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THE IDENTIFICATION OF SEMINAL FLUID AND THE DETERMINATION OF PHOSPHOGLUCOMUTASE SUBTYPE INFORMATION BY ELECTROPHORETIC SEPARATION

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THE IDENTIFICATION OF SEMINAL FLUID AND THE DETERMINATION OF PHOSPHOGLUCOMUTASE SUBTYPE INFORMATION BY ELECTROPHORETIC SEPARATION

By

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ABSTRACT

THE IDENTIFICATION OF SEMINAL FLUID AND THE DETERMINATION OF PHOSPHOGLUCOMUTASE SUBTYPE INFORMATION BY ELECTROPHORETIC SEPARATION

By

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Seminal fluid samples were examined by electrophoretic separations on polyacrylamide disc gels and cellulose acetate to study the usefulness of the polyamines spermine and spermidine as confirmatory markers in the absence of spermatozoa. These separations indicated that spermine and spermidine were poor confirmatory markers for the presence of seminal fluid. They also revealed the presence of a protein band of unknown origin and identity which may be unique to seminal fluid and usable as a confirmatory marker.

The second portion of this study was concerned with the development and testing of a conventional electrophoresis procedure for the determination of phosphoglucomutase subtypes (PGM). The procedure uses an Isogel (FMC) support media with a pH 5.3 phosphate-citrate buffer system and a voltage of 21 v/cm for 4 hours. This procedure provides excellent separation and resolution of the PGM subtype bands as well as information on the erythrocyte acid phosphatase, adenosine deaminase, and adenylate kinase enzyme phenotypes.

Dedicated to:
Anne, Rachel, and Max

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Thanks to my co-workers in the Serology Section of the Metro-Dade Police Department Crime Laboratory for allowing me time off casework to devote my full energy toward development and testing of the PGM Subtyping procedure.

TABLE OF CONTENTS

LIST OF FIGURES

Figure

KEY TO SYMBOLS AND ABBREVIATIONS

ADA: Adenosine Deaminase
AK: Adenylate Kinase
Bis: N,N'-Methylene-Bis-Acrylamide
C: Centigrade
cm: Centimeter
Cleland's Reagent: DL-Dithiothreitol
C: Crosslink
°: Degree
DNA: Deoxyribonucleic Acid
EAP: Erythrocyte Acid Phosphatase
EDTA: Ethylenediamine Tetraacetic Acid
G PD: Glucose-6-Phosphate Dehydrogenase
6
g: Gram
>: Greater than or equal to
JMH/RTC: Jackson Memorial Hospital/Rape Treatment Center
L: Liter
MUP: 4-Methylumbelliferyl Phosphate
ul: Microliter
mg: Milligram
ml: Milliliter
mm: Millimeter
M: Molar
NADP: β-Nicotinamide Adenine Dinucleotide Phosphate
pH: Indicates measure of acidity
%: Percent
PGM: Phosphoglucomutase
PAGE: Polyacrylamide Gel Electrophoresis
PMR: Premixed Reaction Mixture
rpm: Revolutions per minute
RNA: Ribonucleic Acid
SERI: Serological Research Institute
T: Acrylamide
TEMED: Tetramethylenediamine
TCA: Trichloroacetic Acid
UV: Ultraviolet
V: Volts
v/cm: Volts per centimeter

INTRODUCTION

One of the goals of this research was to examine the usefulness of the polyamines spermine and spermidine as confirmatory markers for the presence of seminal fluid when the samples are spermatozoa free. The positive identification of a sample as seminal fluid is a critical determination in helping to solve sexual batteries. Because of this, some background information concerning the crime of rape will be presented prior to discussing the research findings.

The second area of study in this research was the electrophoretic determination of phosphoglucomutase (PGM) subtypes. Several of the electrophoretic procedures which have appeared in the literature were tested to assess their usefulness for determining PGM subtype information. After experiencing difficulty in obtaining usable results and information, these procedures were modified and adapted to create a new procedure.

The determination that a sample does or does not contain seminal fluid will affect the forensic serologist's analysis. Samples determined to be seminal fluid free will

not be analyzed because they will not provide any information of value concerning the seminal fluid donor. Samples determined to be seminal fluid positive can be analyzed to provide additional information such as the PGM subtypes. This information concerning the nature of the samples and any additional information obtained from the analysis will affect the interpretation of the information provided by the analysis of the evidence.

This may then affect the prosecution and defense of the subject by weakening or strengthening their cases and how they will present them to the court during a trial. The additional information may also affect the willingness of the prosecution or defense to go to trial, thus affecting the ultimate disposition of the criminal charges.

BACKGROUND; The Crime of Rape

Rape is a very serious violent crime which has been committed at a greater frequency during the last two decades. Between the years 1972 and 1976, the Federal Bureau of Investigation reported a 21% increase in the number of forcible rapes which were reported to the police.(1) This trend toward an increase in the numbers of reported forcible rapes has continued into the present. During 1985, the number of forcible rapes which were reported to police increased by 7% over those reported during 1984.(2) Furthermore, these statistics are not an accurate representation of the real problem because most rapes are never reported to the authorities.(3) The true forcible rape rate is unknown but some estimates place it between five and ten times higher than the number reported.(4)

Determining the true frequency of occurrence for forcible rape is impossible because of the reluctance of many rape victims to report or discuss the incident. There are a number of reasons for this. There may be a feeling of guilt, shame or embarrassment. The victim may fear that the rapist will retaliate should they report the crime. They may fear the reactions of their friends and family, or they may be totally subdued and frustrated by the legal system. There are undoubtedly other reasons as well.

In recent years, many states have enacted new statutes concerning rape. These new laws are designed to better protect the victims and provide them with more privacy concerning the details of the actual crime and their past personal history. In the past, both of these areas were open to public scrutiny during prosecution of the accused rapist, thus creating a situation in which the victim seemed to be on trial and not the defendant. The new rape laws also have defined the crime in a more realistic way by broadening the scope of what constitutes rape, who may be a victim and who may be a rapist. This has, in turn, made it easier for the state to prosecute this crime when it is reported and a subject is identified.

The Florida statute which proscribes rape provides an example of the new rape laws. This statute removes the term "rape" from the law and renames the crime as "sexual battery". It then defines the term sexual battery and specifically excludes bona fide medical procedures from this definition. The law widens the definition of victim by defining the victim as any individual alleging to have been sexually assaulted. Under many of the old rape laws, men could not be the victim of rape, while under the new law, anyone - male or female - can be a rape victim. The new law also broadens the definition of who can commit a sexual battery. Many of the old laws excluded women from this category. A husband could not be prosecuted for raping his legal wife and males under the age of 14 were considered

incapable of committing this crime. The new law removes these restrictions when describing a possible perpetrator.

A very important aspect of this law concerns corroborative evidence. Under the old rape laws, corroborative evidence or physical evidence of a rape was required to prosecute the crime. If this corroborative evidence could not be demonstrated, then no rape could be proven. The new law doesn't require corroborative evidence to prove that a rape occurred. It recognizes that there may not be any physical evidence present in some sexual batteries. If evidence is present, it is not required that the prosecution use it during the trial even though it may help prove the charges. It is a portion of this corroborative physical evidence, namely seminal fluid, which may be present in a sexual battery with which this scientific study is concerned. The collection, preservation, analysis and interpretation of this physical evidence can help verify that the alleged victim took part in sexual activity. This physical evidence can also provide information about the alleged rapist (subject) and help connect the subject to the alleged victim.

In a sexual battery committed by a post-pubescent male, one type of physical evidence left behind may be seminal fluid. Seminal fluid is the male reproductive fluid. In the normal male, seminal fluid is a turbid white fluid with a volume ranging between 1.5 ml and 5 ml.(5) The fluid is a combination of several secretions and cellular

components which are mixed during the process of ejaculation (ejection of seminal fluid from the male reproductive organ). The ejaculate can be divided into three fractions which correspond to the emptying of the different glands of the genital tract.(6,7,8) The first fraction is the product of the Cowper's gland, Littres gland and prostate gland; the second fraction is contributed to by the ampulla and epididymus and the third fraction by the seminal vesicle.(9) The early portion of the ejaculate (fractions one and two) contain the bulk of the spermatozoa (10) or approximately 75% of the total sperm count.(11) In the normal male the spermatozoa constitute approximately .1% of the total volume of the seminal fluid.(12) This translates to 40-250 million sperm per milliliter in the normal healthy male.(13)

The bulk of the seminal fluid is composed of the secretions from the seminal vesicle (45-80%) and the prostate gland (15-30%).(14) It is because seminal fluid is composed of multiple secretions that its characterization and analysis make it a very valuable tool in the prosecution of rape.

The positive identification of seminal fluid is conclusive evidence that the alleged victim has recently participated in sexual activity in which the partner was a post-pubescent male capable of ejaculating seminal fluid. This helps verify a portion of the alleged victim's allegation of rape. It does not prove in any way that the

sexual activity was rape. There is no physical evidence which can prove rape. The physical evidence may only help indicate that there was sexual activity which may have been forcible.

The other reason the identification of seminal fluid is important is that it contains genetic materials which can be isolated and identified by the forensic serologist. This information is then used to help demonstrate that the alleged rapist could have contributed the seminal fluid sample which was identified. Because seminal fluid can be valuable physical evidence, its collection, preservation and analysis is very important.

COLLECTION AND PRESERVATION OF THE PHYSICAL EVIDENCE

In many large cities there exist rape crisis centers which the victim of a rape may contact for assistance. The purpose of these centers is to provide rape victims with medical treatment and counseling to help them through the rape crisis. The people who work in these centers are specially trained in dealing with the victim so they can determine the circumstances of the assault and properly collect and preserve physical evidence for forensic analysis. One of the first rape crisis centers in the United States was opened in the early 1970's at Jackson Memorial Hospital in Miami, Florida. The Jackson Memorial Hospital Rape Treatment Center (JMH/RTC) provides free, private treatment for all rape victims.

The primary concern of the rape treatment center is the victim's well being. The victim is not required to report the crime to the police in order to receive medical care. This policy recognizes that because many rape victims never report the assault, they never receive medical aid. By not requiring that the assault be reported to the police as a prerequisite for receiving treatment, victims have the opportunity to receive the proper medical care and counseling they will need.

As part of the medical treatment, the victim of a sexual battery is given a complete physical examination. During this examination, notes are made by the doctor

concerning the presence of any physical trauma such as bruises or lacerations. The doctor also collects physical evidence which will be submitted to the crime laboratory for analysis. To aid in the collection of the physical evidence, the JMH/RTC uses a kit which was designed for them by the Serology Section of the Metro Dade Police Department, Crime Laboratory Bureau. The purpose of the kit is to insure that collection and preservation of the physical evidence is done in a manner which will help maintain its maximum forensic value.

The kit is composed of the following items:

A) One red top vacutainer blood collection tube
B) One piece of clean cotton cloth about 1 inch x 1 inch contained in a small cardboard box
C) Sealed paper packages, each containing two sterile swabs
D) Glass microscope slides
E) A ten ml screw top glass tube containing two to three ml of physiological saline

A sample of the victim's blood is collected in a 10 ml red top vacutainer blood collection tube. The red top indicates that the tube contains no preservatives or anticoagulants which may interfere with the analysis of the blood sample. The purpose of this sample is to provide the forensic serologist with information about the victim's blood factors. This information is used for comparison to the information which is provided by analysis of the other physical evidence.

