THE INDIVIDUALIZATION OF FORENSICALLY IMPORTANT PHYSIOLOGICAL FLUIDS

Prepared by

Robert C. Shaler
Research Director and Criminalist

December 1975

Prepared for

National Institute of Law Enforcement and Criminal Justice
Law Enforcement Assistance Administration
U. S. Department of Justice

Grant No. 75N 1990011

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.

This research has been directed toward several ends. Each will expand the criminalists' capabilities for bloodstain analysis, but more important, will enhance the usefulness of this evidence in the court system.

First, statistical studies were completed on three isoenzymes and protein systems of forensic importance: PGM, EAP and Hp. These statistics will provide the forensic scientist the luxury of calculating the frequency with which a particular blood specimen possessing any combination of types for these systems occurs in the population. Although these statistics were obtained in a specific population, they can be used as a part of a national survey.

Second, the successful development of methods for the identification of Gm and Inv(Km) antigens and peptidase A isoenzymes both in bloodstains and seminal stains will enable the forensic scientist to have information which may permit a prediction of the racial origin of his evidence. This would be true not only for bloodstains but also for seminal stains in rape cases.

Third, a technique was developed which will permit the determination of the sexual origin of bloodstain evidence with an approximate certainty of 90%-99%.

Finally, miscellaneous related work was initiated to combine the above so as to derive as much information as possible from a single

stain. Thus, we have found it possible to detect the presence of a drug (dilantin), isolate sex hormones for sexual determinations, and isolate and identify proteins and enzymes for population frequency data and racial determinations. When these methods are complete, it should be theoretically possible to identify the sex, the race, the uniqueness as well as the drug therapy from a single bloodstain.

PREFACE

This study has been concerned with the individualization of forensically important physiological fluids. It has been sponsored by the Law Enforcement Assistance Administration and aims at the development of blood individualizing techniques which will increase the capabilities of the forensic serologist and the utility of bloodstain evidence in the criminal justice system in the United States.

The results of this study will permit the forensic serologist the possibility of determining information about the racial and sexual origin of bloodstain evidence. The statistical information compiled will have immediate application in the courts in Western Pennsylvania and can also be used as a 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.

ACKNOWLEDGEMENTS

The author wishes to express appreciation for valuable consulting assistance provided by Mr. Richard Fox, presently in Ventura County, California, Director, Missouri Regional Criminalistics Laboratory; Dr. Ronald Gilcher, Medical Director, Pittsburgh Central Blood Bank; Mr. Bryan Culliford, Metropolitan Police Forensic Science Laboratory, London; Dr. Cornelius McWright, Director of Biological Research Unit, Federal Bureau of Investigation and Dr. H. H. Takimoto, the Aerospace Corporation. The technical assistance of Dr. Charles Mortimer, Anita Hagins, Denise Neilson, Willard Stuver, Fred Smith and Linda Freed has, of course, been invaluable. Especially acknowledged is the support and encouragement of the late Mr. Charles A. McInerney, Director, Pittsburgh and Allegheny County Crime Laboratory and the Law Enforcement Assistance Administration with special thanks to Messrs. John Sullivan and Joseph Kochanski.

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 initiate a solution to the problems outlined above. In this regard, the research has been divided into three parts.

First, crime laboratories across the country utilize certain isoenzyme systems as a part of their normal routine. The use of these systems is a result of the fact that certain enzyme and protein systems exist as multiple molecular forms. These systems are genetic markers and, thus, biochemically represent the genetic make up of the individual. Although several of these are used routinely by crime laboratories, the statistics used by these laboratories to calculate the uniqueness of a particular bloodstain specimen have been derived from the United Kingdom and other European populations. The results obtained in this research provide a start for a national statistical base. The systems studied, phosphoglucomutase, erythrocyte acid phosphatase and haptoglobin, are systems which are currently used by crime laboratories and for which population frequencies are needed.

For each of these systems, approximately 2000 samples were typed and frequencies for both Caucasian and Black populations were determined. These results provide the Pittsburgh and Allegheny County Crime Laboratory with the capability of calculating the uniqueness of a bloodstain or blood specimen by using data derived from the Allegheny County population as a whole. Thus, this data has been put to immediate use in the criminal justice system in Western Pennsylvania and can also be used at a future time once more data is available nationally.

Second, blood contains information which would permit the potential identification of its ethnic origin. This research has been designed so that information regarding the racial origin of a bloodstain could be obtained. Three systems were specifically chosen for study, all of which have this potential. Peptidase A is a polymorphic enzyme system in which only one of its three types is found in the Caucasian populations.

The rarer types are found in approximately 12% of the black population and thus their identification in a bloodstain would suggest that the particular stain is not of Caucasian origin. In this research we have successfully identified peptidase A in dried blood and also developed methodology which will permit the simultaneous identification of two additional forensically important genetic markers, phosphoglucomutase and adenylate kinase. Thus, by using this newly developed methodology it is theoretically possible to determine that a particular bloodstain could be found in approximately 0.0085% of the population and, in addition, to identify the stain as being from a black person. We have also identified peptidase A in seminal fluid and in seminal stains, permitting the possibility of identifying a seminal stain with regard to its ethnic origin as well as being able to calculate its uniqueness in the population.

Other genetically derived systems which are found in the blood are the Gm and Inv(Km) antigens. These are potentially very discriminating in that their identification, in addition to permitting calculations regarding uniqueness, would also permit a decision to be made regarding the racial origin of the bloodstain. For example, during this research we devised methodology for identifying Gm 1, 2, 4, 12 and Inv(Km) 1 in bloodstains. Thus, if a bloodstain contains both Gm 1 and Gm 2 or neither of them, it cannot have originated from a black person. But the presence of Gm 1 without determining the presence or absence of Gm 2 says nothing concerning the ethnic origin of the bloodstain. The Gm antigens have also been identified in seminal and vaginal stains. This could help to identify a suspect in addition to containing information regarding their racial origin.

Third, blood also contains sex hormones whose relative concentrations should permit a probability calculation to be made regarding the sexual origin of the bloodstain. During the course of this research, we have devised a technique which will permit the determination of the sex hormones testosterone and estradiol in bloodstains. By isolating these sex hormones, testosterone and estradiol, from stains, it was possible to determine their relative concentration, the ratio of which (testosterone/estradiol) permits a prediction regarding the sexual origin of the blood. Approximately 1500 samples have been tested resulting in a 93-99% predictability concerning the sexual origin of the blood. Also, approximately 250 corresponding stains were processed with the same predictability concerning their sexual origin. The sensitivity of the method will permit these analyses to be done on 0.05 ml of blood which is equivalent to one drop.

Other preliminary work was also initiated, the results of which are very promising. The basic technique used in the sexing studies was also utilized for the identification of a drug substance in dried stains. The drug in particular was dilantin (diphenylhydantoin). The selection of this particular drug was based on the availability of commercially prepared reagents and also on the fact that an individual in our laboratory is an epileptic on dilantin therapy. The identification of the drug was successful in a bloodstain made from approximately 1/5 of a drop of blood. The size of this stain was approximately 100 times larger than is necessary for the drug's identification. Thus, it is theoretically possible to identify the presence of other drugs for which reagents are available, including such drugs as morphine, barbituates, LSD, and tetrahydrocannabinol (marihuana).

In addition, it has been possible to separate dilantin from the sex hormones as well as from the genetically derived systems such as the enzymes and other proteins. Theoretically, then, it should be possible to determine the sex, the race, calculate the uniqueness and determine the individual's drug therapy from a single bloodstain made from one drop of blood.

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 systems routinely being used (values to be eventually used in a national statistical study).

II. The development of techniques and procedures for the determination of glutathione reductase and peptidase A isoenzymes and the Gm and Inv(Km) allotypes in blood stains.

III. The utilization of a new concept in forensic blood analysis for the sexing of blood stains.

I. Population Frequencies of the Currently Used, Forensically Important Systems: Phosphoglucomutase (PGM), Erythrocyte Acid Phosphatase (EAP), MN, and Haptoglobin (Hp).

Of the more than 200 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 avaiable for the ABO and Rh systems but not for the MN.[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 but only the isoenzymes phosphoglucomutase (PGM)[3], adenylate kinase (AK)[3], erythrocyte acid phosphatase (EAP)[4], and glutamic pyruvic transaminase (GPT)[5] are being identified. Of the other protein systems available, only the hemoglobins (Hb)[2] and haptoglobins (Hp)[3] are identified.

Presently, of all the systems which have been used forensically to individualize and compare blood samples, there is little or no statistical information for the United States for those most commonly used: PGM, EAP, Hp and MN. Values being used are those derived from the United Kindgom. It is extremely important to obtain these values in the United States since the forensic scientist must be able to present to the court the degree of uniqueness for each of the system variants. The courts are especially interested in having these values obtained for the population in which the crime occurred.

That these frequencies do change from one population to another has been illustrated for the Chinese and Korean population for the immunoglobulin allotypes[6] and for PGM in the German population.[7]

II. The Development of Techniques and Procedures for the Determination of Glutathione Reductase and Peptidase A Isoenzymes and the Gm and Inv Allotypes in Blood Stains.

In expanding the blood individualization capabilities of the criminalist, it was proposed to investigate the applicability of the isoenzyme systems glutathione reductase and peptidase A to dried blood analysis. These

two isoenzyme systems each have three polymorphic forms.[8,9] Although the Caucasian population is predominately Type 1-1, the Black population contains all three types.[8,10]

It was also proposed to incorporate the Gm and Inv (Km) systems into routine use. These are polymorphic serum proteins which are associated with antibody molecules.[11] 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.[12] Presently there are approximately 23 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.[11,12] Like the isoenzymes discussed above, anthropological "races" retain a certain combination of Gm factors. Thus, their value does not lie solely in discriminating among individuals, but also, in estimating the racial origin of a bloodstain.

The forensic applicability of Gm and Inv (Km) types has been established.[13] The stability of the Inv (Km) factor was more than adequately demonstrated by its identification in a 22 year old blood stain.[14] Inasmuch as the immunoglobulins comprise a substantial portion of blood plasma (IgG = 1100 mg%) a small quantity of blood (1-2 µl) should be sufficient for grouping purposes.[13] Examples of occurrence and gene frequencies for a Hungarian population[15] (10,000 people) are:

Gm (1) = 38.4% (0.2150)

Gm (2) = 8.4% (0.0429)

Gm (5) = 93.0% (0.7354)

Inv (1) = 12.1% (0.0624).

The necessity for determining the frequencies on a local level is reflected in the changes in gene frequencies in different parts of China.[16] The same is true in Scotland for Gm(1).[17]

III. Sexing of Blood Stains.

For the determination of the sexual origin of blood stains, workers have utilized cytological techniques to demonstrate the present of the Y chromosome in lymphocytes.[18] Although once the Y chromosome is identified, the origin of the stain is adequately determined as being of male origin, the method does have disadvantages. First, if a negative result is obtained, it does not identify the stain as being female; it only means the Y chromosome could not be found. Second, karyotyping is a technique which requires extensive experience in preparing material and interpreting data. Third, the stain from which these studies are conducted must be of an optimum type (a crust of blood).

