CHARACTERIZATION OF PROTEINS IN HUMAN SEMEN WITH APPLICATIONS TO FORENSIC SCIENCE

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Terrence Wynn Mischler

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CHARACTERIZATION OF PROTEINS IN HUMAN SEMEN WITH APPLICATIONS TO FORENSIC SCIENCE

By

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INTRODUCTION

"By their semen ye shall know them." This statement by Dr. Carl Hartman, in a personal communication to this author, was one of the factors that prompted this research on human semen.

The problem can be divided into two areas. The first is basic research into the electrophoretic and immunological properties of human semen and will serve as a foundation for the remaining work. The second part concerns the problem occurring in forensic science of identifying human seminal stains.

There have been developed, over the years, many means to identify human seminal stains. The finding of spermine, choline, and acid phosphatase have been used for this identification. Each one of these methods has certain drawbacks. Spermine and choline are found in other body fluids; therefore, the finding of these substances only suggests that the stain is semen. The acid phosphatase test has been considered as specific for human semen, but this compound is found in other body fluids and plants, and is heat labile. The only fully accepted way, at present, to positively identify seminal stains is the finding of human spermatozoa on the stain. This too, has a disadvantage as it is based

on the assumption that sperm was originally present in semen and thus would not detect aspermic specimens.

During the last few years immunological techniques have been developed for the identification and characterization of proteins. There has been limited use of these methods for demonstrating organ and species specific proteins in stains from human sources. The results presented here were obtained in an effort to more fully characterize proteins in human seminal stains and to discover the conditions under which they remain immunologically reactive. It is hoped that the results will encourage a greater use of immunology in forensic science.

REVIEW OF LITERATURE

Historical

Leeuwenhoek (1677) was the earliest investigator of semen known to this author. He published for the first time the observation of live human spermatozoa and their movements in fresh semen. His statement that when human semen ". . . had stood a little while, some three-sided bodies were seen in it, terminating at either end in a point; some were of the length of the smallest grain of sand, and some were a little larger . . . ," is the first report of the crystalline substance in semen which later became known as spermine.

John Hunter (1786) stated that ". . . the smell of semen is mawkish and unpleasant, exactly resembling that of the Spanish chestnut; and to the taste, though at first insipid, it has so much pungency as, after some little time, to stimulate and excite a degree of heat in the mouth."

Miescher (1897) pioneered the research on both semen and seminal plasma, especially in fish and his work is reviewed by Mann (1964).

The earliest work concerning the immunological aspects of semen was that of Landsteiner (1899). He injected bull sperm into the peritoneal cavities of guinea

pigs and found that upon removal, they were still motile; but if guinea pigs had received a series of bull sperm injections prior to the final injection, the sperm when removed from the peritoneal cavity were found to be immotile.

Metalnikoff (1899) and Metchnikoff (1900) found that the blood from guinea pigs which had been injected either with their own or foreign sperm, would immobilize sperm of the same species *in vitro*.

Chemical Analysis of Human Seminal Proteins

Goldblatt (1935) found a trace of mucin, nucleoprotein, albumin, globulins and proteose in human seminal plasma. He stated that there were no protamines or histones present. The proteases were not heat-coagulable, passed through a semi-permeable membrane and were not precipitated by trichloroacetic acid.

Huggins, Scott and Heinen (1942) found that protein-like material in seminal plasma amounted to 3.5-5.5 gms/100 ml and about 60% of this consisted of proteoses. These proteoses would pass through a cellulose membrane impermeable to blood serum proteins.

The proteins involved in the coagulation and liquification of semen have received much attention in the literature. Mann (op. cit.) has received the current work on this subject. Rasmussen and Albrechtsen (1960) have

reviewed the literature concerning the fibrinolytic activity of human seminal plasma.

Grant (1959) found by concentrating male urine 4000 times and using immunoelectrophoresis that two faint components arising from the male genital tract could be identified.

Electrophoresis of Human Semen

The advent of electrophoretic techniques has brought about a new dimension for the characterization of the proteins in human semen.

The earliest work was that of Ross, Moore and Miller (1942) in which they used the Tiselius moving boundary electrophoresis. They found five seminal plasma components, which were designated P1, P2, P3, P4 and P5. The component with the slowest mobility was P1 and the fractions increased in migration speed until P5 was reached. They called P1 the proteose fraction. The P2 and P3 components could be divided into a water-soluble and water-insoluble fraction. These had mobilities similar to that of beta and alpha globulins. P4 was identified as a glycoprotein and had a mobility like albumin. The last fraction, P5, was thought to be related to P4 and possibly derived from it.

Ross et al. (1943) using the same methods on "abnormal" seminal plasma found no significant deviation in protein components from that of normal specimens.

Gray and Huggins (1942) also used the Tiselius apparatus. They found four protein fractions having the same mobilities as normal serum albumin, alpha, beta and gamma globulins. They stated that the fractions were identical with those of blood serum.

The development of zone electrophoresis on filter paper has provided impetus for a great deal of research. Mann (op. cit.) has reviewed the literature concerning the application of this technique to the characterization of seminal proteins and has come to the following generalizations. There are 4-5 main fractions which become more sharply defined in semen which has been stored under refrigeration for at least 48 hours. (3 hours, 380 volts, .05 M barbital buffer pH 8.7) The percentages of protein distribution is 6.3% albumin, 15.9% alpha globulin, 41.1% beta globulin, 23.2% gamma globulin and the remaining 13% exhibits no migration.

The most recent report of the electrophoretic analysis of human semen was that of Searcy et al. (1964) using cellulose acetate. They analyzed semen, seminal plasma and washed sperm, after allowing the semen to be refrigerated for 48 hours. A 20 lambda sample of seminal plasma was electrophoresed on 2.5 x 12 cm strips for 3 hours with a current of 1 ma per strip. Oxoid Owen buffer, pH 8.6 was used and the strips were stained with Ponceau S. They reported at least 8 zones of migration with seminal

plasma. A component that moved toward the cathode appeared to have no serum counterpart. There was no fraction having a serum alpha-1 mobility, but there were fractions having serum albumin, alpha-2, beta and gamma globulin mobilities. The albumin was associated with two faster zones of migration. Electrophoresis of whole semen revealed a zone of staining at the origin proteins from washed sperm were observed with mobilities similar to albumin and globulins. These workers contend that the zones of migration, in electrophoresis of washed sperm, were saline-insoluble components on the sperm and the stained origin was due to sperm being electrophoretically immobile.

Immunological Properties of Human Semen

The fact that spermatozoa are antigenic was, as already mentioned, discovered by Landsteiner (op. cit.). Many publications concerning the antigenicity of semen in the older literature are reviewed by Mann (op. cit.). The review presented here covers only the more recent literature.

Ross (1964) carried out the first extensive immunological investigation of human seminal plasma. He prepared an antiseminal plasma and found that it would cause a precipitin reaction with both seminal plasma and blood serum. He also noted that antisera prepared against human serum caused precipitation of seminal plasma.

After Wilson (1954) published some information on autoantibodies there was renewed interest in the antigenicity of semen. He found sperm agglutinins present in seminal plasma and blood serum of 2 sterile men. The sperm of these subjects agglutinated in their ejaculates.

The work of Wilson prompted Weil to continue research on the antigens of human semen. In 1956 Weil, Kotstsevalov and Wilson produced antiserum to both human seminal plasma and seminal spermatozoa. They stated that it was impossible to distinguish between seminal plasma and seminal spermatozoa using immunological techniques. Weil and Rodenburg (1960a) found that spermatocele spermatozoa would not react with antisera produced against seminal plasma or spermatozoa. This gives direct evidence that human spermatozoa acquire antigenicity during their passage through the male genital tract. Weil (1961) stated that the spermatozoa coating antigens do not interfere with the reactivity of the natural antigenic components present on sperm. Thus both testicular and seminal spermatozoa react against antiserum prepared using testicular spermatozoa as the antigen. Weil et al. (1960b) investigated the antigens of spermatocele fluid using gel double diffusion. They reported 4 components; one which is identical to serum albumin and the other 3 seemed also to be derived from blood serum. Weil and Rodenburg (1962) using fluorescein-conjugated antibodies for antiseminal plasma found that human spermatozoa were

stained, but spermatocele spermatozoa were not stained.