The victim provides a saliva sample by removing the piece of clean cotton cloth from the cardboard box and placing it in her mouth until it is saturated with saliva. After saturating the piece of cloth, it is returned to the cardboard box and allowed to dry. The saliva sample is used to provide information about the victim's other body fluids. The information is then used to help interpret the information revealed by the analysis of the remainder of the evidence.

The packages of sterile swabs are used to collect samples from any of the body areas which are alleged to have been involved in the assault. Since most sexual batteries are vaginal, the doctor would use one package of the sterile swabs to swab the vagina for seminal fluid. A second package of swabs would be used to swab the cervix (the upper limits of the vagina) for seminal fluid. The sample is collected by rolling the cotton tip of the swab over the area being sampled. This is done so that the entire surface of the swab absorbs the fluid being collected, and provides a large sample for analysis. After the sample is taken, the wet swabs are used to make smears for microscopic examinations of the sample. This is done by rubbing the wet swab across the surface of a clean glass microscope slide. After the smear is made, the swabs are returned to their original paper package and allowed to air dry away from light and heat. The smears are air dried and then wrapped in gauze for protection.

After the affected areas have been swabbed, the saline contained in the screw top glass vial is used to rinse the vaginal vault. The liquid is then collected and placed back in the glass vial. This vaginal washing is called the vaginal aspirate.

The evidence is then properly labeled and sealed in a paper bag for transport to the crime laboratory. All the samples, excluding the blood standard and the vaginal aspirate, are placed in packages which allow air to flow in and out. This allows the samples to dry quickly, preserving the information which has been collected. The faster the samples dry, the greater their potential for providing information during analysis.

Other evidence which may be collected at the rape treatment center are swabbings and smears of any other area involved in the assault, pubic hair combings, pubic hair standards and the victim's clothing. The clothing which may be the most valuable is the victim's panties. Seminal fluid may drain out of the victim's vagina while traveling to the rape treatment center. The panties may, in some cases, contain more seminal fluid than the vagina, making them a very important source of information about the individual who deposited the seminal fluid sample. As with the rape evidence collection kit, all these items are sealed in paper bags or cardboard boxes and submitted as fast as possible to the crime laboratory. Upon submission to the laboratory, the samples are removed by the serologist and preserved in a

freezer while awaiting analysis. This helps preserve the seminal fluid which may suffer rapid decomposition over time. The faster the samples are preserved and frozen, the more information they may provide during analysis.(15)

The final physical evidence which may be collected and submitted to the laboratory is evidence collected at the crime scene. This evidence is collected by crime scene technicians who are trained in the proper collection and preservation of physical evidence. The evidence which will be collected depends on the details of the crime and varies from crime to crime.

ANALYSIS OF THE EVIDENCE

The rape evidence collection kit and any items impounded at the crime scene are submitted to the crime laboratory where the forensic serologist preserves the samples and does any analysis which may be necessary.

The initial analysis of the evidence is to determine if seminal fluid is present on the samples which were collected at the rape treatment center. If the samples are seminal fluid positive, then further analysis can be performed. This additional analysis may provide information concerning the blood group and enzyme profile of the seminal fluid contributor. Evidence which is seminal fluid negative is of little or no value because it cannot provide any information about the alleged rapist.

The first step in the analysis procedure is to locate and identify any samples which may contain seminal fluid. In the rape evidence collection kits, these items would be the swabbings and the vaginal aspirate. The evidence collected at the crime scene and any of the victim's clothing must be examined visually to locate any stained areas. Seminal fluid is a milky white color when in its liquid form. This liquid, when it dries, leaves a white stain which may be very difficult to visualize on items which have light colorations or are white. Since the stain may be difficult to see on clothing, it is very important for the serologist to note areas which feel stiff in

comparison to the rest of the item. It is also important to watch for areas in which the material appears more wrinkled than the surrounding areas. These variations may indicate that the area may contain a sample which should be tested for the presence of seminal fluid.

A second method which may be used to locate suspected seminal fluid stains is to examine the evidence under long wave ultraviolet light. When a seminal fluid stain is exposed to UV light, it will fluoresce and emit a very pale glow. The area can then be tested by the serologist to determine if the fluorescent activity is the result of seminal fluid being present. A difficulty is that many substances and materials will fluoresce under UV light, so that many stained areas which are located will not contain seminal fluid. Furthermore, the substrate may fluoresce, thus preventing any stains from being visible.

After the suspect stains are located, it must be determined if seminal fluid is present. To demonstrate that a stain is seminal fluid, or contains seminal fluid, several tests are used. The first test is the acid phosphatase test, of which there are many variations, but all rely on the detection of an enzyme called acid phosphatase. Acid phosphatase is an extremely good presumptive marker for the presence of seminal fluid. The enzyme is a product of the prostate gland and is very abundant in seminal fluid (16,17,18), and very persistent in the length of time during which it can be detected.(19,20,21) The detection of acid

phosphatase is a presumptive test for seminal fluid because it is also present in many other biological materials including saliva and vaginal secretions.(22,23) Even though acid phosphatase is not specific for seminal fluid, it is a very good marker for its possible presence. This is because of the high levels of acid phosphatase which occur in the seminal fluid of men with properly functioning prostate glands. The levels of acid phosphatase in seminal fluid are approximately 400 times the levels which can be found in other biological materials.

The majority of tests and procedures for the detection of acid phosphatase are chemical tests which produce a color reaction which may be visualized by the unaided eye. An example of one procedure is the use of sodium thymolphthalein monophosphate as described by Seiden and Duncan.(24) When a sample is being screened for the presence of seminal fluid, a small cutting is removed from the suspected seminal fluid sample. The cuttings, along with a known seminal fluid sample (positive control) and an unstained cutting of the item being examined (negative control), are saturated with a few drops of the sodium thymolphthalein solution. The samples are allowed to stand for five minutes at room temperature, after which time several drops of the color developer are added. As the color developer is added, the sample is observed for the development of a blue coloration. This blue color is a positive test for the presence of acid phosphatase. This

test result is then compared to the positive and negative controls to verify that the test worked properly and that the results were not false-positive for the presence of acid phosphatase.

The samples which are acid phosphatase positive are then analyzed further to conclusively determine if they contain seminal fluid. The best way to demonstrate this is to microscopically examine the sample for the presence of spermatozoa.(Figure 1)

Spermatozoa are the male gametes (25) and are produced in the testes of the normal adult through the process of spermatogenesis.(26) The mature spermatozoan in man has a filoform structure (27), due to the presence of the flagellum or tail which is used for motility. As exhibited in Figure 1, spermatozoa consist of a head, neck, midneck and tail. These cells are unique in their appearance and may be easily identified by microscopic examination of the samples. The presence of spermatozoa is conclusive proof that seminal fluid is present, because spermatozoa does not occur naturally in any other biological fluid. The identification of spermatozoa demonstrates that the sample being examined is either pure seminal fluid or a mixture of seminal fluid with other materials such as the vaginal secretions of the victim.

There are many ways to microscopically examine the smears for the presence of spermatozoa. One of the preferred methods is phase contrast microscopy.(28)

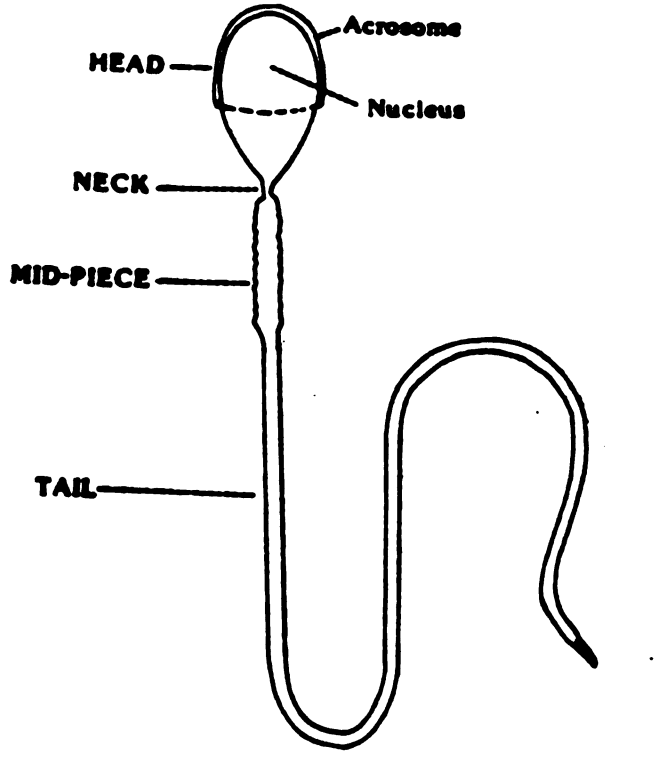

Figure 1: The morphology of spermatozoa.(29)

When biological materials such as seminal fluid are examined using bright field microscopy, there is very little contrast between the cellular matter and the background. This lack of contrast makes it very difficult to visualize and identify what is present in the sample. Phase contrast microscopy bends the light waves being projected through the sample, creating a much better contrast between the background and the cellular materials. Using phase contrast makes visualization of the spermatozoa easier, and reduces the chance that a misidentification may occur. The problem with phase contrast microscopy is that it will not work on samples which are opaque, such as clothing or bedding. These samples can be examined by extracting a small portion of the stain in saline. The eluate may then be placed on a clean glass microscope slide and microscopically examined for spermatozoa using phase contrast microscopy.

In the rape evidence collection kit, the smears which were made from the swabs at the time of the victim's physical exam are examined for spermatozoa. The vaginal aspirate is examined by placing one or two drops of the liquid on a microscope slide and covering the sample with a glass cover slip. The sample may then be examined microscopically for spermatozoa.

What happens when the microscopic examination of the samples which were positive for acid phosphatase fail to reveal the presence of spermatozoa? Until recently, the only way to conclusively identify seminal fluid was to

observe the presence of spermatozoa. This is not always possible, because not all adult men produce spermatozoa. There are many factors which can cause this situation, such as fertility problems, cancer of the testes or blockage of the ducts. This situation is called azoospermia and is an abnormal trait. Azoospermia can be temporary or permanent depending on its cause. Azoospermia can be the result of a vasectomy, which is a surgical procedure designed as a form of birth control. When a vasectomy is performed, the tubes which lead from the vas deferens are severed. The vas deferens is the storage area for the mature spermatozoa. By severing its connection to the ejaculatory duct and the urethra, it prevents the spermatozoa from being mixed with the seminal plasma and creates an azoospermic condition.

Oligospermia or low spermatozoa count may also be the reason that no spermatozoa are seen. Spermatozoa are very fragile after ejaculation and degrade rapidly with time. In the vagina, the major portion of the spermatozoa will be gone within 12 hours, while the acid phosphatase levels of the vagina will be back to normal within 48 hours of sexual intercourse.(30) Positive acid phosphatase tests may be obtained for almost four times as long as positive microscopic examinations for spermatozoa. The rapid decay of spermatozoa, along with a low spermatozoa count may make it nearly impossible to microscopically find spermatozoa on the samples.

Oligospermia is usually the result of fertility problems, high fever, or frequency of ejaculation although there may be other causes. Frequency of ejaculation will affect the spermatozoa count and volume of ejaculate. The more frequently an individual ejaculates, the lower the spermatozoa count and volume of the seminal fluid. This is because the body does not have enough time to regenerate its reserves between ejaculations, thus lowering the amount of spermatozoa and seminal fluid available for ejaculation. It is not unusual to have samples which are acid phosphatase positive and spermatozoa negative. In a study conducted at the Metropolitan Police Forensic Science Laboratory in Great Britain, 1.9% of the seminal stains on swabs examined were azoospermic.(31) When this occurs, other confirmatory tests for seminal fluid must be used. The most frequently used confirmatory marker besides spermatozoa is a protein called p30.