It has been known for some time that major sex characteristics are to some extent dependent upon plasma concentrations of selected androgenic (testosterone) and estrogenic (17β-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 data in Table I[21-23] suggest that by determination of testosterone and 17β-estradiol levels in plasma, a decision could be made determining the sexual origin of the material. It would be expected that methodology could be adapted to express these same type of data in dried blood.

Table I Normal Testosterone and Estradiol Concentrations

	TESTOSTERONE (ng%)	17β-ESTRADIOL (ng%)
Normal male:	300-1200	20-50
Normal female:	20-50	50-28,700*

* These values reflect levels which could be attained during pregnancy and also variations which occur during the menstrual cycle.

Thus by determining the testosterone (T) and 17β-estradiol (E) levels in stains, high ratios of T/E would indicate blood of male origin, while low T/E ratios would suggest female origin. It is conceivable that with abnormal conditions (hypogonadism, etc.), intermediate or aberrant results may be obtained.[24] In these cases, the data would not permit determination of the sexual origin of the blood, but might permit a comparison to be made regarding the hormonal levels in a suspect's or victim's blood versus the stained material.

CHAPTER II. EXPERIMENTAL

I. Population Frequencies for Polymorphic Systems of Forensic Interest

A. Phosphoglucomutase (PGM)

Samples were obtained from either the Central Blood Bank or the County Jail. Each sample was classified as to age, sex, race and date of collection. Several drops of each sample were placed onto cotton sheeting and air dried.

For enzyme analysis the bloodstain samples were cut into 1 x 5 mm sections, placed into a porcelain spotplate, and moistened for 10 minutes with PGM gel buffer (1/10 diluted PGM tank buffer). The moistened threads were inserted into a 1 mm x 20 cm x 20 cm 10% starch gel. The gel preparation[3] consisted of an appropriate weight of starch mixed with a desired volume of gel buffer and heated to a boil. The gel buffer was a 1:10 dilution of PGM tank buffer containing Tris (12.11 g/1), Maleic acid (11.62 g/1) and $MgCl_2$ (2.03 g/1) PH 7.4.

After boiling, the gel was degassed and poured quickly.

Approximately 500 ml of tank buffer were required for each plate in the electrophoresis tank and the buffer was found to maintain integrity through two separate analyses.

Electrophoresis was performed for 22-24 hours at 138 volts in Kohn tanks using cooling plates at 4°C.

The position of the isoenzymes was visualized with an agar overlay. Overlay chemicals should be removed from storage one hour prior to use in order to avoid condensation and subsequent deterioration.

The overlay reaction mixture is prepared as follows. (25 ml) reaction buffer (3.64 g Tris/500 ml pH = 8.0) is added to glucose-1-phosphate

(Wessex Biochemicals) (87.5 mg), $MgCl_2$ (50 mg), NADP (7.5 mg), MTT tetrazolium (2.5 mg), phenazine methosulfate (1.0 mg), and glucose-6-phosphate dehydrogenase (1.0-1.4 units). Agarose (0.5 g) was dissolved in 25 ml H_2O by heating to 90°C. The heated solution was cooled to 65°C and immediately and rapidly mixed with the reaction mixture and then poured onto the starch gel being careful not to form air bubbles. The plate was incubated at 37°C for approximately 2 hours.

B. Erythrocyte acid phosphatase (EAP)

The preparation of samples for electrophoresis is the same as for PGM except that EAP gel buffer containing Clelands reagent is used to moisten the inserts and 2 x 5 mm inserts are put into a 2 mm thick, 10% starch gel. The gel buffer is a 1/100 dilution of the tank buffer which is composed of sodium dihydrogen phosphate (33.8 g/1) and trisodium citrate (32 g/1), pH 5.9.

Visualization of the isoenzyme is accomplished by pouring 10 ml of reaction buffer containing 4 mg of 4-methylumbelliferyl phosphate (Sigma) in 0.05 M citrate buffer, pH 5.0, onto pre-cut filter paper. The moistened filter paper is placed onto the starch gel and the plate incubated for 2 hours at 37°C. The isoenzymes appear as fluorescent bands on a dark background under long wave u.v. light.

C. Haptoglobin (Hp)

For population screening purposes, serum samples were used. This eliminated the need for a gradient gel system which has been found to be necessary for use with dried stains.[2]

The sera were obtained from those samples used for the preparation of the stains mentioned under the PGM section of this report. The sera were

separated from the red cells and 4 drops placed into disposable glass tubes (10 x 75 mm). To each tube was added 1 drop of a red cell hemolysate. This hemolysate was prepared by incubating red cells and toluene (3:1) for 30 minutes followed by centrifugation. The red, lower layer is removed for use with the serum. Hemolysates were also prepared by freezing and thawing of packed cells (3x). One drop of hemolysate and 3 drops of serum constituted the serum hemolysate solution.

Filter paper wicks (1 x 3 mm) were soaked in the serum hemolysate solution and inserted into slots which had been cut in a 1 mm thick, persulfate polymerized 5% acrylamide gel (Cyanogum). The gel buffer was composed of Tris (9.2 g/1), citric acid 1.05 g/1, pH 8.6.

The visualization of the haptoglobin banding patterns was accomplished as follows. A 67% acetic acid solution (7.5 ml) containing (0.24 g) sodium perborate and either (0.075 g) malachite green, (0.1 g) benzidine or (0.1 g) tetramethylbenzidine was poured onto pre-cut filter paper. The paper was laid onto the gel surface at room temperature until staining began (approximately 10 minutes) and then removed. This procedure insured that discernible, not too heavily stained bands are developed. Of the three stains, benzidine proved to be the most sensitive.

II. Identification of Genetic Markers of Potential Use in Identifying the Racial Origin of Blood.

A. Procedural analysis of samples for the presence of Gm and Inv (Km) Factors

1. Titration of Gm antisera. Appropriate antisera were serially diluted (1:2, 1:4, 1:18, 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 rpm for 30 seconds and the agglutination was read macroscopically. The working dilution chosen was the next to the last one which gave a +4 agglutination (see Table II).

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 or Inv (Km) factor. The cells and antisera were incubated for 1 hour at 37°C, centrifuged, washed three times in saline and resuspended in saline to yield a cell suspension of approximately 2%.

3. Identification of Gm and Inv (Km) antigens in serum. Serum to be tested for the presence of Gm or Inv (Km) antigens was diluted 1/10 with saline. One drop of the diluted was mixed with one drop of appropriately diluted antiserum for 30 minutes at room temperature. One drop of sensitized red cells were then added and incubated for 30 minutes at 4°C. The suspension was centrifuged for 30 seconds at 1000 xG and agglutination read macroscopically.

4. Identification of Gm and Inv antigens in bloodstains. Bloodstains were cut from cotton sheets and placed into disposable glass tubes (10 x 75 mm). It was found that the antigens tested could be identified from threads. The size of the thread needed depends upon the particular antigen being sought.

Gm 1: requires a single 1 mm thread

Gm 2, 4, 12: requires a single 3 mm thread

Inv 1: requires a single 1 mm thread

Table II. Titration of Gm Antisera

Antisera Dilutions		Macroscopic Agglutination
Neat (undiluted)		+4
1:2		+4
1:4	working titer	+4
1:8		+4
1:16		+3
1:32		+2

One drop of appropriately diluted antisera was added to the threads and allowed to incubate at room temperature for 2 hours. After removal of the thread, one drop of sensitized cells was added to the tube and permitted to incubate at 4°C for 2 hours. The suspension was centrifuged at 1000 xG for 30 seconds and agglutination was read macroscopically.

5. Seminal and vaginal stains. Since the concentration of IgG is very low in seminal fluid or vaginal secretions, a larger stain must be used. To date Gm 1, 2, 4 and 12 have been determined in both. A (1 cm^2) section of stain was cut, minced into small pieces, placed into a disposable glass test tube (10 x 75 mm), and extracted with a minimal amount of isotonic saline for 2 hours at room temperature. The saline was removed from the stain and concentrated to 1 drop (0.05 ml) using an Amicon miniconcentrator. This drop was analyzed for the presence of Gm 1 antigen as described for bloodstains above.

6. Identification of Gm antigens in seminal fluid. Seminal fluid was obtained from volunteers and permitted to liquify. It was stored frozen (-20°C) until ready for an analysis. One drop of liquified seminal fluid was diluted with 1 drop of isotonic saline or frozen and thawed 4 times before use. Neat seminal fluid is too viscous and does not permit reliable Gm groupings. Two drops or an equal volume of appropriately diluted anti-Gm sera was added to the diluted seminal fluid sample and permitted to incubate at room temperature for 30 minutes. Two drops of a 2% suspension of appropriately sensitized red cells were then added and permitted to incubate at 4°C for 30 minutes. The suspension was then centrifuged at 1000 xG for 30 seconds and the agglutination read macroscopically.

B. Peptidase A

A manuscript has been published.[29]

Appendix A.

C. Glutathione reductase (GR)

At present the glutathione reductase system is not promising for forensic use. Although we have developed methodology for visualization of the enzyme and have determined its viability in a dried state, efforts to identify the various isoenzyme types have not been successful.

III. The Determination of Testosterone and Estradiol in Bloodstains as a Method for Sex determination

A. Preparation of Amberlite XAD-2 Resin Cartridges - New and Used

1. New Cartridges. New cartridges (Brinkmann Drug-Skreen) are received pre-filled but unassembled. Assembly is accomplished by removing cartridge caps, top and bottom, and snapping on sample reservoir. At this point the cartridges must be washed before the sample can be added. The wash procedure is described below.

2. Used cartridges. Cartridges to which samples have been added should dry thoroughly before removing resin. The drying facilitates blowing out the resin by means of an airline placed over the bottom opening of the cartridge.

The cartridges are inverted over a large plastic bag and the resin collected for disposal. All used cartridges were rinsed with acetone to remove residual samples. This was accomplished by placing 50 columns in a metal rack suspended over a sink. The rack was then inverted on a counter-top and the snap-on cap bottoms were replaced. The rack was

turned over again, suspended on suitable supports, and a small wad of glass wool was seated into the bottom opening of each column. Improper seating of the glass wool results in leakage of the resin.

Next, resin was prepared for loading the clean cartridges.

B. Amberlite XAD-2 Resin Wash Procedure

1. Bulk. (100 g) of Amberlite XAD-2 resin is placed in a 600 ml beaker. A Buchner funnel is lined with glass wool which extends up the sides of the funnel, and the funnel placed in a side arm flask with a vacuum attachment.

(300 ml) of methanol is added to the resin and mixed to give a slurry, which is then poured into the funnel. The beaker is rinsed with 500 ml of methanol and added to the funnel.

When the methanol has drained, 500 ml of chloroform, followed by 400 ml of a 1:1 ethanol:water solution is added to the funnel. Vacuum is then applied until the resin is dry. The glass wool is lifted from the funnel by holding the edges which extend up the side of the funnel, and the resin contents poured into a 600 ml beaker. (NOTE: Dry resin beads are difficult to contain if spilled. Be careful. (250 ml) of methanol is added to the washed resin, and a 10 ml pipette is used to add the resin to the columns to measure approximately 5 cm in height.