Rao and Sadri (1960) have claimed the following results. Human semen has at least 16 antigens, 7 of these can be attributed to spermatozoa and 4 to prostatic secretions. They stated that 7 semen antigens were common to blood serum. In a later article, Rao et al. (1961) have reported antigens present on spermatozoa; 4 of these were serologically identical with antigens in seminal plasma and 3 were specific to spermatozoa. These two reports were in abstracted form and an attempt by this author was made to obtain original articles. However, there was no reply to inquiries made of these authors.

Stevens and Fost (1964) found that antiserum prepared by injecting human sperm into rabbits was neutralized by the addition of human seminal plasma. Thus all antigens present in sperm are found in seminal plasma.

Much research concerned with the presence of human blood group antigens on sperm has been reported. The latest work is that of Edwards, Ferguson and Coombs (1964) using the mixed agglutination technique. They showed that the A and B blood group antigens were present on the spermatozoal membrane of secretors; but not on sperm of non-secretors. The report suggests that the soluble blood group antigens in their body fluids become passively attacked to the spermatozoa in the semen.

Vitullo (1965) has demonstrated the presence of A and B antigens in semen stains using the neutralization method. These antigens corresponded to the blood group of the donor. He has told this author that only stains from secretors will show these antigens and at the present time, work is being conducted to determine if the H antigen can be found in the stain.

Immunoelectrophoretic Analysis of Human Semen

Hermann (1959) first used agar immunoelectrophoresis to analyze human seminal proteins and found that fresh seminal plasma can be split into at least 8 fractions; but, after standing for 32-48 hours, 10 fractions can be seen. One fraction migrated the same as serum albumin and precipitated with antiserum to human blood serum. A fraction was seen to precede the albumin and those in the range of the alpha and beta globulins were unstable and poorly defined. The gamma globulin area contained 3-4 lines of precipitation, none of which seemed to be serum gamma globulin. These 3-4 fractions were partly ultrafiltrable and contained mostly proteoses.

Grant and Everall (1959) attempted, using immunoelectrophoresis, to identify the origin of the seminal protein in the genital tract. They stated that prostatic fluids produced 7 precipitin lines and seminal vesicle

fluids produced 6 lines. Two of the prostatic lines were not seen when whole seminal plasma was analyzed. The two most prominent seminal plasma precipitin lines were a beta-2 prostatic and a beta-1 seminal vesicle precipitation. A trace component with an alpha mobility was seen in seminal plasma which corresponded to one in seminal vesicle fluid. The principle post gamma globulin components came from the seminal vesicles. Symons, Rees and Grant (1962) confirmed these results. They added that the testes, the epididymes and the bulbo-urethral glands contribute little, if any to the proteins of seminal plasma.

Klopstock, Haas and Rimon (1963) answered the question of whether or not serum gamma globulin is present in seminal plasma. They used immunoelectrophoresis and a two-origin well system. One well contained seminal plasma, the other well, 2 cm behind, contained pure human gamma globulin. When antiseminal plasma was diffused against this system, the known gamma globulin line fused with an unknown component in the seminal plasma. This is strong evidence that serum gamma globulin is present in human semen.

Searcy et al. (op. cit.) also did some immunoelectrophoretic investigations of human semen. Human serum antiserum produced 4 precipitin lines when diffused against seminal plasma. When antiseminal plasma was used, 7 precipitin lines appeared.

Semen Properties of Lower Animals

There will be no attempt to review the literature concerning seman of lower animals. The reader is directed to the work of Mann (op. cit.) for an extremely complete review on this subject. The specialized field of bull semen has been reviewed by Hunter and Hafs (1963, 1964).

Application of Immunological Techniques to Forensic Science

Farnum (1901) using an antiserum to human semen obtained a precipitin reaction with an extract of a seminal stain 34 days old. This is the first suggestion that immunological techniques could be applied to problems in forensic science.

Muller, et al. (1958a) first described how immunodiffusion tests can be applied to identification of human fluids. These workers listed a number of advantages for this technique: (1) permanent preservation of the results, (2) they are more easily seen, and (3) they do not require prior clarification of turbid materials. The only disadvantage is that results take about a day whereas conventional tests take a few hours. These same authors (1958b) found that spots of sperm, uring, saliva, milk, cerebrospinal fluid, blood and gastric liquid could be identified as being of human origin. Muller et al. (1959) stressed the importance of using the proper antiserum. They stated

that the rabbit should be used and reported injections should be avoided, so as to eliminate species cross-reactions.

In 1959 Weil et al., using the complement fixation test, reported that semen could be detected on female genitalia after intercourse. He used as a positive control vaginal swabs from subjects after artificial insemination. Negative controls were taken from subjects who denied recent sexual activity. This work also indicated no cross-reaction between vaginal secretions and seminal plasma.

Coombs, Richards and Dodd (1963) reported serological identification of seminal stains. They used the Ouchterlony agar diffusion test and stated that extracts of seminal stains taken from cloth were positive. They used anti-seminal plasma adsorbed with serum and boiled saliva. The species specificity of the reaction was also investigated. Dog, ram, rabbit and boar seminal plasma were diffused against the antisemen and only the boar plasma was found to react faintly. These authors concluded by saying the length of time and conditions under which " . . . the specific proteins remain native and reactive is a matter for further experience."

Weil (1961) indicated that antiserum to human semen does not cross-react with the bull, stallion or ram.

Culliford (1964) gave a brief survey of the precipitin reaction and its application to forensic science.

He indicated that antiseminal plasma could be used in conjunction with immunodiffusion and immunoelectrophoresis to identify human seminal stains.

Individualism of Sperm

The statement by Dr. Carl Hartman in the introduction and the article by Hartman, Schoenfeld and Copeland (1964) indicates the possibility of individualizing sperm. These workers believe that sperm can be classified by their morphology and in their article they cited evidence to support this claim. It does not appear that the evidence is sufficient for immediate application to the problem of identifying a subject by his sperm; but, further research in this area may bring about a day when this will be possible.

MATERIALS AND METHODS

Human Semen

The human semen used in this research was supplied by a local physician. The semen was approximately 32 hours old when taken to the laboratory and the major part of this time was spent under refrigeration. When received, the samples were immediately frozen and maintained in the frozen state until they were used experimentally. A pool of 14 specimens was used, the average age of the donors was 28 years and there was no evidence of pathological abnormalities. Merthiolate 1:10,000 (Sodium ethyl mercury thiosalicylate, E. Lilly and Co.) was added as a preservative. The protein concentration was 2.8 percent found by the biuret test of Meites and Faulkner (1962).

Human Serum

Pooled human serum was obtained from a local hospital laboratory. Merthiolate 1:10,000 was added and the serum frozen until needed.

Cellulose Acetate Electrophoresis

The principle of electrophoresis is that a charged ion or particle will migrate toward the electrode having the opposite charge, when placed in an electric field.

A protein is composed of amino acids, some of which have acidic or basic groups. The protein can therefore have either a positive, negative or neutral charge depending on the pH of the environmental solvent. The isoelectric point (pI) of a protein is the pH at which it is electrically neutral and as the pH is raised the positively charged amino acids are neutralized and the protein becomes more basic. A protein like gamma globulin with a pI of 7.2 is mildly negative when compared to albumin with a pI of 4.7 which is strongly negative when both are dissolved in a buffer of pH 8.6. Albumin will consequently migrate toward the anode at a faster rate than gamma globulin because of its greater negative charge; thus resulting in separation of the two proteins. The excellent text by Smith (1963) gives further information on zone electrophoresis of all types.