The use of p30 as a seminal fluid specific marker was first reported in the literature in 1978 by G.F. Sensabaugh.(32) The value of p30 is that it appears to be specific for seminal fluid and, to date, it has not been shown to occur naturally in any other biological fluids including vaginal secretions.(33,34,35) The p30 protein is very stable and persists for long periods of time in both the vaginal environment and in dried samples.(36) The protein is detectable in vaginal fluids for a mean period of 27 hours after coitus compared with a mean period of 14

hours for acid phosphatase.(37) Because p30 protein is a glycoprotein secretion of the prostate gland (38) and since the prostate gland is a major contributor to the seminal fluid mixture, p30 is usually present in high levels making its detection and identification easier. Furthermore, p30 is even present in the seminal fluid of vasectomized men.(39)

There are several procedures which can be used to identify the p30 protein. The procedure which is used most frequently is crossover electrophoresis. The unknown samples are extracted in physiologic saline while a 1% agarose gel is poured. Two parallel rows of wells (holes) are punched in the gel and their cores removed. To one row of wells, the unknown samples are added and to the other row is added anti-p30. Anti-p30 is an antiserum which contains antibodies specific for the p30 protein. Electrophoresis is then performed in a tris buffer system. After completion of the run, the gel is removed and placed in a 1M solution of sodium chloride overnight. The next day, the plate is soaked in distilled water and then dried in an incubator. After the gel has dried, it is stained in Coomassie blue and destained. The gel is then examined for precipitin bands which may have formed between the unknown sample wells and the anti-p30 wells. The band is a positive test result for the presence of the p30 protein, which indicates the sample contains seminal fluid. Figure 2 shows the entire seminal fluid identification and analysis procedure.

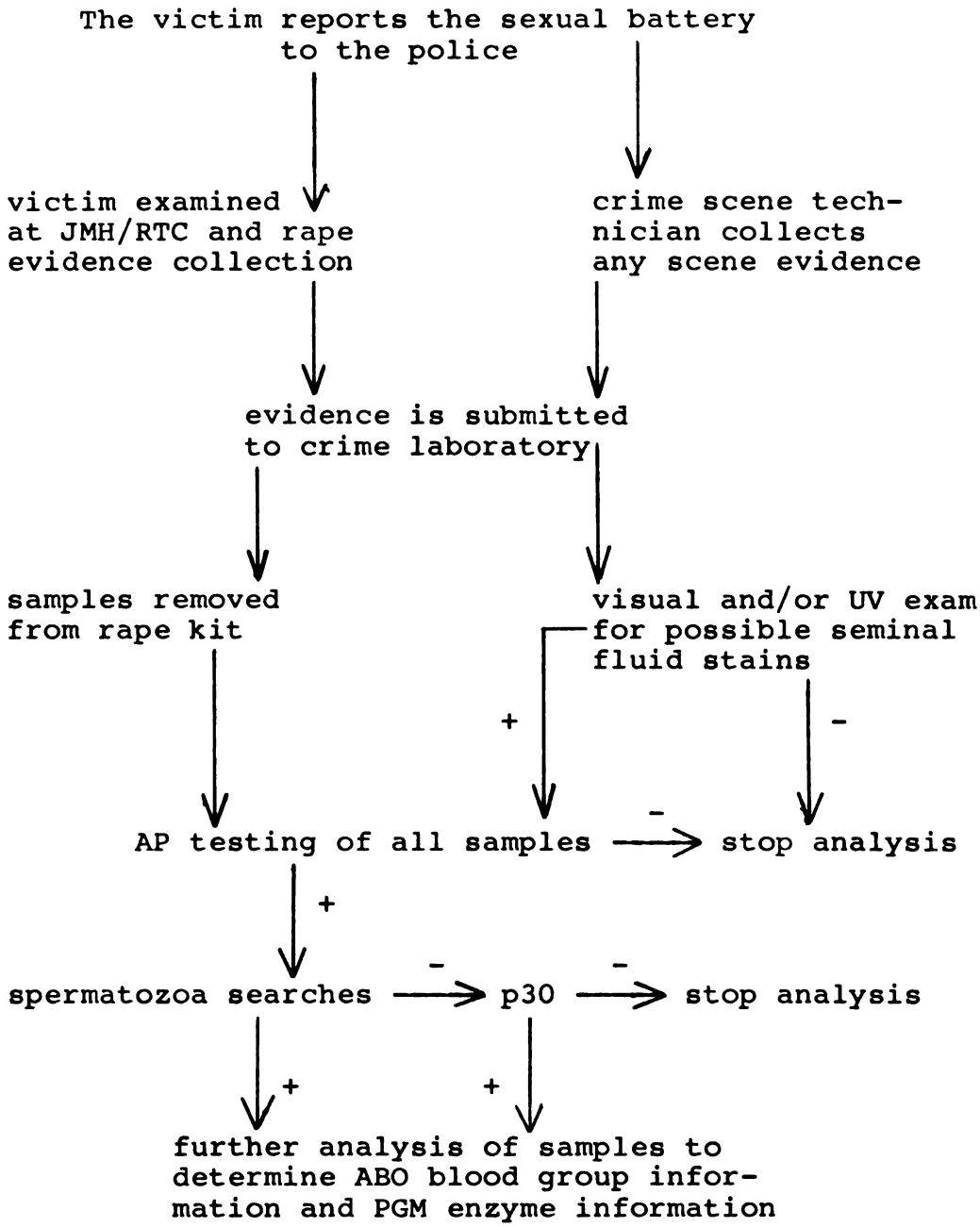

Figure 2: The identification and analysis of seminal fluid evidence.

After all the evidence is examined for samples containing seminal fluid, a determination is made concerning the need for additional analysis. Any samples which are negative for the presence of seminal fluid are of no further value.

The samples which are seminal fluid positive have the potential to provide information about the alleged rapist. These samples can be tested for ABO blood group information. If the seminal fluid contributor is a secretor, analysis of his seminal fluid will provide information about his blood group. A secretor is an individual who secretes blood group information into his other body fluids. These fluids, such as saliva, seminal fluid or vaginal fluid, can then be analyzed to determine his ABO blood group. Analysis of a nonsecretor's body fluids (excluding blood) will not provide information about their ABO blood group. One must keep in mind that approximately 80% of the population are ABO secretors, and that the seminal fluid samples being tested may be mixed with the victim's body fluids. Therefore, interpretation of the blood group information must be compared to blood and saliva standards of the victim and subject to determine who may have contributed the blood group information.

The seminal fluid positive samples are also tested to determine their PGM enzyme information. PGM is an enzyme which is present in the blood of all people, every male's seminal fluid, every woman's vaginal secretions, and is not

affected by an individual's secretor classification. Like the ABO blood group, it is a genetic marker which occurs in several forms, and each individual may only have one of these forms. Testing of the samples may reveal PGM information which can then be compared to the PGM types of the victim and subject for interpretation. To further aid in the interpretation of this information, the three major PGM types can be broken down into 10 subtypes. The subtyping information can be used in several ways, one of which is helping identify samples which may be mixtures of the victim's fluids with the subject's seminal fluid.

The genetic marker information is then used to create a population profile. The profile illustrates who, in the general population, could have contributed their seminal fluid to create the genetic information which was determined. This profile takes into account the victim's genetic markers and any possible mixtures of the victim's fluids with the seminal fluid sample. The profile is then used to generate a population frequency to illustrate the size of the population which could have provided this genetic information. This profile of genetic markers and the population frequency is compared to the subject's genetic markers to determine if the subject is within the contributing population or excluded.

The identification of seminal fluid is very important because it verifies sexual activity and it may provide genetic marker information which can implicate the subject. It is for these reasons that this scientific study on the electrophoretic examination of seminal fluid was done.

SPERMINE AND SPERMIDINE AS CONFIRMATORY MARKERS FOR SEMINAL FLUID

A possible solution to the identification of seminal fluid in the absence of spermatozoa, is to employ electrophoretic analysis of seminal fluid to establish the presence of the polyamines spermine and spermidine, both of which are usually present in seminal fluid.

Seminal fluid is composed of many potentially electrically charged constituents.(40) Therefore, they can be separated by electrophoresis. Two components of seminal fluid are the polyamines spermine ($C_{26}H_{26}N_4$) and spermidine ($C_7H_{19}N_2$). These two substances give seminal fluid its characteristic odor.(41) They occur in variable concentrations within seminal fluid and have been identified as a secretion of the prostate gland in men.(42) An important feature of spermine and spermidine is that they do not occur naturally in vaginal fluid, feces, urine or saliva.(43,44) Another important feature of these polyamines is that they are very stable and can be used for identification of seminal fluid even after long periods of time have elapsed.(45)

Spermine and spermidine can be easily and rapidly separated from the other constituents of seminal fluid and, thus, identify it even in the absence of spermatozoa. The main problem with employing spermine and spermidine to identify seminal fluid occurs in cases in which the

subject's prostate gland is not functioning properly. These polyamines are secreted by the prostate gland, its malfunction may result in no spermine or spermidine being present. Studies also indicate, however, that certain types of prostatic pathology may increase the seminal fluid concentration of these polyamines.(46)

Literature Review:

The following is a survey of the relevant literature concerning the analytical methodology employed in the analysis of polyamines such as spermine and spermidine.

Lewin, Beer and Glezerman (47), while studying the prostatic and vesicular contributions of seminal fluid, developed an electrophoretic technique for the separation of seminal fluid into its constituents. This technique used cellulose acetate as a support medium and provided good separation of the constituent bands. Following electrophoresis of the seminal fluid on the cellulose acetate, the electrophoretic patterns were observed, measured and identified. A series of bands was observed, one of which they labeled the PB band. The PB band was determined to be a product of the early portion of the seminal fluid, and was secreted by the prostate gland. An analysis of the band showed it to contain spermine and spermidine.

While doing a study of urinary polyamine levels for the diagnosis of prostatic cancer, Fair, Wehner and Brorsson (48) found that the prostate gland contained the highest concentrations of polyamines within the human body. It was also found that cancer of the prostate was accompanied by increased levels of these polyamines, especially spermidine. They proposed that the electrophoretic analysis of urine for increased levels of spermidine could be used to diagnose cancer of the prostate. The method which they used for analysis was paper electrophoresis in a citric acid buffer. The procedure was complicated and required an extended period of time and therefore is not deemed suitable for forensic analysis of seminal fluid.

Chen, Forsyth, Buchanan and Holmes (49) determined the amine content of vaginal fluid from women with treated and untreated nonspecific vaginitis. The analysis of the vaginal fluid samples was done using high voltage electrophoresis on a Whatman paper support medium. They also did a second analysis in which thin-layer chromatography was used. Electrophoresis of the amines showed the presence of cadaverine, histamine, putrescine and methylamine in the vaginal fluid of most the untreated cases. In several cases, it was also determined that spermine and spermidine were present. Further analysis of these cases indicated that the presence of spermine and spermidine was due to the presence of seminal fluid in the test samples. It was also determined that spermine and

spermidine are not naturally occurring constituents of vaginal fluid.

Suzuki, Oya, Katsumata, Matsumoto and Yada (50) developed an enzymatic method for the determination of spermine in human seminal fluid. The method depends on an enzymatic reaction between the spermine and the reagent to produce a green stain. The analysis method is easy to do and it yields good results. They proposed using this test instead of the acid phosphatase test because spermine is more stable than acid phosphatase and can be identified in very old seminal stains.