After filling the columns, a small wad of glass wool is packed into the top of each column to prevent the resin beads from floating out and adhering to the reservoir walls. When the excess methanol has evaporated, the columns are ready for use. The caps were removed from the bottoms and the columns placed on a (Brinkmann) vacuum apparatus. Nine columns are run at a time.

Each reservoir contains 25 ml. The reservoirs are twice filled with distilled water and suctioned off to remove residual solvent. When dry, the columns are ready for sample application. The samples are drained through the column by gravity flow (not vacuum). The sample containers are rinsed with isotonic saline and allowed to drain. The reservoirs are rinsed with 25 ml distilled water and vacuum applied to dryness. The next step involves the elution of the samples from the resin.

2. New columns. New pre-filled columns are placed on the Brinkmann vacuum apparatus and rinsed. All solutions are permitted to drain completely through the cartridge before adding succeeding solutions.

Wash Sequence for New Columns

1) 20 ml. MeOH
2) 15 ml $CHCl_3$
3) 10 ml. 50% EtOH
4) 50 ml dist. H_2O

The sample is added to the column and allowed to drip through column without vacuum. Rinse as described in Section IIIB1. (These are allowed to dry, preferably overnight, as water clouded the solution resulting from the succeeding part).

C. Sample Elution - New or Used Columns

The samples are eluted from the XAD-2 Columns with the following sequence of solvents.

1) 5 ml MeOH
2) 10 ml MeOH: $CHCl_3$(1:1)
3) 5 ml benzene

Collect all three fractions in a scintillation vial and evaporate.

During elution, no solvent should be added until the previous solvent has completely dripped through the column.

D. Preparation of Sephadex LH-20 columns

The columns used were New England Nuclear Minicolumns NEA-050. A rack was specially prepared to hold 12 of these columns with a space beneath each for a 13 x 100 mm tube, scintillation vial or a 30 ml beaker. Clean columns were placed in the rack and glass filters (Reeve Angel Grade 934 AH) pushed into the bottom of each to seal the opening using an inverted 2 ml pipette. The bottom and top filters were cut with cork bores.

Sephadex LH-20 (0.50 gm) was weighed into a glass conical vial (Brinkmann), one quantity per column/sample. Benzene:methanol (8-10 ml of 85:15) was added to each vial and then drawn up and expelled from a Pasteur pipette. This was done to insure uniform swelling and hasten clearing and settling in the columns. This step should not be done too early such as the night before or even several hours prior, to prevent absorption of moisture.

The slurry was added to the columns with a Pasteur pipette and allowed to settle. After draining, approximately 10 ml of 90:5:5 iso-octane:benzene:methanol (hereafter abbreviated as IBM) was added to the column. A change of color was noted, as the benzene:methanol solution was replaced on the column by IBM, from whitish to gray.

When the color had changed, the small glass filters were floated through the solvent, and gently placed onto the surface of the Sephadex. Occasionally more than 10 ml of IBM 90:5:5 was required to clear the Sephadex. The sample was added only when the Sephadex had changed color uniformily and the solvent drained completely.

E. Sample addition to Sephadex columns

At this point, when the solvent in the scintillation vials had evaporated to dryness, approximately 0.2-0.3 ml of IBM (90:5:5) was added to each sample vial, which was swirled and capped until ready to be placed on the column. Addition to the columns was accomplished using a Pasteur pipette and dropping the sample onto the column as close to the filter as possible. When adding solvents to the columns, caution must be exercised so as not to disturb the filter on the Sephadex.

F. Steroid fraction collection

IBM 90:5:5 (0.1 ml) was added to rinse the column walls. Immediately 2.0 ml IBM 90:5:5 was added and allowed to drain. When dry, 5.0 ml IBM 90:5:5 was added and the Progesterone Fraction collected in a test tube. When dry, 3.0 ml IBM 90:5:5 was added and discarded. When dry again, 7.0 ml IBM 90:5:5 was added and collected for the Testosterone Fraction. Next, 5.0 ml IBM 90:5:5 was added and discarded. The solvent was then changed to IBM 62:20:18 for the Estradiol Fraction collection. IBM 62:20:18 (1.0 ml) was added and discarded. This was followed by 4.0 ml IBM 62:20:18 which was collected as the Estradiol Fraction. See Figure 1. These fractions were evaporated to dryness.

The following is the procedure for reconstituting the dry fractions for the radioimmunoassay.

G. Radioimmunoassay (RIA) of testosterone, estradiol and progesterone.

1. Sample Size for RIA

   a. Reconstitution of fractions from 1.0 ml serum samples so that the quantity of steroid would be in the same range as if a 100 µl stain had been used.

   1) Reconstitute Progesterone Fraction in 0.5 ml ethanol and take duplicate 50 µl aliquots for RIA.
   2) Reconstitute Testosterone Fraction in 0.5 ml ethanol and take 25 µl duplicate samples for RIA.
   3) Reconstitute Estradiol Fraction in 0.5 ml ethanol and take 100 µl duplicate samples for RIA.

   b. For stains, equivalent to 25-250 µl whole blood, each steroid fraction is reconstituted in 250 µl of ethanol and duplicate 100 µl aliquots taken for RIA.

   c. The duplicate aliquots were placed in separate 10 x 75 mm disposable culture tubes and the ethanol evaporated.

2. Table III shows the procedure employed for the additions to the various 10 x 75 mm tubes for the radioimmunoassay of each steroid.

TABLE III

Tube designation	Assay buffer(ml)†	Sample(ml)	Assay tracer(ml)†	Anti-serum(ml)	Charcoal Suspension(ml)†
Total Count determination	1.1	-	0.1	-	-
Blank	0.2	-	0.1	-	1.0
Zero	0.1	-	0.1	0.1	1.0
Standards	-	0.1	0.1	0.1	1.0
Sample	0.1	Appropriately reconstituted aliquot, dried down	0.1	0.1	1.0

†See below for preparation

For each steroid being quantitated duplicates of the total, blank, and zero tubes plus, 10 standard tubes (0.5-500 pg) are prepared. A standard curve is prepared by incubating all of the samples at 4° for 2-24 hours with the appropriate quantities of buffer, tracer and antiserum as shown in Table III. One can prepare a series of three standards (5, 50 and 250 pg) to check a previous standard curve in the same way. Charcoal suspension is then added with mixing to all but the "total" tubes; all are then allowed to stand for 5 minutes at 4° before centrifuging at 2000 x g for 5 minutes. The dextran-coated charcoal suspension precipitates any unbound antigen and the supernatant is decanted into scintillation vials. Ten milliliters of Riafluor (New England Nuclear) scintillation fluid is added to each vial plus an additional one-half ml of water. The vial is capped, mixed and the resulting clear solution is counted for 5 minutes in a Beckman scintillation counter.

a. Preparation of buffers

The RIA buffers are those described in the New England Nuclear (NEN) literature (24,25,26) which is provided with the specific antisera. Stored at 4°, these buffers may be kept for at least 2 weeks.

1) Testosterone assay buffer is 0.05 M sodium phosphate buffer, pH 7.4, containing 5.0 g bovine serum albumin, Fraction V, and 1.0 g sodium azide per liter.

2) The estradiol and progesterone assay buffers are both the same, being 0.1 M sodium phosphate with 9.0 g sodium chloride and 1.0 g sodium azide per liter adjusted to pH 7.0, and in which 1.0 g gamma globulin, Fraction II, is dissolved.

b. Preparation of dextran-coated charcoal suspension.

1) For the testosterone assay, 9.0 g sodium chloride is added to 1.0 liter of 0.1 M sodium phosphate buffer pH 7.4, followed by 0.25 g of dextran T-70 and dissolved; then 2.5 g of Norit A, neutral charcoal is added.

2) For estradiol or progesterone assays, 0.25 g dextran T-70 and 2.5 g Norit A, neutral charcoal are dissolved in a liter of assay buffer.

c. Preparation of Tracer

The procedures supplied by NEN have lower limits of approximately 15-20 pg, which is not sensitive enough for detecting hormones in blood-stains where a lower limit of 1 pg is necessary. To attain this lower sensitivity requires reducing the mass of tracer used with a corresponding drop in the amount of antiserum required for a 50% binding in the zero standard. The problem with simply decreasing the amount of tracer is that one has too few counts for good statistics in a reasonable counting time. These problems are overcome by the use of tetra-tritium-labeled steroids rather than di-labeled, with about 50% efficiency in counting tritium instead of 30%, and by using half as many total counts. The specific activity of the tetra-labeled NEN steroids is twice that of the di-labeled. Our Beckman LS-100c scintillation counter operates at nearly 60% efficiency for tritium, which is twice what the NEN literature states in their directions, with the decrease in total counts therefore giving a 2 x 2 x 2 or 8 fold decrease in the mass of tracer per assay. A corresponding decrease of approximately 8 fold in the amount of antiserum used enables one to reach the required sensitivity of 1.0 picogram.

The tetra-labeled steriods are made up to be 2500 counts per minute (cpm) (60% counting efficiency) per 0.1 ml of the respective assay buffer.

1) The NEN tetra labeled steroids have a specific activity of 85-105 Ci/mmole. In the case of testosterone 1,2,6,7-$^{3}H(N)$, molecular weight 288.4, this average specific activity of 95 Ci/ mmole makes 2500 cpm equivalent to 5.7 pg. This result is calculated as shown below.

$$2500 \text{ cpm} \times \frac{1 \text{dpm}^{*}}{0.6 \text{ cpm}} \times \frac{1 \text{ µCi}}{2.2 \times 10^{6} \text{ dpm}} \times \frac{\text{Ci}}{10^{6} \text{ µCi}} \times \frac{\text{mmole T}}{95 \text{ Ci}}$$

$$\times \frac{10^{9} \text{ pmole}}{\text{mmole}} \times \frac{288.4 \text{ pg testosterone}}{\text{pmole T}} = 5.74 \text{ pg testosterone}$$

2) A spontaneous degradation of the tetra-labeled steroids upon storage requires that they be purified at least every month by Sephadex LH-20 chromatography. For testosterone or progesterone 2-10 µCi of the steroid are evaporated down, redissolved in 0.2 ml of isooctane: benzene: methanol IBM 90:5:5 and added to an IBM(90:5:5) equilibrated 1.0 g LH-20 sephadex column, 1 cm in diameter. Continued addition of IBM(90:5:5) elutes progesterone in the 5-11 ml fraction and testosterone in the 17-26 ml fraction. Estradiol is purified by evaporating down 2-10 µCi, redissolving in 0.2 ml of IBM (62:20:18) and adding an 0.8 g sephadex LH-20 column equilibrated in IBM (62:20:18). Additional IBM (62:20:18) elutes estradiol in the 7-10.5 ml fraction. The solvent with each purified steroid is gently evaporated under a stream of nitrogen and the purified steroid redissolved in 10 ml of benzene: ethanol, (9:1), and stored at 4°.

---

*cpm: Counts per minute

dpm: Disintigrations per minute

Ci: Curies

3) 10 µl of the purified steroid is placed in a scintillation vial, the solvent allowed to evaporate, scintillation fluid added and the cpm/10 µl determined. From this value, determine the quantity to be taken for 500,000 cpm and place in a scintillation vial. Evaporate the solvent and add 20 ml of the respective assay buffer, mix and allow to dissolve while stored at 4°. This solution is the assay tracer, 0.1 ml of which equals 2500 cmp/5.7 pg testosterone or 2500 cpm/5.4 pg estradiol or 2500 cpm/6.2 pg progesterone.