The electrophoresis was accomplished using the Shandon Universal Electrophoresis Apparatus (Colab No. 2549) and the Vokam Power Unit (Colab No. 2541). The Oxoid cellulose acetate strips were 2.5 x 12 cm (Colab No. 11-53C) and a prepared barbitone buffer with calcium lactate (Colab No. BR-116L) was used. The buffer was made by dissolving 8.8 gm of the mixture into 1 liter of water, giving a pH of 8.6 and molarity of .06. Human serum, semen, seminal plasma and 3 times washed (phosphate buffered saline pH 7.4) spermatozoa were subjected to electrophoresis.

The procedure was to apply 4-5 lambda of a sample in a streak, on the buffer-impregnated strip, one-third the distance from the cathode. A constant current of 1 ma per strip for 1½ hours at room temperature resulted in adequate protein separation. Ponceau S (.2% in 3% trichloroacidic acid-Colab No. C-14-129) was used for initial staining but was found too insensitive for staining of semen electrophoresis. Therefore, Nigrosin (.001% in 2% acetic acid-Colab No. C-14-127) a more sensitive stain, was employed. The Nigrosin staining was accomplished by first submerging the strips in Ponceau S and then in Nigrosin overnight. All the strips were dried, after destaining, by pressing between paper towels using a heavy object for pressure.

Disc Electrophoresis

The disadvantage of cellulose acetate and other types of zone electrophoresis is that separation occurs only by differences in net charge on the proteins. Ornstein and Davis (1962) introduced a modification by adding separation by molecular size, while at the same time ordinary electrophoresis is being preformed. The process is carried out in a polyacrylamide gel column in a vertical glass tube, with the upper end attached to an upper buffer reservoir containing the cathode. The lower end is submerged in a lower buffer reservoir containing the anode. This column consists of three gel layers; the upper layer of large-pore

gel contains the sample, the next layer, a large-pore spacer gel, causes electrophoretic concentration and separation by mobilities of the sample, and the last layer of small-pore gel is where separation by sieving action of the gel is carried out. This sieving process allows small molecules to move faster, while retaining the larger molecules.

The method used was the same as that of Ornstein and Davis with the only change being the addition of TEMED (N,N,N',N'-tetramethylenediamine) to the large-pore gel at a concentration of 1 lambda per ml to cause faster photopolymerization.

The proteins of human serum, semen, seminal plasma and 3 times washed spermatozoa were separated. The sample gel contained 200 micrograms of protein per tube. The only exception occurred in sperm electrophoresis where about .1 ml of packed sperm was mixed with the sample gel in each tube. A constant current of 2 ma per tube for 1½ hours at room temperature produced protein separation. The stain used was Amidoschwarz and a method suggested by Davis was used for destaining.

Preparation of Antiserums

The antiserums were prepared using the alum precipitation method suggested by Dr. L. C. Ferguson. The rabbit antiserums to both human serum and semen were produced by

the same method. The procedure was to mix 5 ml of the sample with 16 ml of water. Then 18 ml of 10% alum (Potassium aluminum sulphate-KAl $(SO_4)_2 \cdot 12H_2O$) was added and the pH adjusted to 6.5 with 5N NaOH. The sediment, which resulted, was washed 3 times with isotonic saline (Merthiolate 1:10,000) and made up to a final volume of 20 ml with this solution. The alum served as an adjuvant for the immunization of the rabbits.

The immunization procedure was as follows: Dutch black-belted rabbits were used and a control bleeding was obtained prior to the initial injection. Two ml of the alum precipitin was injected intramuscularly once a week for 3 weeks in the thigh muscle of each rabbit. The rabbits were bled by heart puncture on the fourth week. This blood and the control blood were allowed to clot at room temperature for 2 hours and then stored overnight at +10°C. The serum was decanted, centrifuged, merthiolate 1:10,000 added and then frozen until needed.

This author received a sample of antiseminal plasma from Dr. A. J. Weil, but unfortunately this did not perform as well as should be expected. The serum looked as if some contamination may have resulted during transportation.

Antiserums Used in Immunological Analysis

The antiserums employed in the immunological investigations of human semen were as follows:

1. Antiserum produced in rabbits to human serum (abbreviated--AHSr).

2. Antiserum produced in rabbits to human semen (abbreviated--AHSp).

3. AHSp adsorbed with an equal volume of human serum (abbreviated--adsorbed AHSp).

   This was prepared to remove antibodies to human serum present in AHSp.

Immunological Double Diffusion

The basic theory of double diffusion was developed by Ouchtertony (1958). A layer of buffered agar, usually in a petri dish, has a number of holes--called wells--punched into it. The arrangement is most often a series of 4-6 wells in a circle around a center well, much like spokes of a wheel around the hub. The antigen is deposited in the center well and the antibody in the outer wells. This can of course be reversed as the need arises. The essential feature is diffusion of antigen and antibody toward each other in the agar. There will be a meeting of the two diffusion fronts and a zone of optimum proportion will result somewhere in the overlapping area. Antigen-antibody combinations will occur in this zone and a white precipitin line appears in the agar indicating that at least one antigen-antibody system is present. Crowle (1961) gives a complete review of all types of immunodiffusion methods and should be consulted for further details.

The following procedure was developed for double diffusion. Five ml of 1% Oxoid Ionagar No. 2 (Colab No. L12) prepared in a phosphate buffer pH 7.4 ionicity 0.15 (merthiolate 1:10,000) was pipetted onto a 2 x 3 inch glass slide (see appendix for buffer formula). The agar was allowed to gel and then placed in a humid atmosphere for 2 hours to age. The slide was laid on a piece of paper, which had drawn on it the appropriate well pattern, and the wells cut with a 10 mm cork borer. The agar was removed using curved forceps, the wells charged with suitable reactants (see results for samples analyzed), and were left to diffuse and precipitate in a humid atmosphere for 3 days at 25°C. The agar was washed one day in phosphate buffered saline pH 7.4 and another day in distilled water to remove protein not precipitated. A moistened piece of Whatman No. 40 filter paper was laid on the agar and the agar was dried to a film in an incubator. Staining was done with Amido Schwarz (see appendix for formula) and destaining accomplished with 5 percent acetic acid.

Three different well patterns were developed for this technique. First, a six-well pattern, having the antigen-antibody wells 8 mm apart for maximum resolution, was employed for preliminary testing of antiserums. Secondly, a three-well pattern was used for the comparison of 3 antigens to 1 antiserum. Finally, a four-well pattern was used

for the major share of this analysis, where 5 mm separated the wells for increased sensitivity.

Immunoelectrophoresis

The technique of immunoelectrophoresis was developed by a number of workers. Williams (1960) has an excellent article giving the theory of immunoelectrophoresis. Essentially this technique employs two independent criteria to separate proteins; first, segregating the proteins by their electrophoretic mobilities and second, identifying them by the immune reaction. The electrophoresis is done on agar-covered slides. Then antibody troughs are cut into the agar parallel to the direction of the electrophoresis. These troughs are filled with the appropriate antisera which diffuses toward the already separated proteins, resulting in precipitation. Thus, proteins are separated first by their migration rate and second by their immune reaction.

The procedure used for immunoelectrophoresis was that of Hirschfeld (1960). The following modifications were made in his method. The electrophoresis was done on the afore-mentioned Shandon Apparatus with a piece of plastic 6 x 6 inches placed on the two center dividers of the chamber. The agar-covered slides (pH 8.6 barbital buffer) were arranged on the plastic and connection with the buffer tank was prepared with buffer-moistened filter paper wicks

(Fig. 1). The antigen wells were charged (see results for samples analyzed) and a constant current of 3.0 ma per slide (8 v/cm) for 90 minutes at room temperature caused separation of proteins. The antibody troughs were filled with antisera and were left to diffuse and precipitate in a humid atmosphere for 2 days at 25°C. The remainder of the procedure was the same as that for the double diffusion.

Seminal Stains

A major concern of this research was to subject human seminal stains to carefully chosen and controlled experimental conditions and determine whether the stain's proteins remained immunologically reactive. The types of conditions and the reason for each will be found in Table I.