Clavert, Montagnon and Brun (51) did a study of the proteins present in seminal plasma. Their method of analysis was electrophoresis on a cellulose acetate support medium. The method and their results were similar to those of the Lewin, Beer and Glezerman study. Their research method differed in that the whole seminal fluid was analyzed. Then an analysis was done of the seminal fluid excluding the prostatic secretions, seminal fluid minus the vesicular secretions, and seminal fluid minus both prostatic and vesicular secretions. They determined that when the prostatic secretions were excluded, the seminal fluid sample lacked the spermine and spermidine constituents, therefore confirming that spermine and spermidine are secretions of the prostate gland.

DISC ELECTROPHORESIS ON POLYACRYLAMIDE GELS

Introduction

Disc electrophoresis on polyacrylamide gels (PAGE) refers to the discoid shape of the protein bands, due to the tubular shape of the electrophoresis cell and the discontinuities in the electrophoretic matrix resulting from the layering of a stacking gel on top of the separation gel.(52,53)

Charged particles and solutes will migrate through a support medium in the presence of an electric fluid. This process is known as electrophoresis. Some molecules have an electric charge because of proton ionization.(54) The particle will have a positive charge if it has less electrons than it has protons and will move toward the cathode (negative pole) of the electrophoresis system, whereas a negatively charged particle will have a surplus of electrons and will move toward the anode (positive pole). The rate of migration of these particles during electrophoresis is variable and depends on several factors such as the net electric charge of the molecule, the temperature of operation, the strength of the electric field, the nature of the support medium, and the size and shape of the molecule.(55) Because all substances are composed of components which may have differing electric

charges, under various conditions, electrophoresis can be used to separate them into their individual components.

In PAGE electrophoresis the gels are composed of acrylamide and the cross-linking agent N,N'-methylene-bis-acrylamide (Bis). The gel is prepared by polymerizing the gel monomer (acrylamide) with the cross-linking agent (Bis) and the appropriate catalyst to form a solid medium. There are two methods of polymerization (gelation) which may be used.(56) Polymerization may be done chemically by the addition of ammonium persulfate and tetramethylenediamine (TEMED) to the liquid gel solution. In the presence of a small amount of oxygen, the addition of these two substances will cause the acrylamide-Bis mixture to solidify. This method of polymerization is usually used when making the separation gel which is the majority of the gel through which the samples will be separated. The other manner of polymerization is photochemical. Photochemical polymerization is achieved by the addition of riboflavin to the acrylamide-Bis mixture and exposure of the mixture to ultraviolet light. This method of polymerization is usually used when adding the stacking gel on top of the separation gel. The purpose of using multiple gels is that the stacking gels, which have a large pore size, will line up the molecules of the sample being separated and the separation gel will then separate the sample's constituents based upon their molecular size. The smaller molecules will move through the pores of the gels more rapidly than the

larger molecules, revealing many well defined protein bands when the gel is stained. The pore size of polyacrylamide gels is inversely related to the acrylamide-Bis concentration. As the Bis concentration increases, the pore size of the gel decreases. The gels can be made from about 3% (large pores) to about 30% (small pores) with the usual range being between 5% and 10%.(57)

An advantage of electrophoresis on polyacrylamide is that the gels are easy to make and they may be stored in refrigerated, airtight packages for extended periods of time. The gels are desirable because they are thermostable, transparent, strong, provide very good sample separation, are relatively chemically inert, and can be made over a wide range of pore sizes.(58) The ability to vary the pore size of the gel lends itself to the separation of compounds which have a small molecular size such as spermine and spermidine.

The disadvantage of electrophoresis using polyacrylamide gels is that the acrylamide and Bis are neurotoxins which are readily absorbed through the skin. The chemical accumulates in the body where it remains permanently to build up in concentration with continued exposure. Because of this, the acrylamides must be handled very carefully to minimize exposure prior to polymerization. After polymerization the gels are no longer hazardous.

The other disadvantage associated with the use of polyacrylamide gels is that the staining and destaining process used to visualize the protein bands is a very long process due to the thickness of the gel. This procedure must be done over several days so the stains and destaining reagents may permeate the gel.

Methods and Materials

I. Gel Preparation

A) Separation Gel (7.184% T, 2.56% C) (59)

Solution A
Dissolve 14.0 g Acrylamide (Bio-Rad)
0.36 g Bis (Bio-Rad)
in 35 ml of distilled water. After all the solids are dissolved, add enough distilled water to bring the solution up to a final volume of 50 ml.

Solution B
Dissolve 9.05 g Trizma Base (Sigma)
12.00 ml 1M Hydrochloric Acid (Ricca)
0.06 ml TEMED (Bio-Rad)
in 25 ml of distilled water. After all the chemicals are in solution, add enough distilled water to bring the solution up to a final volume of 50 ml.

Solution C
Dissolve 0.14 g Ammonium Persulfate (Bio-Rad)
in 90 ml of distilled water. After the solids are dissolved, add enough distilled water to bring the solution up to a final volume of 100 ml.

To prepare the separation gel, mix one part Solution A, one part Solution B, and two parts Solution C. Pour the mixture into borosilicate disc gel tubes which have been sealed at the bottom with parafilm and placed in a level tube rack. The tubes should be filled approximately 3/4 of the way full. Carefully (to avoid mixing) deposit a thin layer of distilled water on top of the mixture. Allow the tubes to stand undisturbed until the acrylamide solution is completely polymerized (1 hour). After polymerization is complete, the stacking gel may then be added.

B) Stacking Gel (3.125% T, 20% C) (60)

Solution A
Dissolve 2.99 g Trizma Base
0.23 ml TEMED
in 20 ml of distilled water. After all the chemicals are in solution, adjust the mixture to pH 6.7 by adding 1M hydrochloric acid (approximately 24 ml). Add enough distilled water to bring the mixture up to a final volume of 50 ml.

Solution B
Dissolve 10.9 g Acrylamide
2.5 g Bis
in 35 ml of distilled water. After all of the solids are dissolved, add enough distilled water to bring the solution up to a final volume of 50 ml.

Solution C
Dissolve 2.0 mg Riboflavin (Bio-Rad)
in 50 ml of distilled water

Solution D

Dissolve 20.0 g of Sucrose (Sigma) in 35 ml of distilled water. After the sucrose is completely dissolved, add enough distilled water to bring the solution up to a final volume of 50 ml.

To prepare the stacking gel, mix one part Solution A, one part Solution B, one part Solution C, four parts Solution D, and one part distilled water. Pour the mixture into the disc electrophoresis tubes which contain the polymerized separation gel. This layer should be approximately 1/4 inch thick. Carefully (to avoid mixing) deposit a thin layer of distilled water on top of the mixture. The stacking gel must be exposed to ultraviolet light to polymerize. Polymerization takes approximately one hour. The completed gels can be sealed with parafilm and stored in a refrigerator for several weeks prior to being used.

II. Buffer Preparation

The tank buffer is a Tris/Glycine buffer. This buffer can be made in a concentrated form and stored under refrigeration for long periods of time. When buffer is needed, the concentrate can be diluted, cooled down to 4°C and used. The tank buffer should be made fresh for every use.

A) Stock Solution (61)

Dissolve 6.0 g Trizma Base
28.8 g Glycine (Sigma)
in 750 ml of distilled water. After the chemicals are dissolved, add enough distilled water to bring the solution up to a final volume of 1 L.

B) Working Solution

The working buffer solution is a 10% dilution of the stock solution. This is made by mixing one part of the buffer stock solution with nine parts distilled water. The diluted buffer must then be cooled to 4°C prior to use. The diluted buffer should be made up fresh for each electrophoresis run and discarded after each use.

III. Electrophoresis Procedure

Electrophoresis of the samples is done in a Bio-Rad electrophoresis cell designed for vertical electrophoresis using polyacrylamide disc gels. The power source is a Bio-Rad model 1420A. All runs are done at room temperature using precooled buffers. The length of the runs is variable, and based on the movement of a bromophenol blue (Eastman) tracking dye which is added to the buffer of the upper electrophoresis cell. The tracking dye is a low molecular weight dye which moves through the gel at a faster rate than most of the proteins which are present in the samples. The dye is used as an indicator of the rate and distance the samples constituents have migrated in the gel.

The dye will not alter or affect the constituents of the sample being separated.

The following procedure is used for the electrophoretic separation of the samples being studied:

1) The polyacrylamide disc gels are placed in the electrophoresis chamber gel holders, with the stacking gel being positioned at the top. The upper buffer chamber in this procedure is the anode (positive pole).

2) The lower buffer chamber (cathode) is filled with cold 10% buffer. The buffer should rise at least 1 cm above the base of the disc gels.

3) The small air space at the base of each disc gel is filled with buffer. This will insure a good electrical contact.

4) The gel holder is gently lowered into the electrophoresis chamber.

5) The large air space in the top of each gel is filled with tank buffer. The gels are now ready for the introduction of the samples.

6) The samples must be mixed with four parts sucrose solution (stacking gel preparation, Solution D). This adds weight to the sample so it will not float out of the disc gel tube. The addition of the sucrose will not alter or affect the electrophoresis results. After the sample is diluted with the sucrose solution, 5 ul are then gently introduced into the upper end of the disc gel.

7) The electrodes are inserted into the electrophoresis chamber. The lower buffer chamber should contain enough buffer so that the end of the electrode is submerged in a minimum of 1 cm of buffer.

8) The upper chamber is gently filled with buffer until its level is 1/4 inch above the top of the disc gels.

9) Four to five drops of bromophenol blue tracking dye are added to the upper buffer chamber. The buffer is gently stirred to mix the tracking dye with the buffer.

10) The lid is placed on the electrophoresis chamber and the power cables are connected to the power supply. The cables should be connected to the chamber so the upper buffer chamber is the anode (positive pole) and the lower buffer chamber is the cathode (negative pole).

11) The power supply is turned on and allowed to warm up for several minutes.

12) The power supply is set to constant voltage and the voltage is adjusted to the desired level. In this study the voltage was set at 240 V.

13) Upon completion of the run, which is either determined by the migration of the tracking dye or the elapsed time, the power supply is turned down to zero and then switched off. Disconnect the power cables and open the electrophoresis chamber.

14) The disc gel tubes are removed from the electrophoresis chamber. The electrophoresis procedure is now complete and the gels must be stained to view the band patterns of the separated sample.

IV. Staining and Destaining

The staining and destaining procedure for polyacrylamide disc gels is very lengthy in comparison to that of other electrophoresis separation media. This is due to the increased thickness of the gel. During staining, the

gel must be totally permeated by the stain, after which the unfixed stain (that which did not bind to any protein) must be removed. Removal of the excess stain, reveals the areas of stained proteins. These areas appear as very thin, well defined discs running the length of the gel.

Three stains were tested during this study. The stains were Coomassie-Brilliant Blue R-250 (Bio-Rad), Crowle's Double Stain for immunodiffusion (Polysciences, Inc.) and Ponceau S (Gelman). The Ponceau S and the Crowle's Double Stain were purchased premade while the Coomassie stain must be made fresh as needed.

A) Stain Stock Solution (62)

Solution A
Dissolve 0.5 g of Coomassie Brilliant Blue R-250 in 35.0 ml of distilled water. After the Coomassie is dissolved, add distilled water until a final volume of 40 ml is reached.