4) If the blank values in the RIA procedure become greater than 100 cpm, it indicates that the tracer requires re-purification.

d. Titration of antisera

Since the mass of tracer was decreased to approximately 1/8 that indicated in the NEN literature, the titer of the antiserum to give 40-50% binding of tracer is determined as follows:

1) To the vial of lyophilized antiserum 10 ml of assay buffer is added and let stand for 30 minutes with occasional gentle stirring to dissolve the solids completely.

2) Prepare 4-fold, 8-fold and 12-fold dilutions of the concentrated antiserum, i.e. 0.1 ml plus 0.3 ml buffer for the 4-fold, 0.05 ml plus 0.35 ml buffer for the 8-fold and 0.025 ml plus 0.375 ml buffer for the 12-fold dilution.

3) Prepare a group of 10 x 75 mm tubes as outlined in Table IV using the prepared assay tracer and the three antiserum dilutions.

TABLE IV

	Tube#	Assay buffer	^{3}H-steroid ~5pg	Dilution of antiserum 4-fold	8-fold	12-fold
Total=	1,2	1.0 ml	0.1 ml	-	-	-
Blank=	3,4	0.1 ml	0.1 ml	-	-	-
	5,6	0.1 ml	0.1 ml	0.1 ml	-	-
	7,8	0.1 ml	0.1 ml	-	0.1 ml	-
	9,10	0.1 ml	0.1 ml	-	-	0.1 ml

4) Mix and allow these tubes to incubate at 4°C for 2 hours.

5) Add 1.0 ml of cold dextran-coated charcoal suspension (with constant stirring) to all tubes except 1 and 2, which serve as duplicate "total" tubes, and allow the tubes to remain at 4°C for 5 minutes.

6) Centrifuge at 2000 x g for 10 minutes.

7) Decant the supernatant into counting vials, add 10 ml of scintillation fluid and count for 5 minutes.

8) Average the values for the blank tubes (3 and 4) and subtract from the averages of all the other duplicate samples. Determine the % tracer bound for each dilution relative to the value of the total tubes by plotting the dilution vs the % tracer bound and determining the dilution which gives 40-50% binding.

9) Dilute the stock solution of antiserum as required to give 40-50% to binding for use in the radioimmunoassay.

e. Preparation of Standards

The standards as supplied by NEN are 100 ng/ml. Other high purity steroids could be used to make stock standard solutions. The range of interest is 0.5 to 250 pg and standards are made as shown in Table V, where the 100 ng/ml solution is the stock.

TABLE V

Solution	Aliquot (ml)	Buffer (ml)	Conc in pg/0.1 ml
A	0.1 Stock	1.9	500
B	0.4 A	0.6	200
C	0.5 B	0.5	100
D	0.5 C	0.5	50
E	0.4 D	0.6	20
F	0.5 E	0.5	10
G	0.5 F	0.5	5
H	0.4 G	0.6	2
I	0.5 H	0.5	1
J	0.5 I	0.5	0.5
K	0.4 J	0.6	0.2

f. Calculation of Testosterone to Estradiol Ratio.

From the scintillation counter data it is now possible to calculate the quantity of each steroid present in the blood stain and then their ratio.

1) The calculation procedure supplied by New England Nuclear is as follows:

For the total recovery standard, the zero standard, each of the other standards, and all samples, average the duplicate values and correct for counting efficiency. The corrected

average blank value is subtracted from the other corrected average values to obtain the net total, standard, and sample values. By then dividing each net standard and sample value by the net total, and multiplying by 100, the percent bound is calculated for each one. A plot of the % bound versus the log of the quantity of the standards yields a standard curve from which the quantities of each sample may be interpolated from their respective percents bound. Successive trials should produce quite similar standard curves as in Figure 2. In the titration of the antiserum, the zero standard was adjusted to have a percent-bound value of 40-50. Any marked variation in the curve is an indication of changed conditions, altered reagents or mishandling.

Using the quantity of each steroid on a standard basis (i.e. per ml serum or per stain), the testosterone to estradiol ratio can be calculated and a prediction of the sexual origin of the blood or bloodstain made.

2) Because of the many calculations involved, it was desirable to write a program and have a computer take the data and read the quantities from the standard curve. The extent to which automated data handling is possible depends on available computer ability.

An accepted means of handling these calculations is to convert ones data to a logit function (28), a plot of which versus the log of the quantity of steroid produces a straight line. From this line, the slope and y-intercept are determined and then any quantity of steroid (S) can be calculated for any data point (F). To convert data to logit function the equation is

$$\text{Logit } Y = \ln \frac{B}{B_o - B} \qquad (1)$$

where B is equal to the percent bound for each sample or standard and is defined as follows,

$$B = \frac{[X(\frac{10}{qx})] - [b(\frac{10}{qb})]}{[T(\frac{10}{qt})]\ [b(\frac{10}{qb})]} \quad [100]$$

where X is the counts per minutes (cpm) for a particular sample, qx is the quench value for the counting of sample X, which for this system in a Beckman LS-100c is near 6.0 for tritium indicating a counting efficiency of near 60%, $X(\frac{10}{qx})$ is the average of efficiency-corrected duplicate samples, b is the cpm for the blank, qb the quench value for the blank and T the cpm of the total with qt its quench value. B_o is the percent of the total radioactivity bound for the zero standard and is as follows

$$B_o = \frac{[Z(\frac{10}{qz})] - [b(\frac{10}{qb})]}{[T(\frac{10}{qt})] - [b(\frac{10}{qt})]} \quad [100].$$

where Z is the cpm of the zero standard and qz its quench value. Substituting these values into equation (1) one obtains after simplification

$$F = \text{logit } Y = \ln \frac{X\frac{10}{qx} - b\frac{10}{qb}}{(Z\frac{10}{qz} - b\frac{10}{qb}) - (X\frac{10}{qz} - b\frac{10}{qb})}$$

$$= \frac{x'-b'}{(z'-b')-(x'-b')} = \frac{x''}{z''-x''} \qquad \text{where}$$

$x' = X\frac{10}{qx}$, $b' = b\frac{10}{qb}$, $z' = z\frac{10}{qz}$, $x'' = x'-b'$ and $z'' = z'-b'$.

On four cycle semilog paper a plot of logit Y(F) on the abscissa versus the quantity of steroid (S) on the ordinate yields a straight line as in Figure 3. The equation of the line is

Log S = m F + b, where the slope m equals $\frac{\Delta \log S}{\Delta F}$ and b, the ordinate intercept, equals log S at logit Y≡F=0 as determined from the standard curve. With these values for m and b the quantity of steroid can be calculated for any F value as the corresponding antilogarithm of log S. After these steps are performed the ratio of quantities of testosterone to estradiol is determined for each sample.

IV. Miscellaneous

A. Detection of Drugs in Dried Blood Stains.

The use of the XAD-2 column described for the isolation of testosterone and estradiol can theoretically be used to isolate drugs in stains as well. Since the concentration of drugs in the blood resulting from therapeutic doses is very small, very sensitive techniques must be employed for their detection. Radioimmunoassay is such a technique. Thus the detection of drugs requires only that an antiserum be available for the particular drug in question.

We have in our laboratory a person on Dilantin (diphenylhydantoin) therapy. Antisera to this drug was obtained and the radioimmunoassay procedure was worked out. The drug was detected in a 10 µl bloodstain. This size stain represents 1/5 of a drop of blood and was approximately 100 times as much bloodstain as required for this particular analysis.

From our results we anticipate that the amount of blood needed to detect diphenylhydantoin would be approximately 1 µl.

Although there are procedural details to be worked out concerning the isolation and fractionation of drugs, it seems that the use of the non-ionic XAD-2 resin for the fractionation of drugs from dried stains is an ideal method since it permits the isolation of the drug fraction

and allows the protein and enzyme fractions to be collected undamaged. Thus far PGM, EAP and haptoglobin have been successfully isolated from the resin.

B. MN System

The absorption-elution technique utilized for the detection of the M and N antigens is described in the pre-publication in Appendix B.

CHAPTER III. RESULTS AND DISCUSSION

I. Population Frequencies for Polymorphic Systems of Forensic Interest

The statistical results of this study are presented in Tables VI, VII and VIII for phosphoglucomutase (PGM), erythrocyte acid phosphatase (EAP) and haptoglobin (Hp), respectively. These results, plus the experimental section for each of these systems, provide a practical approach to the determination of genetic markers in bloodstains for forensic serologists. These data present the forensic serologist with a reliable method for identifying the type for each of these polymorphic systems, as well as providing data which can be used for calculating the uniqueness of a particular blood sample in the population.

The samples for this study 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 date were recorded for each sample. Care was taken to insure that no duplicate samples were taken and that only samples representative of the population were obtained. The jail samples included a significant number of Black persons in the study. Samples were taken from all persons admitted to the jail whether they were released or held.

Table VI shows the results obtained for the PGM system. It should be noted that the values for the Black and Caucasian populations are not the same. This discrepancy must be taken into consideration by the forensic serologist. For example, a stain may be analyzed as being PGM 1-1, but the percentage of this phenotype in the population will be different depending upon the racial origin of the stain.

TABLE VI. Phosphoglucomutase (PGM)

Category	N		Phenotypes 1-1	2-1	2-2	6-2	Phenotypic Frequency 1-1	2-1	2-2	Gene Frequency PGM_1	PGM_2	χ^2	Probability
Caucasian	1253	Observed number	698	487	67	1	0.557	0.389	0.053	0.751	0.248		
		Expected proportion	0.564	0.373	0.062								
		Expected number	706.7	466.4	76.9								
		$\frac{(O-E)^2}{E}$	0.107	0.910	1.285							2.302	0.13
Black	714	Observed number	481	209	24		0.674	0.293	0.034	0.821	0.181		
		Expected proportion	0.674	0.297	0.033								
		Expected number	480.7	211.5	23.3								
		$\frac{(O-E)^2}{E}$	0.0002	0.0295	0.0210							0.0507	0.87

Table VII shows the statistics for the erythocyte acid phosphatase (EAP) system. Again, it must be noticed that there is a difference between the Black and Caucasian population frequencies and the same precautions must be taken in calculating the uniqueness of a particular sample. With this system the identification of the different isoenzyme types is dependent upon the different intensities of certain bands in the zymogram. The interpretation of these bands can become very difficult for certain of the banding patterns, especially for B, CB and C. There are no problems in the identity of BA, A, or CA but it is possible that mistyping could occur for the C, CB or B isoenzymes. Considerable care in the preparation of samples as well as considerable experience is necessary before attempting to utilize this system in case work.