Three different volumes of semen were pipetted on the cloth for each set of conditions. These amounts were .1 ml, .05 ml, and .03 ml. This gradation of semen was to determine any reasonable lower limits, with each set of conditions, for the finding of semen proteins in the stain. The stains were made on 1 cm^2 pieces of knit cotton cloth, dried at room temperature and then subjected to various conditions as described in Table I. Extraction of the proteins was accomplished by submerging the cloth in .2 ml of phosphate buffered saline pH 7.4 (1:10,000 merthiolate) overnight at +10°C.

Fig. 1. Apparatus for Immunoelectrophoresis.

Table I. Experimental Conditions Imposed on Seminal Stains

1. Normal Stain:	Stains were extracted after drying at room temperature to establish the feasibility of extracting the stain's proteins.
2. 42°C Stain:	The dried stains were subjected to a dry heat of 42°C for 30 minutes to determine the effect of subdenaturation temperature on the stain's proteins.
3. 62°C Stain:	The dried stains were subjected to dry heat of 62°C for 30 minutes to demonstrate the effect of slightly supradenaturation temperature on the stain's proteins.
4. 68°C Humidified Stain:	The dried stains were subjected to 68°C for 30 minutes in a humid atmosphere to study the effect of moist heat on the stain's proteins.
5. Washed Stain:	The dried stains were washed vigorously, using a magnetic stirrer, for 5 minutes in soapy tap water and then still moist submerged in the extraction solution. This was performed to determine the effect of washing on the stain's proteins.

The extracts were analyzed using two methods. First, double diffusion tests in duplicate were made on each extract. Second, microscopic examination of each extract was performed to determine the presence or absence of spermatozoa.

RESULTS

Cellulose Acetate Electrophoresis

Electrophoresis of human serum revealed 7 zones of migration (origins indicated by arrow). A pre-albumin, albumin, alpha-1, alpha-2, beta-1, beta-2 and gamma globulin was seen using Ponceau S as a stain (Fig. 2). This technique resulted in 7-8 zones of migration in semen (Fig. 3), using Nigrosin as the stain. These are numbered 1-8 in their order of increasing mobilities. A comparison between known fractions of serum and those of semen shows the following. Two zones of migration (1 and 6) in semen were suggestive of serum albumin and gamma globulin, respectively. There were 1-2 pre-albumins, (7 and 8) in semen, a fraction (5) with an alpha-2 mobility, one fraction (4) with a migration rate of beta globulin, and finally a zone of migration (1) with a post gamma globulin migration rate. The origin (3) was stained.

Electrophoresis of seminal plasma (Fig. 4) produced essentially the same results. The only difference was that the pre-albumin (8) always appeared and the origin (3) did not stain.

Electrophoresis of 3-times washed sperm (Fig. 5) resulted in a stained origin and at least two zones of

