. The evidence was a pair of jockey shorts in which seminal fluid and blood were mixed. The crime laboratory identified the presence of A blood by the absorption elution technique, and also identified the presence of isoenzymes of PGM by electrophoresis. Both the suspect and victim have the same ABO and PGM groupings. The request was made to identify any other blood factors which could differentiate between the suspect and victim. Gm's 1, 4(3) and 12(5) were tested and differences between the victim and the suspect were found in Gm factor number 4(3).

4. The fourth case was from Houston, Texas and the evidence consisted of a seminal stain which was found at the scene of the crime. The case was brought to our attention by the defendant's mother. At that particular time, the evidence was 18 months old. The Houston Crime Laboratory had identified the presence of A antigen in the seminal stain indicating that the seminal stain originated from a person with group A blood. There were two suspects in the case--both of whom were almost identical "look alikes". Both suspects had group A blood and both were secretors. The victim prior to the arrest of the second suspect identified suspect No. one as the perpetrator of the crime. Suspect No. one lived in Pittsburgh, Pennsylvania and had been accused of 20 separate rapes within the past year

in Houston, Texas. The prosecuting authorities were calling him the "commuter rapist". Suspect No. two was from Texas and after being arrested for shooting a security guard confessed in an unsolicited manner to all of the rapes including the one where the evidence was being analyzed. He could describe the apartments for each of his victims. The criminalist from the Houston crime laboratory brought the evidence to this laboratory and together we tested for the presence of Gm factors in the seminal stain. Factors 1, 2, 4(3), 10(13) and 12(5) were tested. Factor No. 12(5) was different in the two suspects. In this particular instance because of the difference in relative concentrations it was not possible to use the Gm factors as a method of distinction between the two suspects from the evidence seminal material.

5. The next case was from Woodbury, New Jersey. A homicide case in which the evidence was two weeks old. The evidence in this particular case was brought to our laboratory by the New Jersey State Police. The evidence considered was a pair of jockey shorts which were stained with a mixture of blood and seminal fluid. The blood was present from the the victim, seminal fluid from the suspect. The New Jersey State Police laboratory identified the genetic marker in the ABO group system, EAP, PGM and AK as being the same as the victim and suspect. The request was made by the prosecutor's office that differences between the victim and the suspect blood be identified if possible. The MN blood group system was tested. The suspect was identified as group M and the victim was identified as group MN. Since the MN blood group is not found in seminal fluid the presence of MN on the jockey shorts suggests that the origin of the blood on the jockey shorts could not have been from the suspect. It could have

been from the victim. In addition, GM typing was done. The tests showed that the victim had Gm types 1 and 2. The suspect had Gm type 1 and did not have Gm 2. The seminal fluid on the jockey shorts was found to contain Gm 1 and not 2. This is consistent as being from the suspect. The blood contained on the jockey shorts was found to contain Gm types 1 and 2 consitent with the victim's.

6. The next case was from the Pennsylvania State Police. The evidence was two months old and involved a rape case in which the suspect had been arrested and the victim had a pair of green slacks on which there was a seminal stain present. The Pennsylvania State Police laboratory identified the presence of secretor blood group A in the seminal stain. The evidence was brought to the laboratory in order to determine whether the suspect could have been the person who committed the crime. The seminal material from the suspect was tested for the presence of Gm factors 2, 4(3) and 12(5). The seminal stain on the victim's slacks was also tested for the 1, 2, 4(3) and 10(13). Some differences were found between the suspect's seminal fluid and the seminal stain found on the victim's slacks. It would be impossible for that suspect to have been the perpetrator of the crime. Two other suspects were arrested for the same crime. Seminal analyses done on the individuals proved to be inconsistent with the seminal stain found on the green slacks. Thus, it was impossible for each of these additional suspects to have committed the crime.

7. The next case involved a seven year old fingerprint found on the side of a car. The evidence was brought to the laboratory by the Public Defender's Office as a result of analysis carried out by the Pennsylvania State Police laboratory. The Pennsylvania State Police laboratory claims to have identified the presence of human blood on the fingerprint. From

the amount of blood claimed to have been present by the criminalist, it would have been impossible to identify either presumptiously or conclusively that blood was present.

8. This laboratory received a letter from a man in jail requesting that an outside and independent analysis be conducted of blood evidence which was involved in his homicide case. At this particular time the defendant is in jail with his co-defendant friend convicted of a murder which he claims he could not have committed. After discussion with the defense council and the defendant's expert witness, it was apparent that the blood group analysis submitted by the Pennsylvania State Police Laboratory was either in error or not conducted at all. Unfortunately, in this particular case the qualifications of the defense expert to analyze the blood evidence were not appropriate for the work for which he was commissioned. The techniques used by the defense expert to corroborate the work done by the Pennsylvania State Police laboratory indicated a profound lack of knowledge concerning the detectability of dried blood group factors in an aged stain. The method that he used was antiquated and his interpretation of the results are not surprising in view of the fact of the the method he used, and his lack of expertise in forensic serology. The defense expert was a toxologist. The work done by the Pennsylvania State Police laboratory in which the blood group AB was identified presumably by the absorption elution and absorption inhibition tests were either in error or not done at all. It is quite possible that the work was never done since pictures taken of the physical evidence by the defense expert (the only steps taken by the defense which indicated any degree of competence at all) indicated that the evidence had not been analyzed at all by the Pennsylvania State Police laboratory analyst.

9. Evidence was brought to this laboratory by the criminalist of the Trenton, New Jersey State Police crime laboratory. The evidence was two weeks old at the time. The criminalist had already identified the presence of blood group A, as determined by the absorption elution technique, and PGM, EAP and AK, by electrophoretic technique, were the same between the victim and the suspect. The evidence was brought to this laboratory in order to determine whether or not analyses of other blood group factors could differentiate between the victim and suspect. Several blood group systems were analyzed. The Gm blood group systems were analyzed for the presence of 1, 2, 4(3), 10(13) and 12(5). No differences were found between the victim and the suspect. Carbonic anhydrase II also was analyzed and no differences were found between victim and suspect. Differences were found in the haptoglobin phenotypes of the individuals.

Each of these cases which have been described above are instructive. First, in some instances an obvious lack of advanced capabilities of some of the crime laboratories involved. For example, it seems almost inconceivable that the Medical Examiner's Office of New York City is incapable of conducting an analysis beyond the ABO system. Second, in some of the cases the laboratory involved such as the New Jersey State Police laboratory have indicated an advanced ability to handle blood evidence. However, as in some cases even their advanced capabilities are not sufficient in order to determine differences in blood between suspect and victim. Thus as a result of research funded by LEAA these capabilities do exist and in these particular cases differences were found. Third, in two separate cases two separate laboratories demonstrated an alarming incapability to properly analyze blood evidence. These cases demonstrated a lack of expertise on the part of the analyst in terms of understanding the tests that were being performed, theory behind the tests and practical limitations. It is unfortunate that

situations like this exist because in both of those cases innocent people have gone to jail. Although it is undesirable to let criminals go free it is much better that they go free than to incarcerate an innocent person. The research which has been funded by LEAA has demonstrated the most practical of all applications - that of being utilized in the criminal justice system in this country. In each instance in the cases described above, where it was feasible, the criminalist who did the original work in the local crime laboratory was brought to this laboratory and trained in these advanced techniques.

END