Table VIII shows the statistics compiled for the haptoglobin system. Again, as with the PGM and EAP systems, there are differences in the values for the Black and Caucasian populations. One facet of haptoglobin analysis which must be of concern to the forensic serologist is the possibility of having a Hp_o, no haptoglobin present in the individual, and therefore none in the sample. Should this situation occur it must be interpreted with caution since the inability to demonstrate haptoglobins by electrophoretic technique does not necessarily indicate the presence of the Hp_o genotype but may instead represent degradation of haptoglobins in that particular sample. If it is known that the victim's blood is of the Hp_o type, then the corresponding stain would not be expected to give a discernible banding pattern. The same caution in interpreting Hp 2-1 M types must be exercised. This is true since the identification of

TABLE VII. Erythrocytic Acid Phosphatase (EAP)

Category	N		Phenotypes A	BA	B	CA	CB	C	RA	Gene Frequency p^A	p^B	p^C	χ^2	Probability
Caucasion	1239	Observed number	145	526	491	20	57							
		Phenotypic frequency	0.117	0.425	0.396	0.016	0.046			0.337	0.632	0.031		
		Expected proportion	0.114	0.426	0.399	0.021	0.039	0.001						
		Expected number	140.7	527.8	494.9	25.9	48.5	1.2						
		$\frac{(O-E)^2}{E}$	0.131	0.006	0.031	1.34	1.490	1.2					4.198	0.38
Black	718	Observed number	39	239	426	2	11	0	1					
		Phenotypic frequency	0.054	0.333	0.593	0.003	0.015	0	0.001	0.222	0.767	0.009		
		Expected proportion	0.049	0.341	0.588	0.004	0.014	0.0001						
		Expected number	35.4	244.5	422.4	2.9	9.9	0.1						
		$\frac{(O-E)^2}{E}$	0.366	0.124	0.031	0.279	0.122	0.1					1.022	0.91

TABLE VIII. Haptoglobin(Hp)

Category	N		Phenotypes 1-1	2-1	2-1M	2-2	0	Phenotypic Frequency 1-1	2-1	2-2	Gene Frequency Hp_1	Hp_2	χ^2	Probability
Caucasion	1263	Observed Number	185	555	26	495	2	0.146	0.460	0.392	0.38	0.62		
		2-1 and 2-1M combined		581										
		Expected proportion	0.144	0.471		0.394								
		Expected number	182.4	595.1		485.5								
		$\frac{(O-E)^2}{E}$	0.038	0.335		0.186							0.559	0.46
Black	721	Observed number	206	293	66	148	8	0.286	0.498	0.205	0.535	0.454		
		(2-1)+ (2-1M)		359										
		Expected proportion	0.286	0.486		0.206								
		Expected number	206.4	350.2		148.6								
		$\frac{(O-E)^2}{E}$	0.001	0.221		0.002							0.224	0.65

this Hp type is accomplished by recognizing that certain 2-1 bands are not present. Thus, although one might expect all haptoglobin bands to disappear at the same rate, their disappearance from a stain is also dependent upon the sensitivity of the staining technique. Thus, the more concentrated bands in a degrading Hp 2-1 type may be the only ones visualized, but the sample may appear as a Hp 2-1 M thus changing drastically the population frequency. Where possible, serum samples should be run for comparison. For example, if a serum sample of the victim or suspect is available, this should be used for typing purposes. The determination of the Hp type of the stain should then be compared to this to determine the viability of the haptoglobins in that stain. It is probably very dangerous to positively identify 2-1 M in stains.

II. Identification of Genetic Markers of Potential Use in Identifying the Racial Origin of Bloodstain Evidence.

A. Gm and Inv(Km) Antigens.

The method used for the identification of the Gm and Inv types in blood and physiological stains is the absorption inhibition technique. The technique devised is a modification of the manufacturers recommendations based on the ability to detect the presence of the weakest antigen in dried blood.

Table IX illustrates the incubation time required to obtain a 4+ agglutination with the weak antisera prepared commercially. Thus, any problems encountered with this system are largely concerned with quality of antisera available.

TABLE IX. Incubation Times for Random Lots of Commercial Anti-Gm Sera

Anti Sera	Incubation time (4°C)
Gm_2	40 minutes
Gm_{12}	80 minutes
Gm_1	120 minutes

For this work, commercially prepared, pre-diluted, lyophilized, antisera for Gm 1, 2, 4, 12 and Inv(Km) 1 were purchased from Behring Diagnostics. It was determined that the reconstituted antisera has a very short shelf life (7 days) and should not be thawed from the frozen state more than once. It was also determined that the dilution of the manufacturer is sufficient for forensic purposes, provided the expiration date of the antisera has not passed.

Behring Diagnostics supplied, gratis, raw (undiluted) anti Gm 1 sera. It was found that this antiserum has a shelf life (unfrozen and undiluted) of at least 6 months - to date, see Table X. The appropriate dilutions for forensic purposes can be determined by titration as previously described at which time its shelf-life decreases to 7 days at a maximum. For the antiserum supplied a dilution of 1:16 was found to be satisfactory for grouping purposes. The working dilutions must be determined prior to each usage.

The great versatility of this system is the ability to identify these antigens in stains other than blood. Thus far successful groupings have been made in seminal fluid, seminal stains, and a vaginal stain. The problem encountered with these stains and fluids is the relatively small amount of gamma globulin present. Thus larger quantities of stain must be eluted. We have routinely been using 1 cm^2 stain eluted in saline and then concentrated to a drop using an Amicon miniconcentrator.

The sensitivity of this method is very great. For example, for Gm 1 a single 1 mm thread or 1 µl of serum is required for grouping purposes. The other Gm antigens, 2, 4, and 12, require approximately 2-4 µl of serum or a 2-4 mm single thread. The Inv 1 (Km) antigen requires approximately 2 µl of serum or a 1-2 mm single thread of bloodstain.

TABLE X. Gm Antisera Shelf Life

	Date	Working Titer	Working titer Shelf Life
Raw Anti-Gm_1	6/1975	1:32	unfrozen storage
	11/1975	1:16	unfrozen storage
	2/1976	1:4	unfrozen storage
Commercial (Reconstituted)		-	unfrozen storage
Commercial (Reconstituted)		-	frozen storage

The persistence or viability of these antigens within the time span of this project was determined. The Gm 1 antigen was found to be viable for at least one year. For the other Gm antigens, 2, 4 and 12, and the Inv(Km) 1 antigen, the determination of the viability was not accurate since sufficient antisera was not available.

B. Peptidase A

The discussion of the procedures for the determination of peptidase A and the statistics can be found in the pre-publication in Appendix A.

That essentially none of the Caucasian population is found to have the peptidase A 2-1, 2-2 or 3-1 types automatically gives this system great utility in the determination of the racial origin of a bloodstain. The methodology was developed so that PGM and adenylate kinase (AK) could be determined at the same time as peptidase A. Thus, in addition to the possibility of determining the racial origin of a stain, other statistical information can be determined at the same time. In an ideal situation a stain could be identified as coming from a Black individual with a probability of being found in 0.0085% of the population.

It was also found that peptidase A could be identified in seminal stains but not in seminal fluid which had been stored at either -20°C or +4°C for more than 1 week. The viability of this enzyme system in seminal stains is at least 2 weeks.

C. Glutathione Reductase (GR)

Although several different electrophoretic systems were devised for the separation and visualization of the GR isoenzymes, none proved satisfactory. Two systems for visualization were found to be successful but none of the systems used was capable of separating the different isoenzymes.

III. The Sexual Determination of Blood

The extraction of steroids from a bloodstain can be accomplished in greater than 90% yield. Several successful methods were employed, including solvent extraction. Although satisfactory isolation of the steroids resulted with an organic solvent extraction procedure, the other forensically important factors, i.e. enzymes and proteins, were destroyed.

With the interest of the forensic scientist in mind, a technique using a non-ionic resin (Amberlite XAD-2) was used. Using columns filled with this resin, drugs, drug-like substances and hormones (testosterone and estradiol) are immobilized on the column while the other forensically important factors, i.e., enzymes, proteins, and metals pass through undamaged.

We have found that the steroid hormones, testosterone and estradiol, can be separated from the acidic and neutral drugs by solvent elution. Thus, should drugs be present in the stain, they too can be identified. The steroid fraction was isolated and then fractionated using Sephadex LH-20.

The concentration of testosterone and estradiol was determined using a radioimmunoassay procedure.

Approximately 1500 serum samples were analyzed. It was found that the T/E testosterone/estradiol) ratio varied between 60 and 0.02 such that values above 3.51 could be identified as being male and values below 3.51 could be identified as female.

That the probability of sexual predictions is not 100% is interesting. Some of the samples gave values which were not in the T/E range expected. For example, several male bloods were found to have values of approximately 0.2 while several female bloods were in the range significantly above 3.51. All these samples were run on a blind trial basis without regard

to the state of health or the sexual preferences of the individuals. Had any pathological state of the individual been known, some of the aberrant results might have been explained.

Approximately 250 stains corresponding to the serum samples were also processed. The stains were 6-8 months old and the predictability was the same as for the serum samples for males and females. One interesting result from the stain analysis was that the T/E ratio dividing males from females, Figure 4, was 1.0 instead of 3.51 for serum. The predictability for males and females was the same: 92.7% for females and 90-98% for males (Table XI.) The ratio discrepancy is difficult to explain, but there may be two factors involved. The first could involve the age of the stain (6-8 months). Since the overall T/E ratio is lower in the stains, the concentration of testosterone is either lower or the estradiol is higher. In the former case, it may be possible that testosterone degrades over a period of time, although the chemistry of the substance would not indicate that this would be so. For the latter case, an increase in the concentration of estradiol could also be explained on the basis of cross-reacting substances which may be present in the red cell or an enzymatic conversion is taking place. Since the stain contains lysed red cells whereas serum does not, the presence of a cross reacting substance in red cells would give an abnormally high value for estradiol and consequently a lower T/E ratio. These possibilities are being investigated.

IV. Miscellaneous Results

A. Detection of drugs in dried blood

We decided to take advantage of the fact that one of the laboratory personnel is an epileptic on dilantin therapy. Thus, we worked out the

TABLE XI. Bloodstain Data

T/E	Percentages		Predicability
	Male	Female	
Above 2.0	56.2	1.1	98.9% male
1.0-2.0	37.5	10.4	89.6% male
Below 1.0	7.3	88.5	92.7% female

radioimmunoassay for dilantin in serum and utilized the procedure for the determination of the drug in a bloodstain. The smallest stain for which the procedure was attempted was 10 µl or 1/5 of a drop of blood. It was found that this size of stain is approximately 100 times larger than is necessary for the identification.

Since antisera are available for other drugs, i.e. morphine, LSD, THC and the barbituates, procedures for their detection in bloodstains is also a possibility.

The use of the XAD-2 resin to isolate the drugs permits the detection of the presence of the drug without destroying the other forensically important factors in the stain.

B. The MN system

Although the absorption-elution technique is adequate for identifying the presence of the M and N antigens, problems inherent in the system can lead to mistyping if they are not understood.

It is known that uncertainties arise as a result of the greater sensitivity required by forensic serology. Thus the weak anti-N sera available is not always strong enough to detect the N antigen present in a given blood stain. Also, by using the absorption elution technique, one cannot distinguish between MN and cross-reacting M stains (Mx).

Since it is possible to distinguish MN from Mx in whole blood, an approach was taken to duplicate the conditions used in whole blood grouping for use in the absorption elution technique.

Anti-N sera (Ortho) was concentrated in volume five times (Titer increased two fold). The absorption elution test normally utilized bovine serum albumin (BSA) with the indicator cells to increase the sensitivity of the reaction.