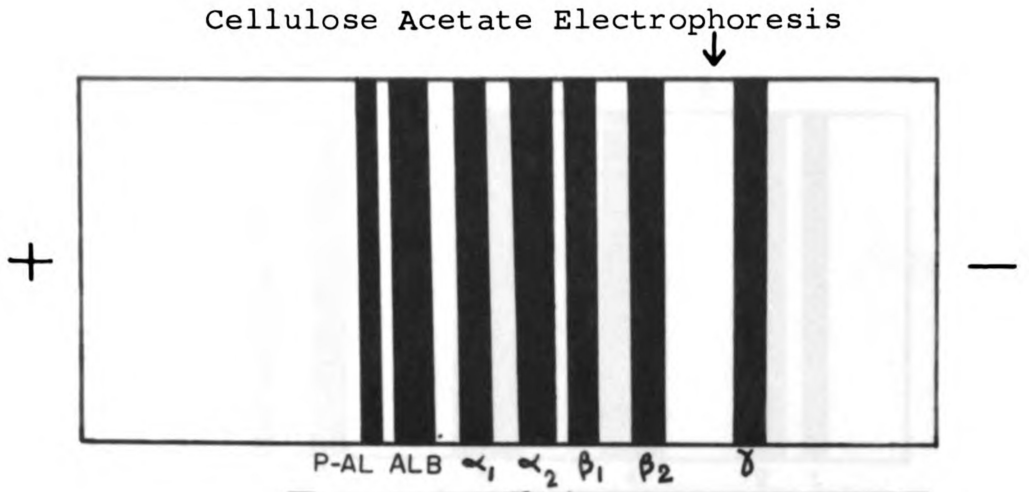


Fig. 2.
Human Serum

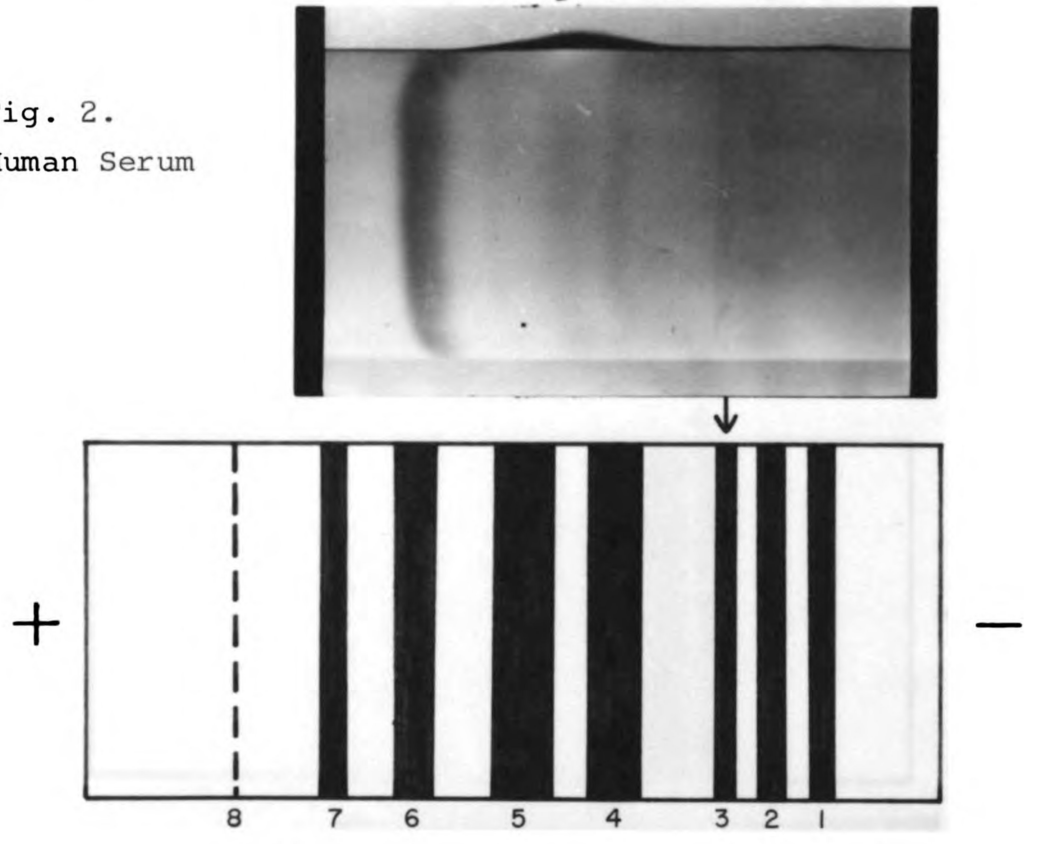


Fig. 3.
Human Semen

Cellulose Acetate Electrophoresis

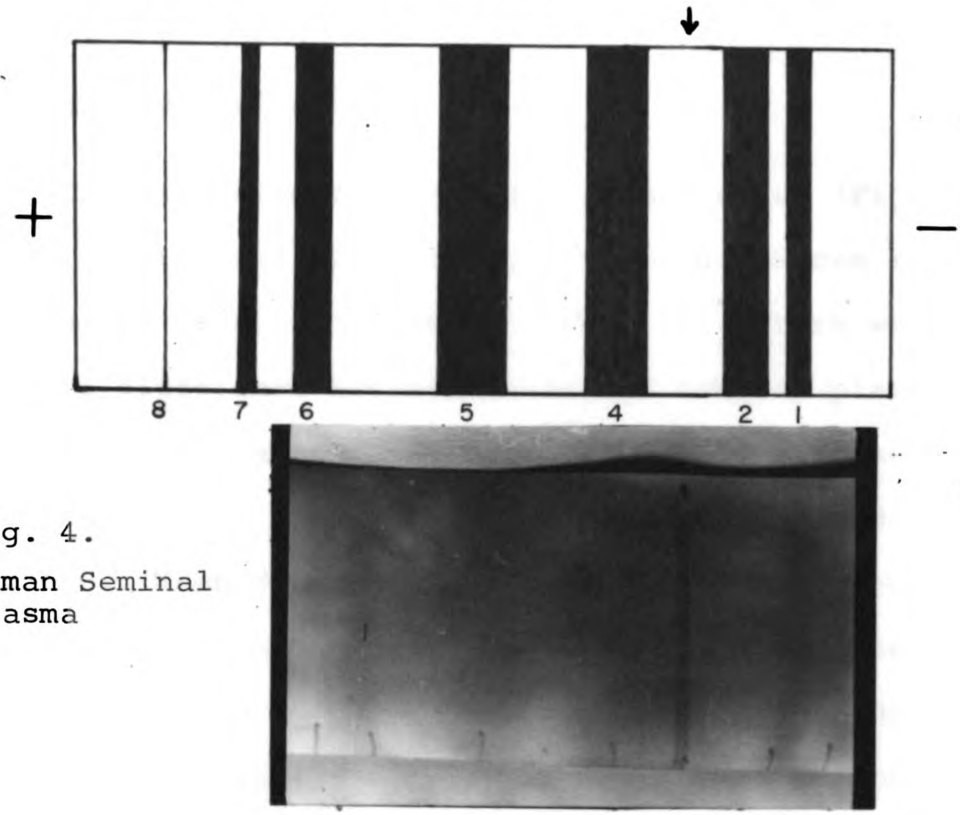


Fig. 4.
Human Seminal Plasma

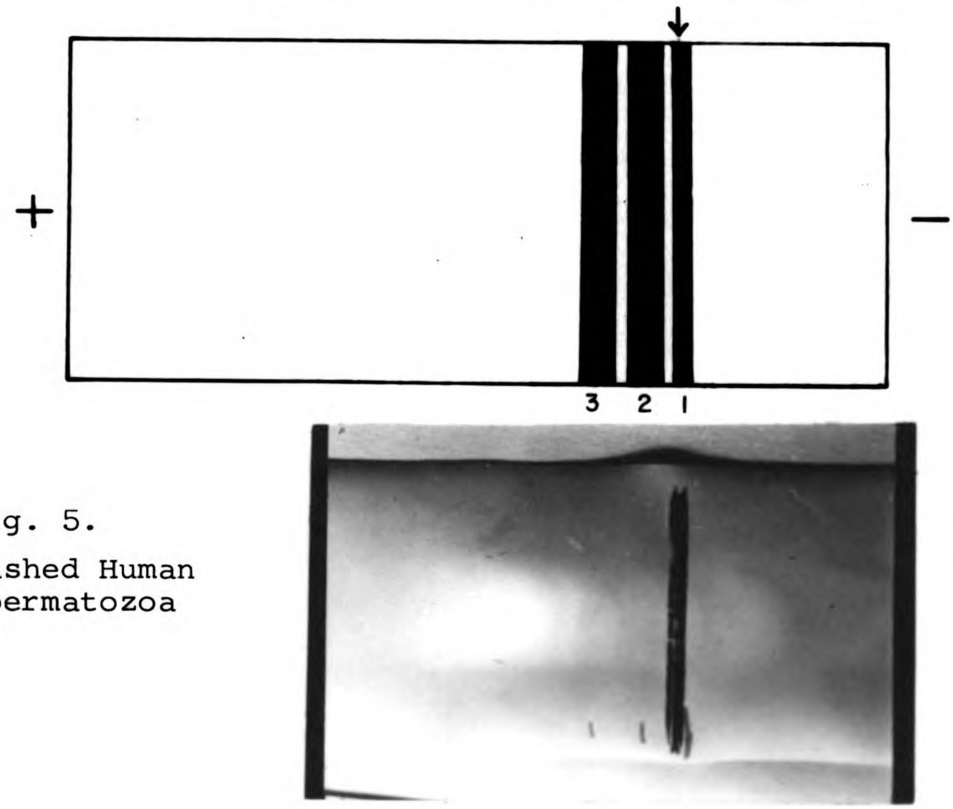


Fig. 5.
Washed Human Spermatozoa

migration in the direction of the anode.

Disc Electrophoresis

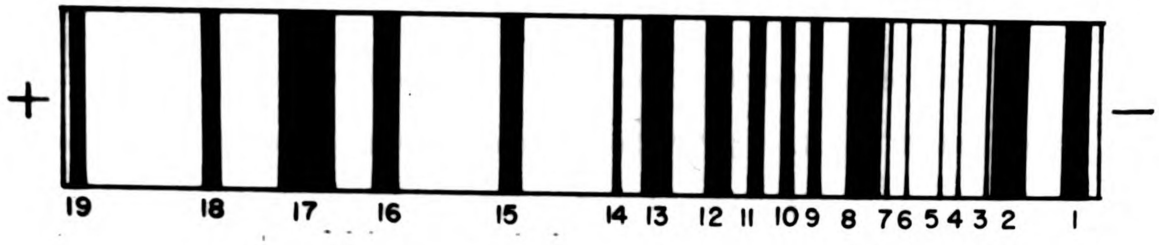
The disc electrophoresis of human serum (Fig. 6) resulted in at least 19 zones of migration. Human semen produced at least 10 fractions (Fig. 7). There was no observable difference between semen and seminal plasma electrophoretic patterns. Fraction No. 10 in semen electrophoresis had the same mobility as serum albumin No. 17 in Figure 6. When 3-times washed human spermatozoa were subjected to disc electrophoresis at least 10 components were observed (Fig. 8). It was impossible to distinguish between semen, seminal plasma and washed sperm with regard to their patterns of protein separation by disc electrophoresis.

Preliminary Testing of Antiserums

Double diffusion analysis of sera from each rabbit immunized to produce AHSp revealed that rabbit number 1 sera produced at least 6 precipitin lines when reacted with human semen (Fig. 11). Therefore, this rabbit serum was used for the remainder of the immunological investigations of human semen. Control serum taken from rabbits prior to immunization proved negative.

Rabbits which produced AHSr showed no significant difference, therefore, pooled serum was used. Again control serum taken from rabbits prior to immunization proved negative.