Solution B
Dissolve 12.5 g of Trichloroacetic Acid (TCA) in 80.0 ml of distilled water. After the TCA is dissolved, add distilled water until a final volume of 100 ml is reached.

B) Staining Solution

Mix 2 ml of the stain stock solution with 40 ml TCA solution.

All three of the protein stains contain trichloroacetic acid (TCA). The purpose of the TCA is to fix the proteins which are present in the gel. If the proteins are not fixed, they will diffuse out of the gel during the staining process.

The following staining and destaining procedure is used:

1) The polyacrylamide gels are carefully removed from the tube. This is done by forcing water between the edge of the glass tube and the gel with a syringe. The gel will slide out of the tube. When handling the gel be very careful not to damage it.

2) The gel is placed in the stain for a minimum of 24 hours or until it is totally permeated by the stain.

3) The gel is placed in the appropriate destaining solution to remove the excess stain. As the destaining solution becomes dark with the excess stain, it should be replaced by fresh destaining solution. This is continued until all the excess stain has been removed from the gel. The gel may then be stored in distilled water for an indefinite period of time.

The Coomassie Blue and Crowle's stains are removed from the gels by soaking them in a mixture which was 50% methanol (Mallinkrodt), 40% distilled water, and 10% glacial acetic acid (Mallinkrodt). After the majority of stain is removed, the gels are transferred to a mixture which is 88% distilled water, 7% glacial acetic acid, and 5% methanol for removal of the remaining stain.

The Ponceau S stain is removed by soaking the gels in a 5% solution of glacial acetic acid, which was replaced as necessary until the destaining process was completed.

V. Results

The initial sample separations are done at a constant voltage of 250 volts for valuable lengths of time, depending on the migration of the tracking dye. The samples which were run were human serum, human hemoglobin, and human seminal fluid. The human serum samples and the human hemoglobin samples were obtained from the blood bank at Edward W. Sparrow Hospital, Lansing, Michigan. The human seminal fluid samples were provided by the Laboratory of Clinical Medicine which obtained the samples from male donors being tested for fertility problems. Since the seminal fluid samples were submitted by men with suspected fertility problems, the following criteria was used to select the seminal fluid of "normal" fertile males.

1) Seminal fluid is a white-grey opaque coagulum which is viscous.

2) pH 7.2 to pH 7.8

3) The seminal plasma should initially coagulate and subsequently liquefy within 10 to 30 minutes.

4) A total volume of 1.5 ml to 5.0 ml.

5) Normal motility of the spermatozoa:
   a) The majority ($\geq$70%) of the normal spermatozoa show fast movement.
   b) The majority ($\geq$70%) of the normal spermatozoa show forward movement.
   c) The majority ($\geq$70%) of the normal spermatozoa show some type of movement.
   d) The majority (>70%) of the normal spermatozoa should be motile after two hours.

6) A spermatozoa count between 40-250 million spermatozoa/ml.

7) The majority ($\geq$90%) of the spermatozoa should not be agglutinated initially or after two hours.

8) Normal spermatozoa morphology:
   a) The majority ($\geq$70%) of the spermatozoa should be normal forms.
   b) Less than 4% of the spermatozoa should be abnormal or immature forms.
   c) The seminal plasma should not contain large numbers of foreign cells as leukocytes, bacteria and/or fungi.

9) The viability of the spermatozoa must be $\geq$65%.

The criteria used were the same as the standards which were set by the Laboratory of Clinical Medicine, Lansing, Michigan to assess the seminal fluid samples of its patients. The information concerning each of these samples was provided with the samples for assessment by this researcher.

The seminal fluid samples which were selected for this study were centrifuged for 10 minutes at 3000 rpm to remove the cellular components. The seminal plasma was divided up into portions and stored at -70°C until needed.

The initial electrophoretic separations were done to determine the best electrophoretic separation parameters for this study and to determine what sample band patterns would

appear on 7% polyacrylamide gels. The final initial area of comparison was to compare the banding patterns of the seminal fluid samples to those of the plasma and the hemoglobin so that bands which may be unique to the seminal fluid could be identified.

After obtaining an understanding of the sample band patterns and a feel for the basic electrophoresis procedure, the samples were run against laboratory prepared mixtures of spermine and spermidine. This was done to help identify which bands in the gels were spermine and spermidine. The laboratory prepared spermine and spermidine standards were run simultaneously on cellulose acetate to aid in the determination of their correct concentrations. All the standards which were developed contained too high a concentration of spermine and spermidine for the cellulose acetate study of seminal fluid, but these increased levels were useful in the polyacrylamide disc gel studies.

A comparison of the gels used to separate the seminal fluid samples with the gels used to separate the spermine and spermidine standards failed to reveal the presence of any spermine or spermidine bands. Since the length of time during which the electrophoretic separation was done was being determined by the movement of the tracking dye, it is possible that the samples were being electrophoresed for too long. If the time of electrophoresis is too long, portions of the sample being separated can be run off the end of the gel and lost. In the case of spermine and spermidine, this

is possible because their molecular weight is lower than that of the tracking dye and they would move through the gel faster than, and in front of, the tracking dye.

To test for this possibility, the length of electrophoresis was timed and decreased to determine at which point the spermine and spermidine bands could be identified on the disc gels. The point at which the bands could be identified on the disc gels would be used to set the length of time during which the electrophoretic separations of the seminal fluid samples could be done. Decreasing the length of time during which the samples were electrophoretically separated failed to reveal the presence of the spermine and spermidine bands on the disc gels.

Since shortening the run time of the electrophoretic separation failed to reveal which bands were the spermine and spermidine, different protein stains were tested. The use of the different stains could possibly identify different band patterns, because of the differences in their staining sensitivity. It was possible that the spermine and spermidine were not being stained by the Coomassie-Brilliant Blue, which was being used. The additional stains which were tested were Ponceau S, which was used to stain the seminal fluid samples being electrophoretically separated on cellulose acetate, and Crowle's Double Stain for immunodiffusion. The gels stained with these dyes failed to reveal the presence of the spermine and spermidine bands.

Spermine and spermidine are very small molecules. The smaller the molecule, the faster it will migrate through the polyacrylamide disc gels. The gels being used were 7% acrylamide which indicates that the pore size of the gel is large. In this particular study it is possible that the pore size of the gel was too large to trap the spermine and spermidine within the gel matrix, thus explaining the failure to identify these bands on all the previous electrophoretic runs.

The pore size of acrylamide gels can be decreased by increasing the acrylamide concentration of the gel. The acrylamide concentration may be increased as high as 30%, creating gels which have very small pore openings in the gel matrix. These gels are used to study substances with very small molecular sizes such as DNA and RNA. To test for the possibility that a smaller pore size in the gel matrix would help in the identification of the spermine and spermidine, new gels were made. The new gels had a higher Bis to acrylamide concentration, which decreased the pore size of the gel matrix. The solutions and procedures used to make these gels are as follows:

Gel Preparation (63)

Solution A
Dissolve 11.47 g of Trizma Base in
50.0 ml of distilled water plus
28.92 ml of 1M Hydrochloric Acid
add enough distilled water to bring the solution up to a final volume of 100 ml.

Solution B
Dissolve 1.92 g of Trizma Base in
50.0 ml of distilled water plus
25.6 ml of 1M Phosphoric Acid
add enough distilled water to bring the solution to a final volume of 100 ml.

Solution C
Dissolve 38.0 g Acrylamide and
2.0 g Bis in
70.0 ml of distilled water. After the chemicals are dissolved, add enough distilled water to reach a final volume of 100 ml.

Solution D
Dissolve 5.0 g Acrylamide and
1.25 g Bis in
70.0 ml of distilled water. After the chemicals are dissolved, add enough distilled water to reach a final volume of 100 ml.

Solution E
Mix 0.6 ml of a 10% solution of Ammonium Persulfate
10.0 ml of a 0.02% solution of Riboflavin Phosphate
89.0 ml of distilled water

Mix the above stock solutions as prescribed in Figure 3 to produce the desired gel. The procedure for making the gels and polymerization is the same as was described earlier.

SOLUTION	SEPARATING GEL			STACKING GEL
	5%T 5%C	10%T 5%C	15%T 5%C	
Monomer C or D	1.25 ml C	2.5 ml C	3.75 ml C	10 ml D
Buffer A or B	2.5 ml A	2.5 ml A	2.5 ml A	5 ml B
Distilled Water	5.0 ml	3.75 ml	2.5 ml	None
Catalyst E	1.25 ml	1.25 ml	1.25 ml	5 ml
TEMED	20 ul	20 ul	20 ul	10 ul

Figure 3: PAGE composition menu.(64)

The study using the disc gels with smaller pore sizes was identical to the one which was done on the 7% acrylamide disc gels. Electrophoresis was carried out over variable times based upon the movement of the bromophenol tracking dye. The gels were stained with the same three protein dyes and all the known seminal fluid samples were run versus the laboratory standards of spermine and spermidine.

The study which was done on the disc gels with the smaller pore sizes resulted in the same findings as the study using the 7% acrylamide gels. The spermine and spermidine bands could not be isolated and identified.

IV. Conclusions

The electrophoretic separation and analysis of the seminal fluid samples on polyacrylamide disc gels failed to provide any information concerning the location or identification of spermine and spermidine. The failure to located and identify the two polyamines is probably the result of their small molecular size. The small molecular size of these substances allows them to migrate rapidly through the pore matrix of the gel, making it impossible to confine them within the gel and create identifiable bands patterns upon staining. If this is the case, then separation of seminal fluid on polyacrylamide disc gels is

not suitable for the isolation and identification of spermine and spermidine.

The other possibility is that the spermine and spermidine may have been negatively charged. The molecular charge of spermine and spermidine is determined by the tank buffer. If these polyamines have a negative charge in this buffer system, they will migrate toward the anode which has a positive charge. If these compounds migrate toward the anode, they will not migrate through the disc gel and the gel will not exhibit any band patterns when stained, because the spermine and spermidine will not be present in the disc gel. To determine if this was the reason for which no results were obtained, it would be necessary to reverse the polarity (switch the direction) of the electrophoresis run. This possibility was not thought of at the time the research was being done and was never examined.

ELECTROPHORESIS ON CELLULOSE ACETATE MEMBRANES

Introduction

Cellulose acetate membranes may be used as a support medium for the electrophoretic separation and examination of biological fluids such as seminal fluid. These membranes are composed of cellulose, which is a complex polysaccride.(65) The cellulose is acetylated with acetic anhydride to produce a membrane with a three-dimensional network of interlocking pores.(66) These membranes can be purchased as a film or with an inert support backing such as mylar. The purpose of the backing is that cellulose acetate is very brittle when it is dry; the backing helps prevent the film from cracking and breaking.

Cellulose acetate has many desirable properties such as pore size, density and thickness which can be controlled during production of the membrane. Each of these properties is determined by the degree of acetylation (67), which is easily controlled. This results in many different types of cellulose acetate membranes for electrophoresis which exhibit a good degree of uniformity from batch to batch and even between those of different manufacturers.

An additional advantage of cellulose acetate is that electrophoresis is faster on cellulose acetate than on most other mediums. The generation of heat during electrophoresis is usually not a problem, manipulations are

easier than on other mediums and they can be rendered clear for scanning of the band patterns by a densitometer. Electrophoresis on cellulose acetate is also fairly inexpensive compared to electrophoresis on other support media. The equipment is inexpensive, the buffers are easy to make or they may be bought premeasured or mixed and the applications are very broad. The disadvantage of electrophoresis on cellulose acetate is that it lacks sensitivity in comparison to electrophoresis on other mediums. This means that larger sample sizes are required to achieve the desired results. Because the advantages outweigh the problem of lack of sensitivity, the second phase of this research was done on cellulose acetate.