Although the data obtained were very preliminary, the results are suggestive of the following.

1. Using concentrated anti-N preparations, a much faster, more conclusive reaction with N antigen is obtained even with optimized stains up to 1 year old.
2. The differentiation test between Mx and MN, utilizes protein (i.e., tube test uses washed cells while the slide test uses whole blood). Thus, by eliminating BSA from the indicator cell suspension in the absorption elution reaction, it became clear that commercially prepared anti-N will not react well with old stains (1 year) and not at all with cross-reacting (Mx) M stains. The concentrated (5x) anti-N sera (same lot number as unconcentrated anti-N) reacts well with both Mx and old stains. By adding protein back to the indicator cells, the weaker anti-N preparation now reacts with both the fresh Mx and old N stains.
3. It seems possible that by utilizing both the concentrated and unconcentrated anti-N preparations, both with and without the presence of BSA in the indicator cells, a test could be devised which would permit a differentiation between Mx and MN stains. Also, by using the concentrated anti-N preparation, a better reaction with the N antigen occurs, even with older stains (1 year).

CHAPTER IV. CONCLUSIONS

It is concluded that the results of this research can be used immediately in the Criminal Justice System in the United States. This research has provided the criminalist and forensic serologist with the capability of determining the uniqueness of a blood specimen in the black or white population for the PGM, EAP and Hp systems.

In addition, by identifying the peptidase A isoenzyme type and the Gm and Inv(Km) factors present in a bloodstain, it may be possible to identify the ethnic origin of the stain.

A technique was developed for determining the sexual origin of a bloodstain by analyzing the testosterone/estradiol ratio in the stain.

Finally, preliminary results have indicated that drugs can also be identified in bloodstains without losing the capability of identifying the other forensically important factors present in blood.

Thus, this research will not only expand the technological capabilities of the forensic scientist but also increase the usefulness of bloodstain evidence as an aid in the criminal justice process.

CHAPTER V. BIBLIOGRAPHY

1. Stedman, R., J. Forensic Sci. Soc. 12, 379 (1972).
2. Giblett, E., Genetic Markets in Human Blood, Blackwell Pub. Co., 1969.
3. Culliford, B. J., The Examination and Typing of Bloodstains in the Crime Laboratory, PR 71-7 U.S. Department of Justice, Law Enforcement Assistance Administration, 1971.
4. Wraxall, B. and Culliford, B., personal communication.
5. Welch, S. G., J. Forensic Sci. Soc. 12, 605 (1972).
6. Schanfield, M. S., Hum. Hered. 22(2), 144 (1972).
7. Kneiphoff, H. and Nagel, U., Aerztl. Lab. 16(11), 354 (1970).
8. Lewis, W. H. P. and Harris, H., Nature 215 (1967).
9. Kaplan, J. D. and Bentler, E., Nature 217, 256 (1968).
10. Lewis, W. H. P., Nature 230, 215 (1971).
11. Grubb, R. and Laurell, C. B., Acta Pathol. Microbiol. Scand. 39, 390 (1956).
12. Ropartz, C., Lenior, J., and Rivat, L., Nature 189, 586 (1961).
13. Planque, J., Ruffie, J. and Ducos, J., Toulose Medical J. 62, 685 (1961).
14. Blanc, M., Görtz, R. and Ducos, J., C. R. Soc. Biol. 167(5), 77 (1973).
15. Rex-Kiss, B., Z. Morphol. Anthropol. 64(1), 15 (1972).
16. Schanfield, M. S., Hum. Hered. 21, 628 (1971).
17. Iza, H. and Martan, M., Hum. Hered. 21, 628 (1971).
18. Renard, S., J. Forensic Sci. Soc., 11(1), 15 (1971).
19. Roenner, P. and Hall, H. Endokrinologie, 58(2), 264 (1971).
20. Doerner, G., Stahl, F., Rohde, W., Hall, H., Roenner, P., Gruber, D. and Heeter, U., Endokrinologie 61(2), 317 (1973).
21. Furuyama, S., Mayes, D. M. and Nugent, C. A., Steroids, 16(4), 415 (1970).
22. Hillier, S. G., Brownsey, B. G. and Cameron, E. H. D., Steroids, 21(5), 735 (1973).
23. Boon, D. A., Keenan, R. E. and Slaunwhite, W. R., Jr., Steroids, 20(3), 269 (1972).
24. Pal, S. B., Clin. Chim. Acta, 33, 215 (1971).

25. New England Nuclear Testosterone [^{3}H] Radioimmunoassay PAK.

26. New England Nuclear Estradiol Radioimmunoassay PAK.

27. New England Nuclear Progestrone Radioimmunoassay PAK.

28. Rodbard, David, Clin. Chem. 20, 1255-1270 (1974).

29. Neilson, D. M., Shaler, R. C., Stuver, W. C., Mortimer, C. E., and Hagins, A. M., J. Forensic Sci. 21(3), 510 (1976).

LEGENDS TO FIGURES

Figure 1. Steroid separation on Sephadex LH-20.

Figure 2. Testosterone Standard Curve.

Plot of % Bound -vs- log [testosterone, pg].

Figure 3. Logit Y Standard Curve

Plot of Logit Y -vs- pg steroid

Figure 4. Bloodstain Data.

Ratio of Testosterone/Estradiol (T/E) -vs- Donor Age.