Disc Electrophoresis



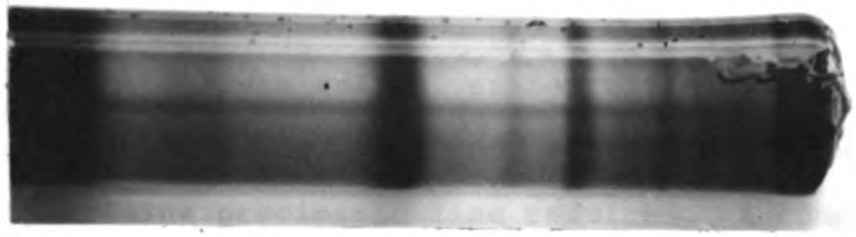


Fig. 6.
Human Serum

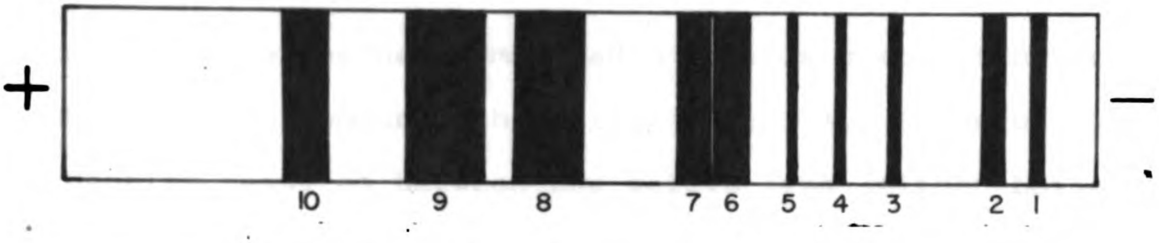


Fig. 7.
Human Semen

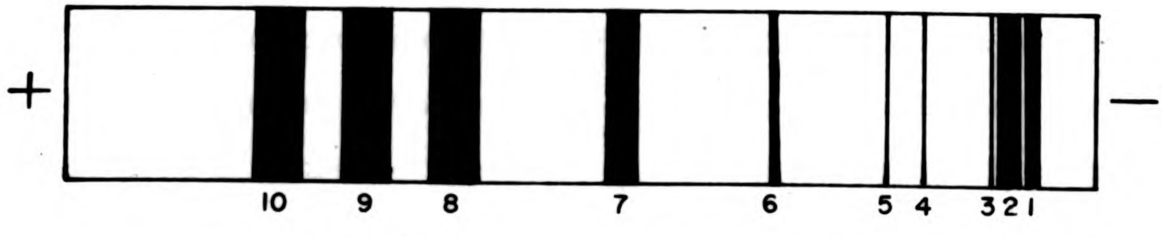


Fig. 8.
Washed Human
Spermatozoa

Immunological Controls

1. When adsorbed AHSp was diffused against human serum no precipitation resulted (Fig. 12). This indicated that the adsorption of antibodies to serum was complete.

2. When pooled human saliva, either boiled (15 min. in a boiling water bath) or untreated, was diffused against adsorbed AHSp a faint precipitin line resulted. A comparison of semen and saliva, with adsorbed AHSp, was undertaken to determine whether semen had a visible precipitin line which was in common with saliva (Fig. 12). No serological identity resulted between the saliva line (either boiled or untreated) and any of the 4 visible semen components. Therefore no further adsorption was considered necessary.

3. Neither human urine nor extracts of cloth used to make seminal stains, when diffused against adsorbed AHSp, resulted in any precipitation.

It is concluded that adsorbed AHSp is organ specific for human semen. The literature indicates this antiserum has a high degree of species specificity. Therefore, it can be stated that with proper controls, adsorbed AHSp is both species and organ specific for human semen.

Double Diffusion of Human Semen

Human semen was analyzed using the four-well pattern and the three basic antiserums (Fig. 13). At least 5

antigenic components could be seen when AHSp was diffused against undiluted semen. Those which were in common with serum were determined by the diffusion of AHSr against the semen and this resulted in at least 4 lines of precipitation. Finally, adsorbed AHSp indicated that at least 4 antigens were specific to human semen. Normal rabbit serum was diffused against the semen as a control.

The semen was diluted 1:10, 1:100, 1:500 and 1:1000 to determine the sensitivity of this procedure and were analyzed the same as above. Table II gives a summary of the results. The patterns of precipitin lines obtained in each of the five tests were similar.

Table II. Double Diffusion Analysis of Human Semen

Dilutions of Human Semen	Number of Precipitation Lines		
	AHSr	AHSp	Adsorbed AHSp
Undiluted	4	5	4
1:10	3	6	4
1:100	2	4	2
1:500	2	1	1
1:1000	2	1	1

Immunoelectrophoresis

Immunoelectrophoresis of human serum using AHSr resulted in precipitin patterns which corresponded to published reports. The immunoelectrophoresis of human semen was accomplished using the three aforementioned

antisera. When AHSp was diffused against the electrophoresed semen at least 10 lines of precipitation resulted (Fig. 9). These were numbered 1-10 in their order of increasing mobilities. AHSr was diffused against the electrophoresis of human semen to determine which of these components were in common with human serum (Fig. 10). This resulted in at least 5 precipitin lines that were given numbers which correspond to similar fractions found in Figure 9. Revealed were components similar to serum albumin (10), gamma globulin (1) and other serum globulins (X, 6, 9). Fraction X did not appear to have a common component in immunoelectrophoresis of semen using AHSp. The analyses of semen using adsorbed AHSp caused at least 5 lines of precipitation. They appeared to correspond to precipitin lines numbered 2, 3 or 4, 5, 7 and 8 in Figure 9, and thus, these were specific to human semen.

Seminal Stains

The extracts of seminal stains were analyzed by employing the methods described in the double diffusion analysis of human semen. The results obtained from the .05 ml stains were typical of those obtained from all stains and therefore are presented in Figures 14, 15, 16, 17 and 18. Results of analysis of all stains are summarized in Table III.

Immunoelectrophoresis

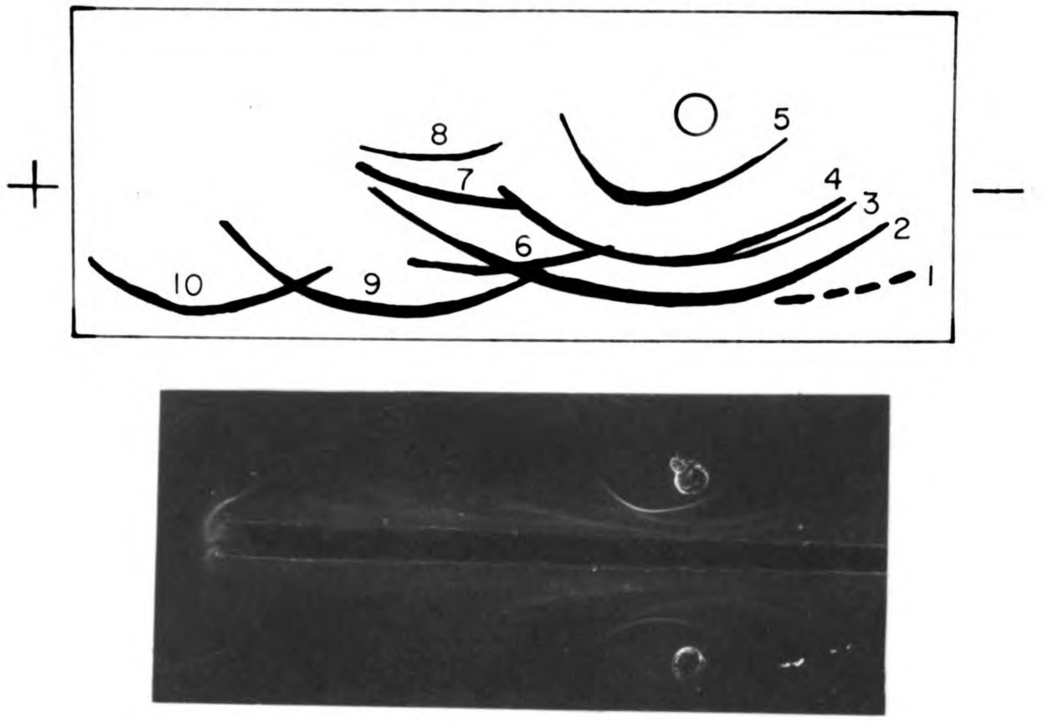


Fig. 9. Human Semen with AHSp.