Methods and Materials

I. Procedures

All sample separations were performed on Titan III cellulose acetate (Helena) which has an inert mylar backing for support. Prior to use, the cellulose acetate plates were soaked in the electrophoresis tank buffer for 15 minutes. To do this, a beaker is filled with approximately 250 ml of tank buffer. The cellulose acetate is slowly lowered into the beaker allowing the buffer to migrate along the cellulose acetate by wick action. Care should be taken to avoid the trapping of air in the pore matrix of the

cellulose acetate. If air becomes trapped (small white areas will be visible), the cellulose acetate should be discarded. After the cellulose acetate has been soaked in the tank buffer, it is ready for application of the samples.

The samples analyzed on cellulose acetate were a serum control (Dade), liquid seminal fluid, used neat and prepared as described in the previous section, reconstituted seminal fluid stains which were made by drying the liquid seminal fluid samples on clean pieces of cotton cloth and a laboratory produced standard of spermine and spermidine. The samples were applied to the cellulose acetate with a Super-Z applicator and Cellulose Acetate Template (Helena) using the following procedure:

The Super-Z electrophoresis tank (Helena) is filled with cold buffer. The buffer used for this procedure is HR buffer (Helena) which is a tris-sodium barbital buffer with a pH range of 8.6 to 9.0. Two wicks (2" x 5") made from filter paper are wetted by submerging them in the tank buffer. One wick is folded over each support bridge, being sure they make good contact with the buffer and that there are no air bubbles trapped under the wick. The inner chambers of the electrophoresis tank are filled with ice, which provides cooling during electrophoresis. The electrophoresis tank is ready for use.

The wells of the sample application plate are filled with approximately 10 ul of each sample using a Drummond Microdispensor. The Super-Z applicator is lowered into the

wells on the sample application plate several times. The first sample application using the Super-Z applicator is applied to a blotter to determine if the samples are being applied evenly, if not, the last two steps are repeated. The cellulose acetate is removed from the beaker of tank buffer and gently blotted between two pieces of filter paper to remove any excess buffer (do not allow the cellulose acetate to dry). The cellulose acetate plate is placed in the aligning base, cellulose acetate side facing up, aligning the edge of the plate with the black line labeled "cathodic application". The samples are applied to the plate by lowering the Super-Z applicator into the sample wells three times and then transferring the applicator to the aligning base. The Super-Z applicator is lowered down to make contact with the cellulose acetate and held for five seconds, prior to release. The application of the samples is repeated two more times in order to deposit a sufficient quantity of sample for analysis. The more concentrated samples may not need multiple applications. If this occurs, these samples can be removed from their wells on the applicator plate prior to reapplication of the other samples.

The cellulose acetate is placed in the electrophoresis tank face down and a quarter is placed on it as a weight to insure contact with the wicks. The chamber is covered with its lid and the power cables are attached to the power supply. Electrophoresis is anodic (towards the anode) at

180 V for periods of 5 minutes and 15 minutes. This was done to examine which length of time provided the best results. As will be explained, both lengths of time may be suitable for examining different constituents of seminal fluid.

After completion of electrophoresis the cellulose acetate is stained in Ponceau S (Gelman) stain for serum proteins and hemoglobin using the following procedure:

The cellulose acetate is placed into the dye solution by quickly sliding it under the liquid to submerge it totally. The cellulose acetate should remain in the dye solution for three minutes. The cellulose acetate is removed from the dye and drained well to minimize the carryover of dye to the 5% acetic acid destain bath. The bath should be gently agitated while the cellulose acetate is in it. After two minutes, the cellulose acetate is removed and drained. This step is repeated in two more acetic acid baths. After the third acetic acid bath, the cellulose acetate is removed and drained. The cellulose acetate is placed in a bath of absolute methanol for two minutes and gently agitated. The methanol bath is repeated one time prior to removal and draining of the cellulose acetate. The unbound stain should be totally removed from the plate. The cellulose acetate is placed in Clearing Aid (Helena) for five minutes. This reagent transforms the opaque white background of the cellulose acetate to clear. The cellulose acetate is removed from the clearing solution

and placed on a hot tray in a drying oven for five minutes. The plate is then ready to read in a densitometer.

I. Results

The initial electrophoresis runs on cellulose acetate were done to determine the best length of time for the run and to see what the seminal fluid band patterns looked like in comparison to a serum sample which was run as a control. The initial runs were done for lengths of 5 and 15 minutes. The 15 minute runs provided the best separation of the negatively charged portions of the seminal fluid and serum samples. The negatively charged materials migrate toward the anode. These negatively charged constituents appeared to comprise the majority of the materials in the samples as indicated by the near absence of any band patterns between the origin and the cathode. Based upon the better separation and the anodic migration of most of the constituents making up the samples, a 15 minute run time was determined to be best.

An area of interest on the cellulose acetate plates which were electrophoresed for 15 minutes was a band which migrated slightly off the origin toward the anode. This band, when stained with Ponceau S dye, would only partially accept the stain. The partial acceptance of the stain produced a band which was visible to the unaided eye by virtue of it having a different coloration than the other

materials stained with Ponceau S. The other unique aspect of this band was it did not occur in the serum samples which were being run on the plates as a control. The serum samples stained with Ponceau S did not exhibit any band patterns in the position at which this band occurred. Since this band does not occur in serum, it is possible that this material may be seminal fluid specific. Because of the possible unique nature of this band, the research which was conducted on the dried seminal fluid stains was oriented toward demonstrating the presence of this material and its potential as an indicator of seminal fluid.

The second phase of this study of seminal fluid samples on cellulose acetate was the identification of the bands which corresponded to spermine and spermidine. To aid in this, a standard was created which was a mixture of 1 ml of human serum, 4 mg of spermine tetrahydrochloride (Sigma) and 4 mg of spermidine trihydrochloride (Sigma). This sample was electrophoresed along with known seminal fluid samples and a serum control for 15 minutes and stained with Ponceau S. A comparison of the band patterns produced by the three samples failed to reveal any bands between the origin and the anode which may be spermine and spermidine. A review of the literature revealed why. The polyamines spermine and spermidine are basic and have a positive charge in barbitol buffers (68) such as the Helena HR buffer. The polyamines, being positively charged, will migrate toward the cathode. The samples are applied 2.5 cm from the

cathode and 5 cm from the anode, leaving very little room for cathodic migration before hitting the wick and running off the edge of the plate. With this in mind, several plates were electrophoresed for 5 minutes after which the area between the origin and the cathode was examined. The spermine and spermidine were found to be present cathodic to the origin. The 15 minute electrophoresis time is too long for the identification of spermine and spermidine using this procedure. The resolution to this problem is a run time of 5 minutes or a move of the origin to the anodic end of the plate, both of which could result in the loss of the unique band which was previously discussed.

The remainder of the research on cellulose acetate was oriented toward this unique band and demonstrating that it is present in dried seminal fluid as well as fresh liquid seminal fluid samples. When biological fluids dry, some of their constituents may be lost forever. This is because the fluid changes chemically upon drying causing the loss or destruction of some of its constituents. In forensic serology, the majority of the biological fluids which are examined have been dried. It is for this reason that the only information which is of value is that which survives the drying process unchanged.

For this portion of the study, the seminal fluid samples were split into two equal portions. One portion was applied to clean cotton cloth and allowed to air dry at room temperature. The other portion was retained in liquid form

and frozen at -80°C until used. The purpose of this was so that a comparison could be made of the band patterns from liquid and dried seminal fluid samples of the same source.

Prior to electrophoresis, the dried seminal fluid stains must be eluted from the cloth in a manner which will provide the largest quantity of the sample. The following elution techniques were examined to determine which would recapture the largest quantity of sample.

A) Extraction in 0.85% saline

A 2 cm sample of the stain was cut from the cotton cloth and placed in 5 ml of 0.85% saline for one hour at room temperature. At the end of one hour the cloth was removed from the saline and discarded. The liquid extract was then used as a sample and electrophoresed as in the procedure which was previously discussed. Samples extracted by this method failed to show any band patterns on the cellulose acetate. This extraction procedure was then repeated with incubation at 2°C and 37°C, both of which provided the same results. Failure to achieve any patterns after electrophoresis on cellulose acetate may indicate that the seminal fluid samples did not elute off the cotton cloth or that the quantity which did elute off the cotton cloth was too dilute for detection.

B) Extraction in 0.85% saline and concentration of the sample

The previous elution procedure was repeated at temperatures of 2°C, room temperature and 37°C. Prior to discarding the piece of cloth, an effort was made to wring it out to retain as much of the eluate as possible. The eluate was then concentrated in a Minicon B-15 clinical sample concentrator (Amicon). The sample, after filtration, was approximately 50 times more concentrated than it was prior to being concentrated. Electrophoresis of these samples provided very weak band patterns which were unreadable.

C) Extraction in 0.85% saline at 2°C for variable time lengths

The previous extraction technique was repeated except all the extractions were done at 2°C and the time length of the extraction was changed. Extractions were done for 2, 4, 8 and 16 hours, after which the samples were concentrated to 50 times their original concentration. These samples produced band patterns which were detectable with a densitometer, but they were still very weak.

D) Extraction in pH 7.5 phosphate buffered saline

The samples were extracted in 5 ml of phosphate buffered saline with a pH of 7.5 at 2°C, room temperature and 37°C for four hours. After the extraction was complete, the samples were concentrated to 100 times their original concentration. Electrophoresis of these samples provided very weak band patterns on cellulose acetate.

E) Extraction in pH 7.3 phosphate buffered saline

The samples were extracted in 5 ml of phosphate buffered saline with a pH of 7.3 at 2°C, room temperature and 37°C for four hours. After the extraction was completed, the samples were concentrated to 100 times their original concentration. Electrophoresis of these samples provided weak but readable patterns in cellulose acetate. The extracts which were done at 37°C provided the best results.

A comparison was then done of the densitometer charts of the samples extracted at 37°C in pH 7.3 phosphate buffered saline to the charts of the corresponding liquid seminal fluid samples. These comparisons indicated that most of the materials present in liquid seminal fluid and detectable on cellulose acetate survived the drying process and were extractable. The comparison also indicated that only a small portion of the dry seminal fluid was being

reclaimed during the extraction process. One substance which did not extract well was the unique band which was identified in the liquid samples because of its failure to accept the Ponceau S dye. Since this substance could not be clearly identified after extraction of the seminal fluid from the cloth, one must wonder if it survives the drying process and, if it does survive the drying process, what is the best extraction method.

III. Conclusions

The electrophoretic analysis of seminal fluid on polyacrylamide disc gels and cellulose acetate to identify spermine and spermidine is not feasible. The polyamines spermine and spermidine have such small molecules with a high electric charge that they are not easily captured by electrophoresis on polyacrylamide disc gels. Electrophoresis on cellulose acetate lacks the sensitivity necessary to identify any band patterns from dilute samples. This can be a major problem since neat seminal fluid is a rarity in the analysis of rape evidence. The seminal fluid is almost always mixed with the vaginal secretions of the victim. These secretions dilute the seminal fluid sample and this will create problems for the electrophoretic examination of the sample on cellulose acetate.

The unique band which was identified, is the only finding of potential value which was identified by the study of seminal fluid on cellulose acetate. This substance may be seminal fluid specific, which means it could be used to conclusively identify the presence of seminal fluid when no spermatozoa is identified. To determine if this band is seminal fluid specific, several avenues of research should be explored.