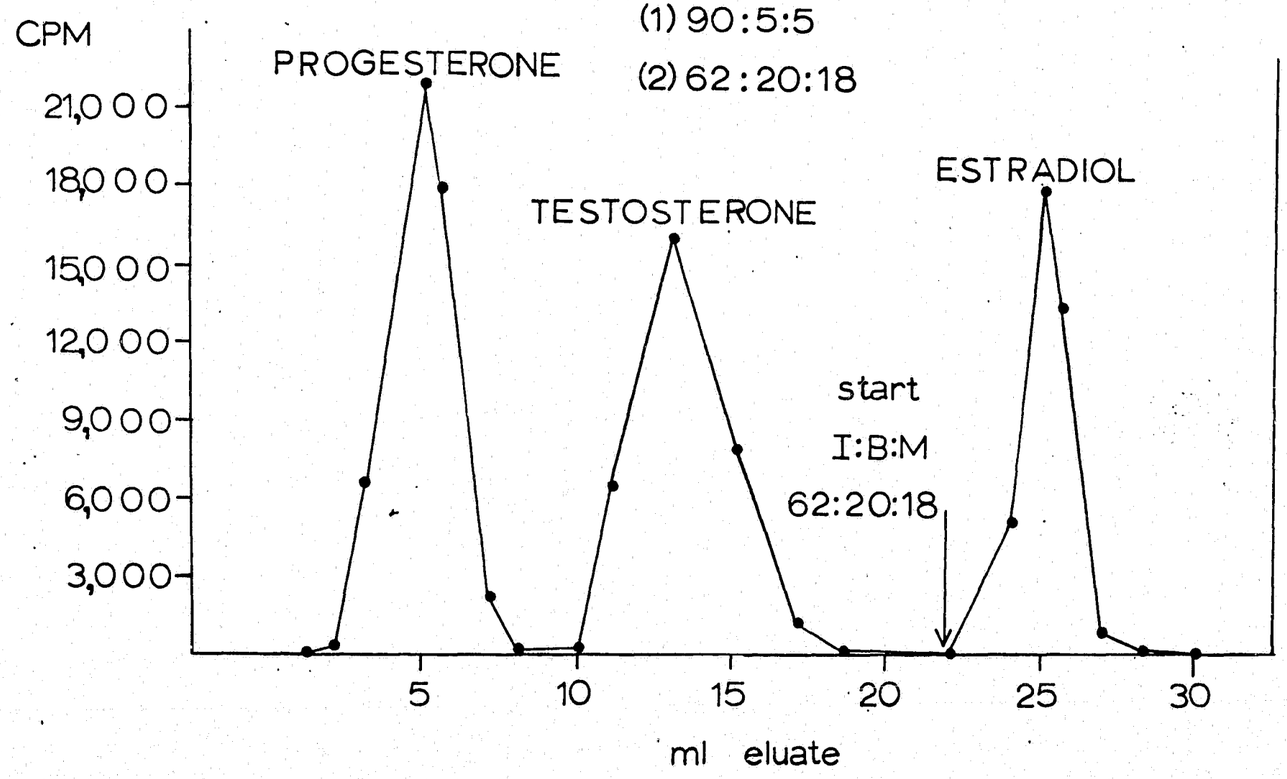
STEROID SEPARATION

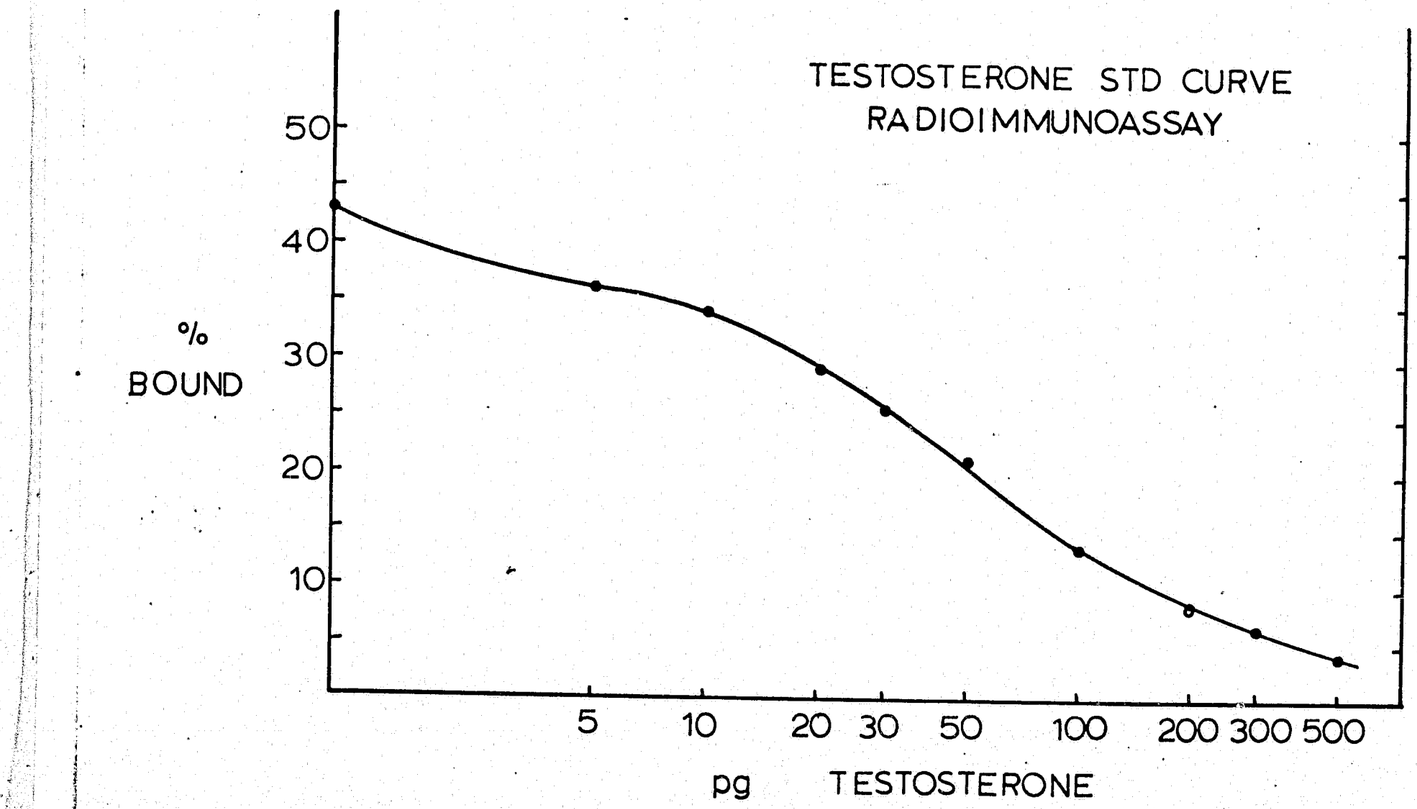
Sephadex LH - 20, 0.5g

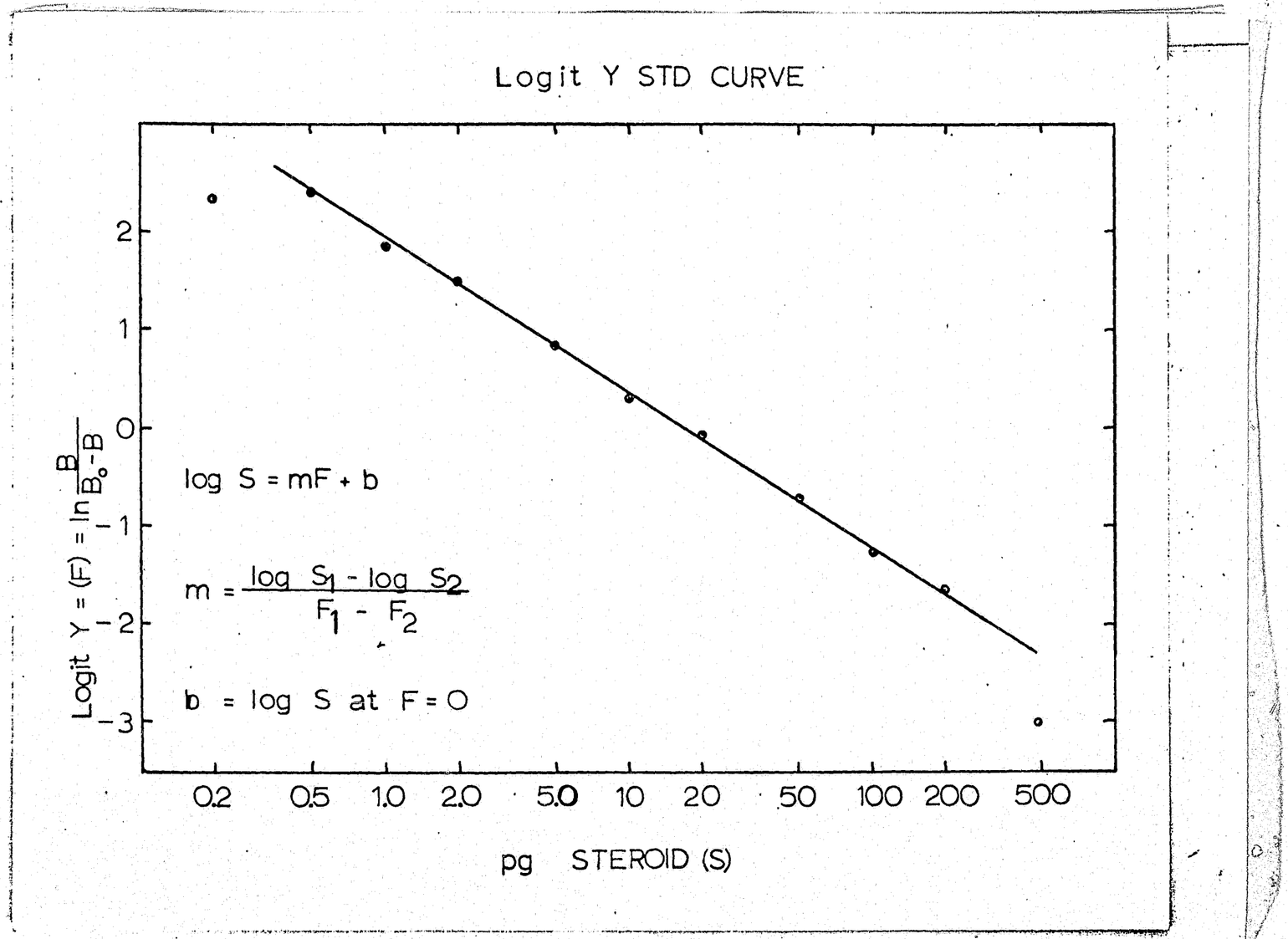
Isooctane: Benzene: Methanol

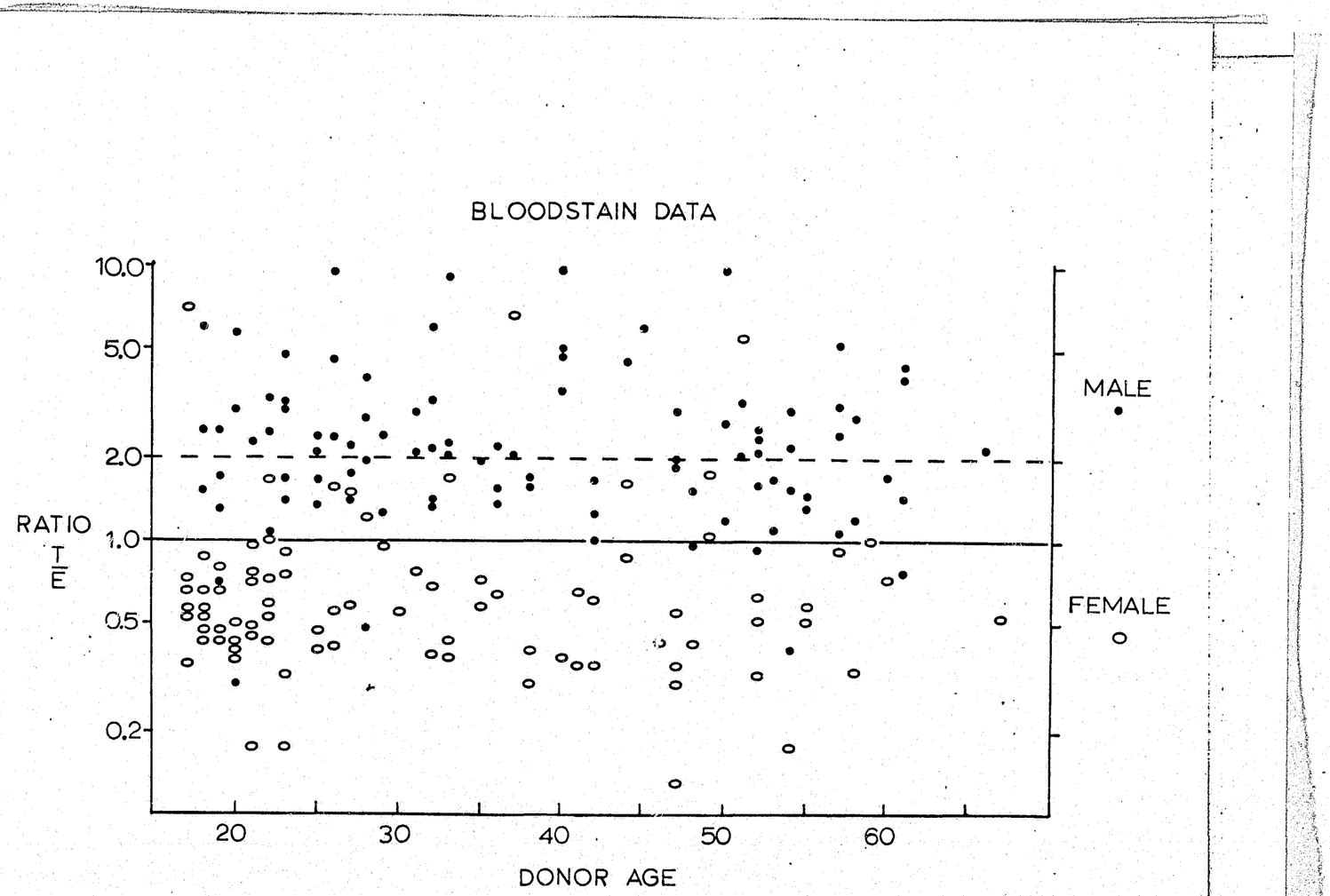
(1) 90:5:5

(2) 62:20:18




TESTOSTERONE STD CURVE
RADIOIMMUNOASSAY
%
BOUND
50
40
30
20
10
5
10
20
30
50
100
200
300
500
pg TESTOSTERONE


Logit Y STD CURVE
2
1
0
−1
−2
−3
Logit Y = (F) = ln B/B₀-B
log S = mF + b
m = log S₁ - log S₂ / F₁ - F₂
b = log S at F = 0
0.2
0.5
1.0
2.0
5.0
10
20
50
100
200
500
pg STEROID (S)


BLOODSTAIN DATA
10.0
5.0
2.0
1.0
0.5
0.2
RATIO
T
E
MALE
FEMALE
20
30
40
50
60
DONOR AGE

SUMMARY

The technique for identifying the presence of phosphoglucomutase, adenylate kinase and erythocytic acid phosphatase as well as the ABO and MN antigens in the same small bloodstain is present.

THE IDENTIFICATION OF HEMOGLOBIN, PGM, AK, EAP, ABO, MN, AND SPECIES IN A SMALL BLOODSTAIN

Robert C. Shaler* Ph.D., Anita M. Hagins, B.S.M.T., and Charles E. Mortimer, Ph.D.
University of Pittsburgh, Department of Chemistry

William C. Stuver, M.S. and Denise M. Neilson, M.S.
Pittsburgh and Allegheny County Crime Laboratory

Ralph Plankenhorn, M.S.
Pennsylvania State Police, Greensburg, Pa.

Forensic bloodstain analysis has taken advantage of certain blood components, i.e., enzymes, proteins and antigenic markers. These exist as biochemically inherited traits allowing a calculation to be made as to the uniqueness of the blood sample in the general population. Theoretically it should be possible to classify stains to a very small segment of the population, however, the utilization of these inheritable systems depends on the quality and quantity of stain material.

Studies of the chemical nature of the ABH, MN and Rh antigens on the red blood cell demonstrate the water insoluble nature of these substances.[1,2] The forensic utility of this was demonstrated by Whitehead and Brech[3] when they were able to do ABO and species identification on the same fragment of dried blood.

This report is an extension of their work and is concerned with the chemical identification of hemoglobin, species specificities, antigenic components ABH and MN, Erythrocytic enzyme systems PGM, EAP and AK on bloodstain threads approximately 7 mm. This work is the product of two independent, simultaneous studies and is presented as a joint report.