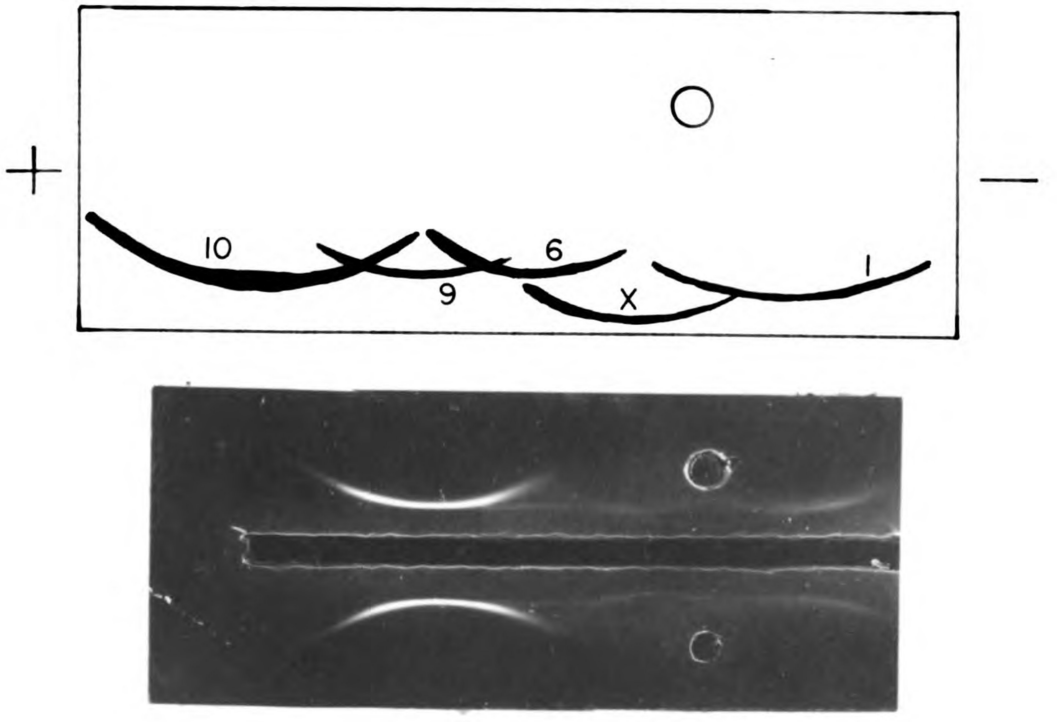


Fig. 10. Human Semen with AHSr.

Fig. 11. Preliminary Test of AHSp

1. Rabbit No. 1
2. Rabbit No. 2
3. Rabbit No. 3
4. Rabbit No. 4
5. Control serum
6. Pool--Rabbits No. 1 and 2

C. Human semen

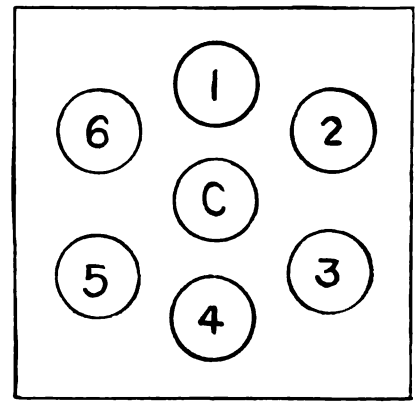


Fig. 12. Immunological Controls

1. Human serum
2. Human semen
3. Human saliva

C. Adsorbed AHSp

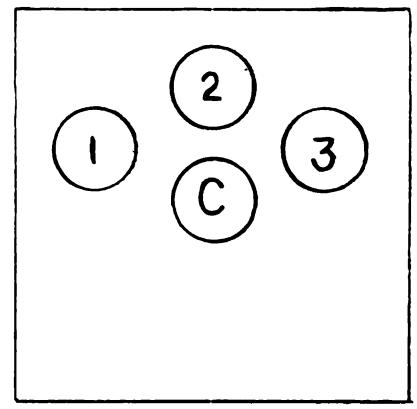
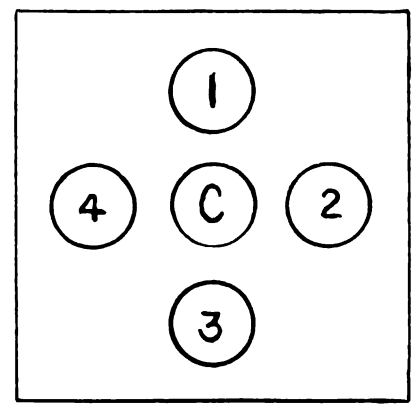