First, the band must be identified as to its chemical composition and properties. After the band is identified, its site of origin within the male reproductive tract must be determined. Determination of these facts will further indicate if the substance is seminal fluid specific or not. If the substance appears to be seminal fluid specific, a procedure must be developed for the identification of this substance in seminal fluid stains. After development of a viable identification procedure, samples of other biological fluids must be analyzed to demonstrate that this band is not naturally occurring in anything other than seminal fluid.

DETERMINATION OF PHOSPHOGLUCOMUTASE SUBTYPES

Introduction

Phosphoglucomutase is an isoenzyme which is routinely used in determining information about the enzyme profile of an individual. Isoenzymes are proteins which are the same but occur in multiple molecular forms. As in the red blood cell groups, each individual will only exhibit one of these forms and that form of the enzyme will be the same regardless of the tissue or body fluid which is examined. These multiple enzyme forms may be determined and identified by electrophoretic separation of the sample. The best source of isoenzyme information is blood. Blood contains dozens of isoenzymes which can be electrophoretically determined. Some of these enzymes are present in other body fluids such as seminal fluid and vaginal fluid. These fluids may be electrophoretically separated to determine their isoenzyme information. Once such enzyme is phosphoglucomutase (PGM). PGM information is present in several of the body's fluids, such as the blood, seminal fluid and vaginal fluid. PGM can be separated into three major phenotypes (Figure 4) by low voltage electrophoresis on 10% starch gels. The three major PGM phenotypes and their population frequencies for whites in Miami, Florida are: PGM 1: 60.1%, PGM 2-1: 33.1% and PGM 2: 6.8%.(69) It has been demonstrated that each of these PGM phenotypes

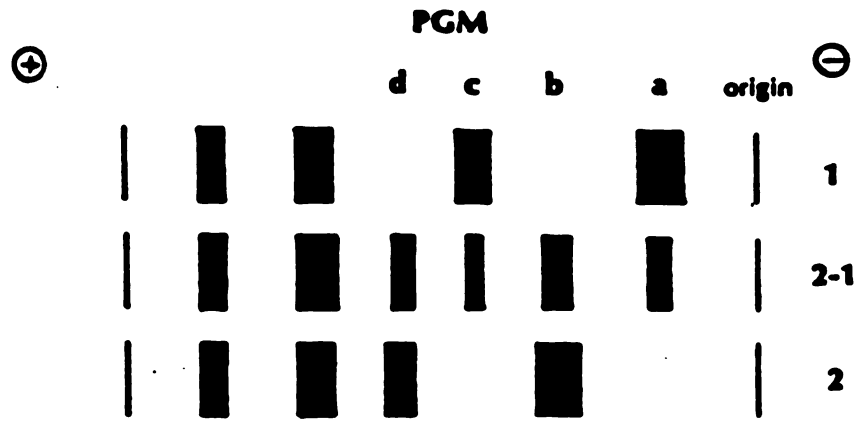

Figure 4: PGM phenotypes.(70)

may be further divided into subgroups by low voltage electrophoresis on agarose (71,72) or by isoelectric focusing.(73,74) PGM subtyping is valuable because it divides the three major phenotypes into ten phenotypes, each of which has a lower population frequency than the three major forms. The PGM subgroup group phenotypes and their population frequencies for whites in California are shown in Figure 5.

The most important benefit of PGM subtype information is the higher potential for identifying possible mixtures of body fluids contributed by several individuals, such as seminal fluid with vaginal fluid. When a man ejaculates into the vagina of a woman, there is a mixing of their body fluids. The PGM information present in the woman's fluids may mask the seminal fluid donor's PGM information, especially if the woman is a PGM 2-1. Prior to the development of the PGM subtyping technology, seminal fluid samples from the vagina of 2-1 women were of little or no value unless the victim and seminal fluid donor were different ABO blood groups. The ability to determine PGM subtypes increases the chances of identifying mixtures because the victim and seminal fluid donor may be different PGM subtypes. When this occurs, the PGM subtype of the seminal/vaginal fluid mixture may then be compared to the victim's PGM subtype (obtained from analysis of the victim blood standard), to predict the possible subtypes of the seminal fluid donor. This information is then compared to

PGM Phenotype	Subtype	Population Frequency(75)
PGM 1	1+	39.6%
	1+1-	16.7%
	1-	2.7%
PGM 2-1	1+2+	23.1%
	1+2-	6.8%
	1-2+	3.7%
	1-2-	1.8%
PGM 2	2+	2.9%
	2+2-	2.2%
	2-	0.5%

Figure 5: PGM subtype phenotypes and population frequencies.

the PGM subtype information of the subject (obtained from analysis of the subject's blood standard) to determine if he is in the group of possible seminal fluid contributors. Because there are ten PGM subtypes, this situation occurs much more frequently than when determining the PGM major phenotypes. Figure 6 illustrates the improved analysis benefits of PGM subtyping technology.

At present, several problems exist with the conventional methods for determining PGM subtypes. In tests done using a phosphate-citric acid buffer system (76) at pH 5.5 and tests of an acetic acid buffer system (77) at pH 5.3, there were problems in obtaining readable band patterns. Tests of a phosphate-citric acid buffer system which used a 1% isoelectric focusing gel (78) provided good results, but were difficult to reproduce. This system also exhibited poor separation between the PGM 2+ band and the more anodic PGM bands and poor separation between the PGM 2- and 1+ bands.

Another area of consideration in the analysis of blood and body fluids is sample quantity. Limited sample size restricts the amount of information which may be determined about it. This is because each test expends a portion of the sample, continually decreasing the sample quantity available for the next test. Electrophoresis systems which provide information on multiple enzyme groups are preferable to systems which provide information on only one enzyme group because large sample quantities are not necessary.

Victim PGM Phenotype	Seminal/Vaginal Fluid Mixture PGM Information	Possible PGM Phenotype of Semen Donor
PGM 2-1	PGM 2-1	PGM 1 PGM 2-1 PGM 2
PGM Subtype 1-2+	PGM Subtype 1+1-2+	PGM 1+ PGM 1+1- PGM 1+2+

Figure 6: The effects of PGM subtyping on the analysis of sexual assault evidence.

It has been demonstrated that modification of the phosphate-citric acid buffer system by lowering the pH and using isoelectric focusing agarose provides PGM subtype information that has a good band pattern separation and reproducible results. It has also been demonstrated that the same plate can be developed to determine the Adenosine Deaminase (ADA), Erythrocyte Acid Phosphatase (EAP) and Adenylate Kinase (AK) enzyme information. These three enzymes also occur in multiple forms and are also useful in the analysis of blood samples. They are not useful in the analysis of semen and other body fluids because of very low concentration in these fluids or because they only occur in the blood.

Methods and Materials

Buffers

Tank Buffer:

The tank buffer is a phosphate-citric acid buffer composed of 19.21 g of citric acid, anhydrous (Sigma) dissolved in 800 ml of distilled water. The buffer is then adjusted to pH 5.3 at 23°C by the addition of approximately 35 g of sodium phosphate, dibasic (Sigma) and brought up to a final volume of one liter.

Gel Buffer:

The gel buffer is a phosphate-citric acid buffer composed of 0.48 g citric acid, anhydrous dissolved in 800 ml of distilled water. The buffer is then adjusted to pH 5.3 at 23°C by the addition of approximately 0.75 g of sodium phosphate dibasic and brought up to a final volume of one liter.

Gel and Plate Specifications:

The gel is composed of 1 g of Isogel (FMC Corp.) dissolved in 100 ml of gel buffer and poured onto a glass plate which is 24 cm long and 25 cm wide. The plate should have borders which are 1 cm wide and 1 mm thick. The gel should be allowed to solidify completely before being placed on a cooling plate.

Samples and Electrophoresis:

The blood samples and body fluid samples were obtained from regular case evidence which were in the laboratory for routine analysis. The samples were placed in slots which were cut into the gel 7 cm from the cathode and then wetted with 2 ul of 0.01M Cleland's Reagent (Sigma).

Electrophoresis is performed at a constant temperature of 5°C and a voltage of 21 v/cm for four hours with the samples run anodically.

Development:

The reaction buffers and reaction mixtures used for development of the EAP, ADA and AK enzymes are the same as those specified in Culliford (79) and Wraxall.(80)

EAP

Reaction Buffer:

Dissolve 0.96 g of citric acid, anhydrous and 0.4 g of sodium hydroxide (Sigma) in 80 ml of distilled water. After the solids are totally dissolved, bring the buffer up to a final volume of 100 ml. This buffer has a pH of 5.0 at 23°C.

Reaction Mixture:

Mix 4 mg of MUP with 10 ml of the reaction mixture. Saturate a piece of 1 mm Whatman filter paper which measures 25 cm long and 8.5 cm wide with the reaction mixture.

AK

Reaction Buffer:

Dissolve 1.20 g trizma base and 0.4 g of magnesium chloride (Sigma) in 80 ml of distilled water. The buffer is then adjusted to pH 8.0 at 23°C by the addition of 1:1 hydrochloric acid (Fisher) and brought up to a final volume of 100 ml.

Reaction Mixture:

Dissolve 55 mg of AK PMR (SERI) and 1 mg of G PD hexokinase (Sigma) in 15 ml of reaction buffer. This 6 mixture is light sensitive, it must be kept in a dark place until ready for use.

Dissolve 0.3 g of agarose Type I (Sigma) in 15 ml of distilled water. After the agarose is totally dissolved, cool the solution to 60°C. Pour the reaction mixture into the agarose and apply it to the enzyme plate immediately.

ADA

Reaction Buffer:

Dissolve 0.355 g of sodium phosphate, monobasic (Sigma) and 0.19 g of sodium phosphate, dibasic in 80 ml of distilled water. After the solids are dissolved, bring the buffer up to a final volume of 100 ml. This buffer has a pH of 7.0 at 23°C.

Reaction Mixture:

Dissolve 30 mg of ADA PMR (SERI), 35 ul of xanthine oxidase and 35 ul of nucleoside phosphorylase in 15 ml of reaction buffer. This mixture is light sensitive and must be kept in a dark place until ready for use.

Dissolve 0.3 g of agarose Type I in 15 ml of distilled water. After the agarose is totally dissolved, cool the solution to 60°C. Pour the reaction mixture into the agarose and apply it to the enzyme plate immediately.

Development Procedures:

The EAP is developed first in the area covering from the origin, 8.5 cm toward the anode by covering this area with the Whatman filter paper soaked in the reaction mixture. The AK overlay is positioned at the origin running

4 cm toward the cathode. This is done by placing plastic borders around the area, pouring the reaction/agarose mixture into it and allowing it to solidify. The ADA overlay is positioned from the edge of the anodic wick, 5 cm back toward the origin. The same procedure is followed as in the AK overlay. The plate is then placed in an incubator adjusted to 37°C for 20 to 30 minutes, after which the EAP and AK results may be read.

The EAP is visualized by exposing the developed area of the plate to ultraviolet light. During this procedure, the AK and ADA enzyme areas should be covered to shield them from the ultraviolet light. The AK results are visible under white light.

After recording the EAP and AK results, the PGM subtypes can be developed. The procedure for preparation of the PGM subtype reaction buffer and mixture is described by Sutton and Burgess.(81)

PGM Subtypes Development

Reaction Buffer:

Dissolve 18.0 g trizma base, 2.0 g magnesium chloride and 1.0 g histidine mono hydrochloride (Eastman) in 400 ml of distilled water. The buffer is then adjusted to pH 8.0 with 1:1 hydrochloric acid and brought up to a final volume of 500 ml.