EXPERIMENTAL:

A. Treatment of Blood Samples.

1. A whole blood sample was divided into two aliquots, one, approximately 2 drops, being dried onto cotton sheeting. These stains were used for PGM, AK and EAP determinations according to Culliford[4] as modified by Marone[5] and Wraxall.[6] The red blood cells in the second aliquot were washed in isotonic saline three times and suspended at a concentration of approximately 4%. ABO and MN determinations were made using the tube technique according to specifications of the antisera manufacturer. In addition, a small portion of the whole blood from the second aliquot was used to check for cross reacting M by the slide technique.[7]

2. Bloodstains from case material were also used. If the stains were not already dried on threaded material, they were dissolved in saline and redried onto cotton threads.

B. Simultaneous Determination of EAP, PGM, AK, ABO and MN.

1. Preparation:

Six threads (5-7 mm) were cut from a section of bloodstain material moistened with saline. Two were inserted into a starch gel for PGM and AK determination,[4,5] two were inserted into another gel for EAP analysis,[6] one thread was placed into an agar well for species determination by immunodiffusion,[8] and the last was placed onto a microscope slide for identification of blood by microcrystallography (Takayama crystals of hemoglobin).[9] The threads were removed from the starch gel either during the electrophoretic run (1 hour) or after its completion. These threads were teased into individual fibers and mounted onto a ceramic ring slide. Control threads were prepared from selected bloodstains obtained in a general screening program[10] by drying onto pre-washed cotton sheets. The threads were mounted with nail polish as in Figure #1 and the appropriate controls added to detect incomplete washing of the fibers (MN determinations).

2. Enzyme determinations:

a. PGM and AK

Electrophoresis was carried out for 22 hours at 6.5 V/cm at 4°C in a 1 mm, 14% Starch gel prepared in 0.1 M Tris, .01 M EDTA, 0.1 M Maleic Acid, .01 M $MgCl_2$, pH = 7.4 take buffer diluted 1:10. The PGM side of the gel was stained 1-2 hours before the AK using the agar overlay technique at 37°C described by Culliford.[4]

b. EAP

Electrophoresis was for 4 hours at 400 volts (20 V/cm) in a 0.245 M sodium dihydrogen phosphate, 0.15 M trisodium citrate, pH = 5.9 tank buffer diluted 1:100. The plate is stained with 4 mg of 4-methylumbelliferyl phosphate dissolved in 10 ml 0.05 M Citric acid/NaOH pH = 5.0. The solution was absorbed onto Whatman 3 mm filter paper and laid on the gel surface covering an area between the two wicks. The plate was incubated at 37°C for 1-2 hours.

3. MN determination: absorption elution

One drop of antisera (neat) anti M and anti N (Ortho) was placed in each well in rows I and II, Figure #1, respectively. For some tests, a drop of a 22% solution of BSA was added to the fibers

in row III, while in other runs nothing was added.

A modified absorption-elution technique of Howard and Martin[11] was utilized. Absorption was for 35-45 minutes at 4°C, washing was accomplished with two 10-15 minute cold saline immersions. Elution was at 56°C for 15 minutes. Agglutination was observed after 12 and 24 minutes using a 0.2% suspension of indicator cells.

At the completion of the MN determination, the ceramic ring slide was immersed in a beaker of saline at 56°C and allowed to incubate for 15-30 minutes. The slide was blotted dry and then used for the ABO determination.

4. ABH determination - absorption elution:

The absorption-elution technique was performed the same as for the MN determination described above. It was blotted dry and one drop of saline was added to each fiber and placed in a moisture chamber for 15 minutes at 56°C.

One drop of a 0.2% cell suspension of appropriate indicator cells (A_1, B and O) was added to rows I, II and III respectively. Agglutination was observed after 10 and 20 minutes.

RESULTS:

A. Interpretation of MN Results.

The interpretation of the absorption-elution results after MN determination should provide no problems provided the analyst is cognizant of the inherent problems with the system.

First, the N determination is normally manifested as a weaker response, generally attributed to dosage effects and weak antisera. Thus, the time for complete agglutination of N bloods is longer than for M or MN.

Second, there is the problem of cross reaction, - M blood cells which react with anti N sera. In wet blood determinations this presents no problem since the cross reaction is not normally observed within the time restriction of the tube method. However, the sensitivity of the absorption-elution technique will indicate the presence of the N antigen in a cross-reacting M. Differentiation between MN and cross-reacting M's is possible since the former agglutinate the indicator cells faster. Cross reacting M whole blood can be detected using a slide method.[7]

B. Interpretation of ABO Results.

Problems associated with ABO grouping after MN determinations are a result of incomplete washing of antisera. Thus, by the incorrect choice of controls or indicator cells, confusing results may occur. That is, observed agglution in the ABO system may be attributed to the reaction between M and/or N sites on the indicator cells and remaining antisera from the previous MN testing. It is necessary to have controls which will demonstrate agglutination with the proper A, B, or H antigen as well as demonstrate incomplete washing of the anti M or anti N from the previous determination.

The problem of incomplete washing arises particularly with M stains. That is, stains which contain M antigen. Since the anti M sera is usually of a higher titer, and the absorption-elution method is a very sensitive technique, it will detect very small quantities of the residual antisera. Thus, if the situation arises in which the analyst is dealing with a B, M stain, it could be possible to mistype it as an AB, M. The use of proper controls would indicate that complete washing had taken place. A typical case of a B, M stain is

indicated in Figures 2 and 3. The setup of the ring slide is the same as in Figure 1. Figure 2 shows a typical pattern of agglutination for an M stain. Agglutination will be observed where the M antigen is observed in the unknown and controls containing the M factor. Figure 3 illustrates the same M stain taken through the ABH determination. Complete washing, Figure 3A, of the treated fibers after M determination in Figure 2 shows that agglutination is found in the proper controls. Row I shows agglutination for the A antigen control and no others. The unknown shows agglutination in Row II indicating the presence of the B antigen. Again, the controls show the expected agglutination indicating a group B stain.

Figure 3B illustrates the same blood in which incomplete washing occurred. In Row I, agglutination was observed in the A,N control as well as the B,M control. The unknown gives strong agglutination (an M stain) as well as the M (B,M) control. It is interesting that no agglutination took place in the MN control. Generally, the MN controls (or MN stains) will not indicate incomplete washing because the number of M antigenic sites available in MN stains is significantly less. Row II illustrates the same problem. The unknown shows agglutination indicating the presence of the B antigen. The controls in Row II confuse the situation. The M control shows agglutination as does the B, N. Again, the MN shows nothing. The results permit no conclusions concerning the ABH antigens present in the stain.

In situations where incomplete washing has been demonstrated, i.e., confusing results are obtained, the slide may be rewashed in a beaker of saline at 56°C for 15-30 minutes and the procedure repeated. This should be sufficient to elute most, if not all, the residual antisera.

It is appropriate at this point to discuss the use of indicator cells. Once the MN grouping of the stain has been determined, the indicator cells for the ABH determination can be chosen in order to eliminate or minimize the effects of incomplete washing. For example, the use of N or MN indicator cells will greatly reduce the occasion for confusing results since the reactivity of the N antigen is not observed in incompletely washed stains.

DISCUSSION:

The determination of multiple genetic markers from the same fragment of dried blood presents obvious advantages for the forensic serologist. First, it permits the derivation of a significant amount of information from a relatively small sample of stain material. Second, although electrophoretic procedures are time-consuming, the time during electrophoretic runs is not wasted.

The use of the above procedures depends on the quality of the stained material being examined. If the stain is fairly heavy, then a minimum amount of material will be required. More material may be required if the stain is light. Obviously, light and heavy refer to subjective amounts since fabrics of different types (cotton, polyester, etc.) retain different amounts of bloodstains. The analyst should add various quantities of blood to different types of fabric to pre-determine what quantity of blood works best for them. This becomes than a matter of relating (visually) to "case" stains in order to determine how much stain is needed. Generally,

if there is enough material to perform any one of the individual examinations mentioned, the multiple analysis approach could be considered.

Other observations which have been made are concerned with problems which have been encountered.

In one instance a very high titer anti N sera was obtained and as a result a different wash procedure has been used successfully. The first wash was as described under the methods section. The second wash was for ten minutes in a beaker of cold saline which was gently rotated.

While collecting stains at crime scenes, sometimes it is necessary to dissolve the stain in saline and then absorb the liquid onto cotton threads. It has been found that these reabsorbed stains are viable up to ten days.

With regard to enzyme systems, there seem to be problems in obtaining data from some stains. These include stains found on certain metal surfaces. For example, on iron surfaces all systems are not reliable. The use of EDTA will not alleviate the problem. Stains found on automobiles which have been used on salted streets give no results with EAP, faint PGM's, while AK presents no difficulty. From glass surfaces it is difficult to obtain EAP results. Thus, the material from which the stain is taken will dictate to some extent the number of systems which can be analyzed reliably.

ACKNOWLEDGEMENTS:

This work was supported by the Law Enforcement Assistance Administration Grant No. 75N1-99-0011.

REFERENCES

1. Liotta, II, M. Quintilianti, L. Quintilianti, A. Buzzonetti and E. Giuliani, 1972, Vox Sang. Vol. 22, 171-182.

2. R. H. Kathan and A. Adamany, 1967, J. Biol. Chem. Vol. 242, No. 8, 1716-1722.

3. P. H. Whitehead and A. Brech, 1974, J. Forens. Sci. Soc. Vol. 14, 109-110.

4. B. J. Culliford, "The Examination and Typing of Bloodstains in the Crime Laboratory", U. S. Department of Justice, Law Enforcement Assistance Administration, National Institute of Law Enforcement and Criminal Justice, PR 71-7, December 1971, 106-145.

5. Peter M. Marone, unpublished results.

6. B. Wraxall, personal communication.

7. Willard C. Stuver, unpublished results: One drop of whole blood is added to each cavity of a two well microscope slide. One drop of anti M is added to one of the wells and one drop of anti N is added to the other. The slide is rotated and cross reaction will be evidenced by agglutination within 30 seconds. In the tube method, washed erythrocytes are used and no cross reaction is discernable within 10 minutes.

8. Willard C. Stuver, unpublished results: The agar diffusion plate (60 x 15 mm plastic petri dish) is composed of 0.5% Ionagar prepared with 7.0 gm sodium barbiturate (Veronal), 1.1 gm diethyl barbituric acid and 1.0 gm calcium lactate per liter of water. The wells are no further than 6 mm apart.

9. P. L. Kirk, 1953, "Crime Investigation", Interscience, New York, 659-660.

10. LEAA Grant in Aid "Individualization of Forensically Important Physiological Fluids", Grant No. 75N1-99-0011.

11. H. D. Howard and P. D. Martin, 1969, J. Forens. Sci. Soc., 9, 28.

LEGENDS TO FIGURES:

Figure 1. Illustrates the proper use of controls for combined MN and ABH determination using the absorption-elution technique.

Figure 2. Illustrates the agglutination observed for a typical M stain for the absorption-elution technique.

Figure 3A. Illustrates complete washing in the determination of the B antigen after M determination using the absorption-elution procedure. The controls are set-up as in Figure 1.

Figure 3B. Illustrates incomplete washing in the determination of the B antigen after M determination using the absorption-elution procedure. The controls are as in Figure 1.

END