Reaction Mixture:

Dissolve 90 mg PGM PMR (SERI), 2 mg NADP, 25 mg EDTA trisodium (Sigma) and 1 mg G 6 PD in 20 ml of reaction buffer. This mixture is light sensitive and must be kept in a dark place until ready for use.

Dissolve 0.4 g agarose Type I in 20 ml of distilled water. After the agarose is dissolved, cool the solution to 60°C. Pour the reaction mixture into the agarose and apply to the enzyme plate immediately.

The position of the PGM subtype overlay is the same area which was developed for EAP. The overlay pouring procedure is the same as that for the AK and ADA enzymes. After pouring the overlay, the plate should be placed in a 37°C incubator for 60 to 90 minutes or until the band patterns have developed to a sufficient density.

Results and Discussion

PGM Subtypes (Figure 7):

The pH 5.3 phosphate-citric acid buffer system provides very good resolution and separation of the PGM bands. The separation which occurs between the PGM 2+ band and the more anodic bands is excellent, but diminishes with each usage of the tank buffer. Due to this effect, the tank

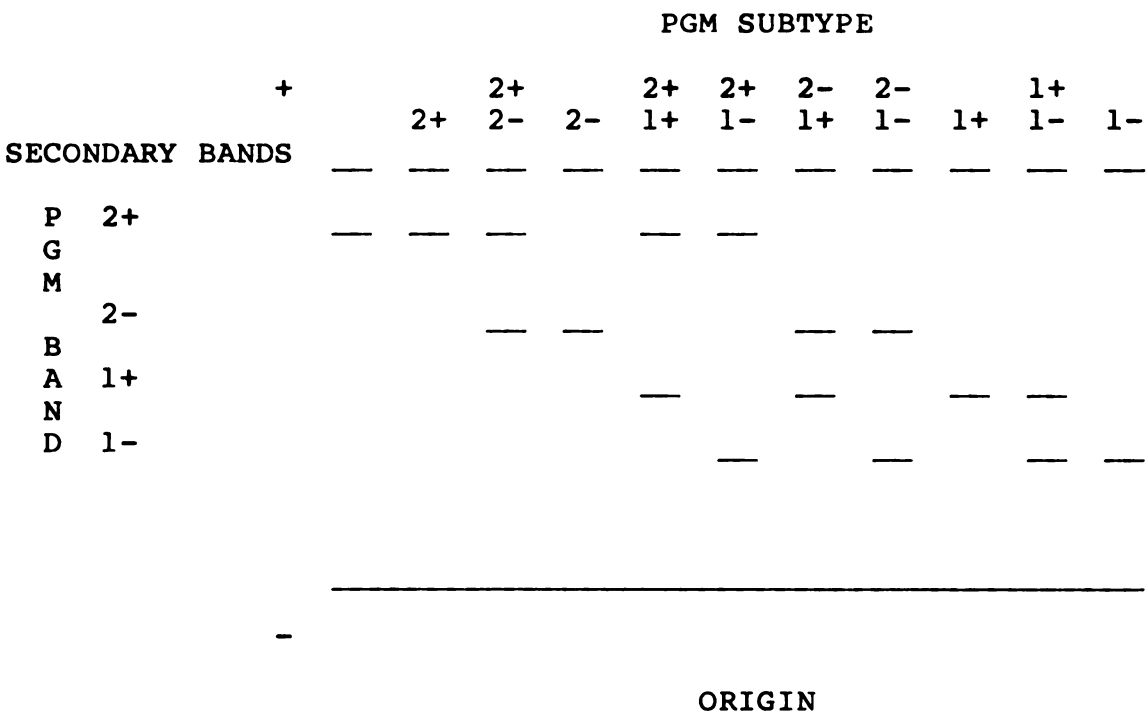

Figure 7: PGM subtype band patterns.

buffer should be used a maximum of three times and then discarded.

When determining the PGM subtypes of samples it is recommended that the major PGM type be determined in advance. This aids in the reading of the subtype bands and prevents samples from being misread. This is very important in samples from individuals who are PGM 1+ or PGM 1- because these samples may exhibit an additional band which occurs in the PGM 2 region and may be interpreted as a PGM 2+ band. The band is located midway between the positions of the 2+ and 2- bands and can be identified by the use of proper standards and knowledge of the sample's major PGM type. The nature of this band is unknown at this time. It may be a degradation product which is evidenced by the failure of the band to occur in fresh blood samples.

EAP (Figure 8):

The resolution and separation of the EAP band pattern is extremely good. The band patterns are very sharp with a strong intensity which provides for good visualization under ultraviolet lighting. Due to the intensity of the band patterns, the degradation band which occurs anodic to the primary B band is more easily visualized and may be misread as an R band. This problem is minimized when 0.01M Cleland's Reagent is applied to the samples prior to electrophoresis. It is essential to use minimal amounts of

the Cleland's Reagent, because it migrates to the position of the AK 1 band and, when used in excess, will obscure this band.

ADA and AK (Figure 8):

The resolution and intensity of the band patterns are comparable to that which occurs when analysis is done using the pH 5.5 phosphate-citric acid buffers and a starch gel.(82)

Conclusion

The pH 5.3 phosphate-citric acid buffer system and use of a 1% isoelectric focusing support gel provides information on four enzyme groups simultaneously. This minimizes the quantity of sample expended during analysis, while maximizing the enzyme information obtained. Care should be taken in determining the PGM subtype and EAP enzyme information when using this system. If the enzyme character of the sample is in question, the sample should be examined by isoelectric focusing and, if this methodology is unavailable, it should be reported out as inconclusive. It should also be taken into consideration that multisystems such as this may provide information concerning several enzymes, but it may not be the best or clearest way to determine each of the individual enzyme types being

identified. This may lead to errors in its identity of the enzyme types, so great care should be taken when reading these enzyme plates.

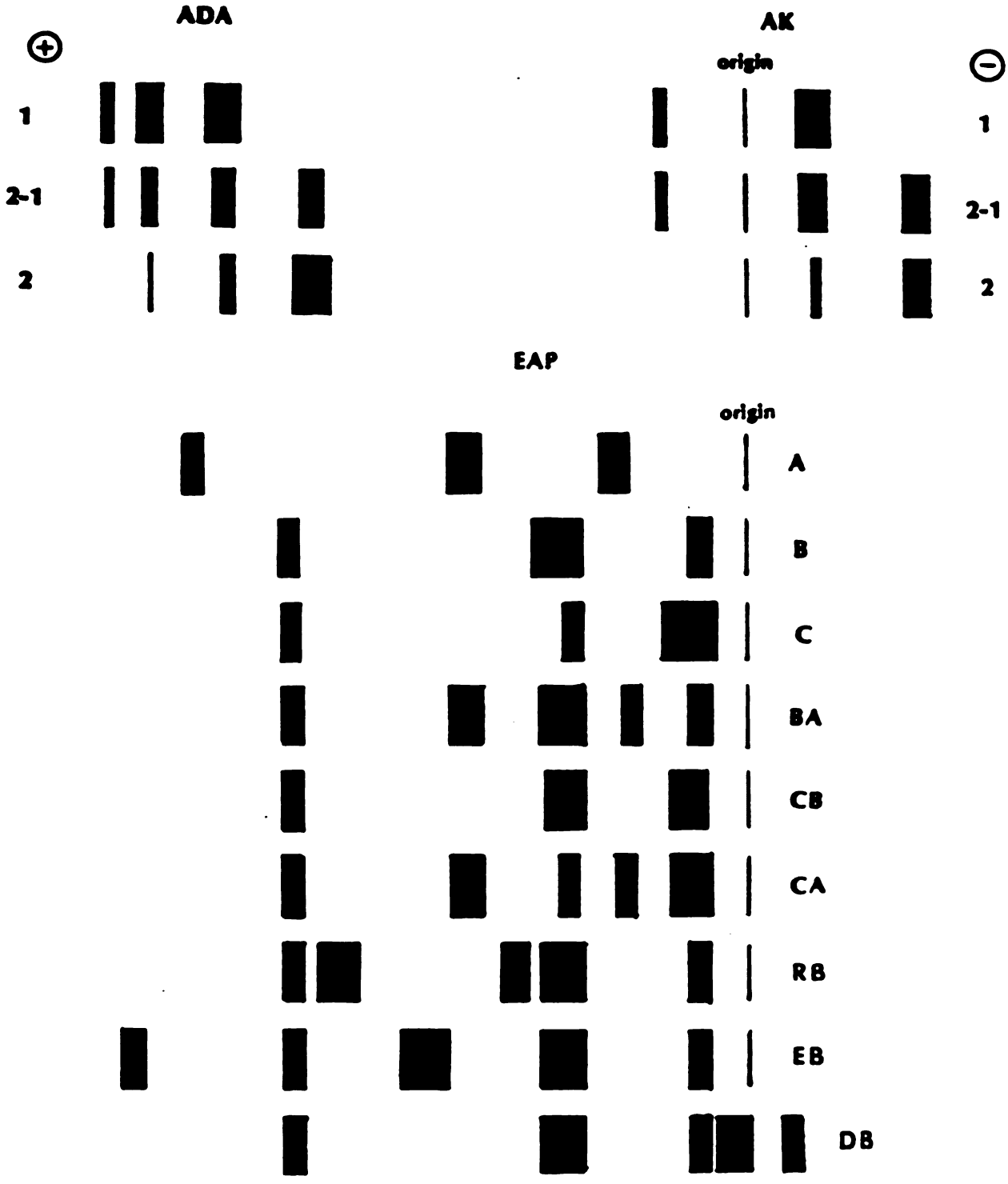

Figure 8: ADA, AK, and EAP band patterns.(83)

SUMMARY

Seminal fluid samples were examined by electrophoretic separations on polyacrylamide disc gels and cellulose acetate to assess the usefulness of the polyamines spermine and spermidine as confirmatory markers. The electrophoretic separations indicate that spermine and spermidine are poor confirmatory markers for seminal fluid. Electrophoretic separation of samples on cellulose acetate revealed the presence of a protein which may be specific for seminal fluid. At present, the identity and origin of this protein are unknown making it a potential area of future research.

The second portion of this research study involved the modification and development of an electrophoresis procedure for the determination of PGM subtypes. The procedure which was developed is done on 1% Isogel in a pH 5.3 phosphate-citrate buffer system at a voltage of 21 v/cm for 4 hours. This electrophoresis methodology provides clear reproducible results as well as information on the EAP, ADA, and AK enzyme phenotypes of the samples.

The additional information which is obtained by the determination of the PGM subtypes may affect the interpretation of the evidence and how it relates to the victim and the alleged rapist. This more in-depth interpretation may, in turn, affect the prosecution and defense of the subject by strengthening or weakening their cases. It can also affect the planning concerning the

presentation of the case to the court and what should be emphasized during that presentation. It also may cause either side to be unwilling to go to court by providing information which is contradictory to the position being taken, thus affecting the ultimate disposition of the case.

If the subject is convicted of the criminal charges, the methodology which was used, as well as the information which was determined, may affect the appeals process. Scientific evidence and the expert opinions arrived at as a result of the analysis are subject to judicial review. If the courts decide that the methodology and/or opinions are flawed, the conviction may be overturned resulting in a new trial and exclusion of either the methodology or expert opinions from future criminal cases, thus affecting the future prosecution or defense of all cases.

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