Fig. 13-18. Analysis of Human Semen and Semen Stains

1. AHSr
2. AHSp
3. Adsorbed AHSp
4. Control

C. Fig. 13 - Human semen

Fig. 14-18 - Extracts of different stains



Immunological Double Diffusion

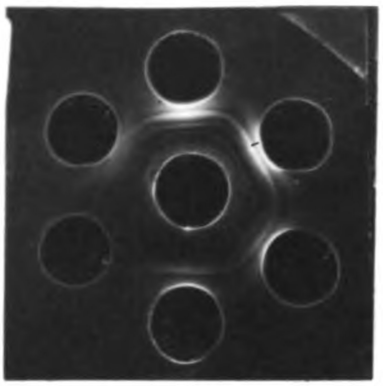


Fig. 11. Preliminary Testing

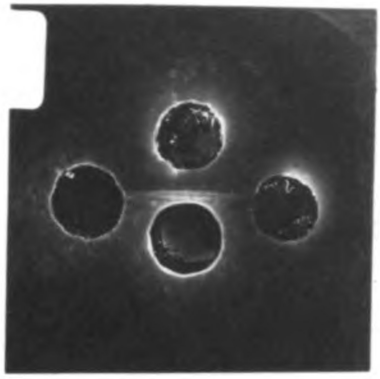


Fig. 12. Controls

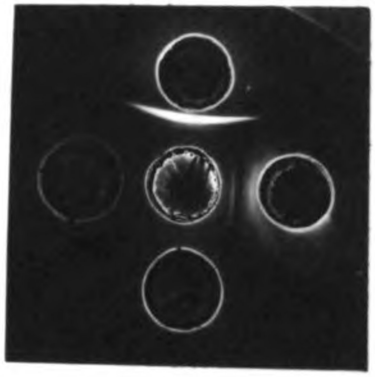


Fig. 13. Human Semen

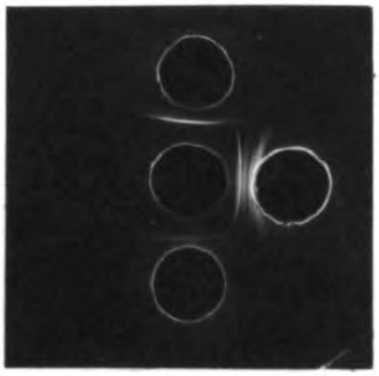


Fig. 14. Normal Stain

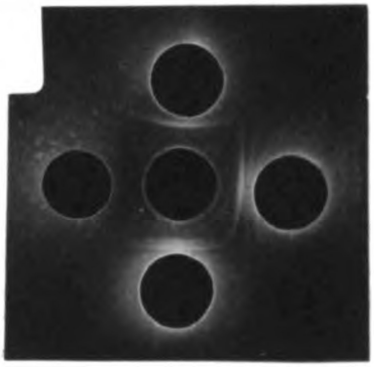


Fig. 15. 42°C Stain

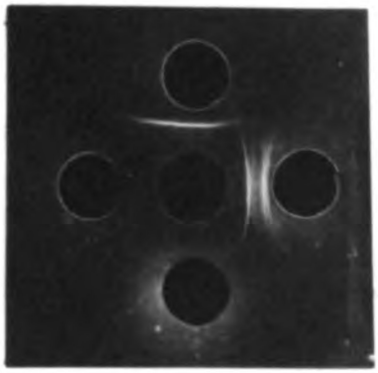


Fig. 16. 62°C Stain

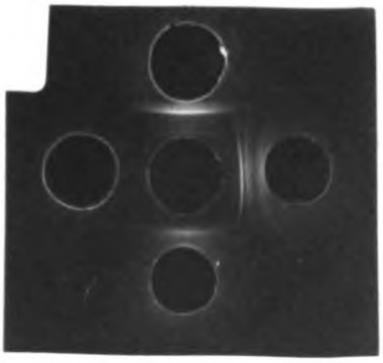


Fig. 17. 68°C Humidified Stain

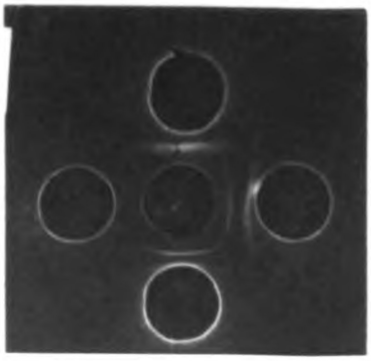


Fig. 18. Washed Stain

Table III. Analysis of Human Seminal Stains

Stain	Number of Precipitation Lines AHSr	AHSp	Adsorbed AHSp	Presence of Sperm
Normal Stain				
.1 ml	2	5	3	yes
.05 ml	3	6	3	yes
.03 ml	2	6	3	yes
42°C Stain				
.1 ml	4	6	2	yes
.05 ml	2	5	2	yes
.03 ml	3	3	2	no
62°C Stain				
.1 ml	3	6	1	yes
.05 ml	1	8	1	yes
.03 ml	1	6	1	no
Humidified Stain				
.01 ml	2	3	3	yes
.05 ml	3	5	3	no
.03 ml	4	6	4	no
Washed Stain				
.1 ml	2	3	3	yes
.05 ml	2	3	3	yes
.03 ml	2	3	3	no

It can be seen that for each experimental condition and semen volume immunological reactions resulted with each of the three antisera, and except for the 62° stain, at least 2 lines of precipitation were always observed. There was never any reaction with the control serum. A similarity of precipitin patterns was seen upon analysis of all the stains. The .05 ml stains illustrate these patterns. Table III shows the great degree of consistency within each experimental condition of the number of precipitin lines obtained by the three antisera. Also there was observed, between the experimental conditions, a similarity in the number of precipitations obtained by the three antiserums. Finally, as shown by Table III, spermatozoa were found in the majority of stain extracts. The only exceptions were stains which had small amounts of semen pipetted on the cloth.

DISCUSSION

The results of the cellulose acetate electrophoresis confirmed, by a different procedure, those obtained by Searcy et al. (1964). It is rather interesting to note that results reported here were found using one-fourth the sample size and employing Nigrosin as a stain. The staining at the origin in both semen and sperm electrophoresis is thought not only to be due to immobile sperm, but also to cellular debris present in the semen. Scarcy et al. postulated that the zones of migration obtained from electrophoresis of washed sperm were due to saline-insoluble components on the surface of the sperm. The possibility also exists that proteins from within the sperm might be involved.

Disc electrophoresis for the analysis of human semen has not, to this author's knowledge, been reported. This is the first attempt to separate, in this way, the proteins of human semen. The ability to increase the resolution of semen and seminal plasma components to at least 10 zones of migration indicates the greater sensitivity of this technique. The results of electrophoresis of washed sperm seem to suggest that what was removed from the sperm is either the same or very similar to components present in

seminal plasma. Stevens and Fost (1964) support this suggestion by showing that the sperm share their antigenic components with seminal plasma. Our results also suggest that the proteins being removed by electrophoresis of sperm could be the spermatozoal coating antigens (SCA) proposed by Weil et al. (1956, 1960a). At the same time the serum proteins found in spermatocele fluid by Weil et al. (1960b) could also be attached to the sperm, thus they too could be removed by electrophoresis. Therefore it is possible that any components found on the sperm would have a common factor present in the seminal plasma. Finally, these serum proteins might be the natural antigenic endowment that Weil (1961) believes are present on the sperm.

The results reported here and those obtained by others do not eliminate the possibility that proteins from within the sperm could somehow contribute to these results. Thus the question that still remains is, just where is the original location of the antigenic components on the sperm? A great deal more research is needed to completely characterize the proteins involved with the human spermatozoa.

The characterization of human semen proteins, using immunological techniques, produced a great deal of information. The possibility arises that there could be present in saliva, trace amounts of a heat-stable protein that is held in common with human semen but absent serum. This factor probably exists in semen only in limited amounts.

This is shown by the comparison of semen and saliva using adsorbed AHSp, in which no visible serological identity resulted between the semen precipitin lines and the one saliva line. Research is needed to fully identify and characterize this saliva factor.

The double diffusion analysis of undiluted semen, using the four well pattern, demonstrated at least 5 antigenic components. This could be increased to 6 components by either diluting the semen 1:10 or by increasing the distance between reacting wells to 8 mm; thereby increasing resolution by either reducing the width of precipitin lines or by causing them to be spread appart. These 5-6 components can be resolved into 4 factors which are common to serum and 4 which are specific to semen by the use of AHSr and adsorbed AHSp respectfully. This also demonstrates that, in a complex antigenic system, the number of precipitin lines only indicates the least number of antigen-antibody systems present. It should be noted that in analysis of 1:10 dilution of semen only 3 components common to serum can be seen. This demonstrates the importance of having enough antigen present to react with its corresponding antibody. Finally, as Table II denotes, precipitation occurred in all dilutions of semen, thus demonstrating test sensitivity of at least 1:1,000 dilutions of semen.

At present the most elegant immunological technique is that of immunoelectrophoresis. The results obtained in

analysis of semen generally agree with reports already published. There is an increase in components resolved by this method over those found by double diffusion. This is an indication of the increased separation of components brought about by electrophoresis prior to immunodiffusion. The majority of the precipitin lines are in the alpha-2 and beta range as first shown by Grant and Everall (1959). The use of AHSr definitely shows fractions indicative of serum albumin and gamma globulin as demonstrated by Hermann (1959) and Kopstock et al. (1963), respectively. There does not seem to be any prealbumin as Hermann (op. cit.) observed. Disc electrophoresis also produced at least 10 fractions. It is impossible to state that these two techniques resolve the same proteins, because two different criteria are being used to develop and characterize them. But it is probable that these two techniques do resolve the same or similar fractions.

Double diffusion was used to investigate the proteins in human seminal stains. The reasons for using this technique were indicated by Muller et al. (1958a) cited in the Review of Literature. It was possible to identify all stains as human semen, no matter what experimental conditions were imposed on them or the amount of semen that was pipetted on the cloth. This was shown by the fact that adsorbed AHSp always produced a precipitin reaction. AHSr and AHSp reacted in all tests, thus demonstrating that

non-organ specific proteins could be found in all extracted stains. These results lead this author to believe that semen amounts lower than .03 ml could be extracted and identified, as this amount always reacted strongly. A reasonable lower limit of semen, .03 ml, that could be found on the stain was established, but the absolute minimum of semen on a stain that could react was not determined. This work extends that of Coombs et al. (1963) by showing the conditions under which the proteins in the seminal stain ". . . remain native and reactive." Finally, similar precipitin patterns and numbers of precipitation lines within and between conditions showed the specificity of the results.

The conditions imposed upon the seminal stains demonstrated that the stains' proteins remain immunologically reactive over a variety of conditions. This is seen by the fact that not only did the normal and 42° C stains retain their reactivity; but stains subjected to temperatures normally in the range of protein denaturation, either dry heat (62° C stains) or moist heat (68° C humidified), also remain immunologically reactive. Finally, washing the stained cloth in soapy water failed to destroy this reactivity of the stains' proteins, thus demonstrating the ability of the proteins to withstand washing and still remain immunologically reactive.

The species specificity of the various methods of testing were not checked. However, Muller et al. (1959), Weil (1961), and Coombs et al. (1963), all indicate a high degree of species specificity for AHSp. The need for species typing would not seem too frequent in human semen stain identification, but if were presented, adequate controls would have to be conducted at that time. Finally, a decision arose of just what animals to test and where to stop testing.

This research demonstrates that, within the conditions imposed on the stains and with the amounts of semen originally present, the seminal proteins will retain their immunological reactivity. Therefore, it is believed that human seminal stain proteins will remain immunologically reactive over a great variety of environmental conditions and semen amounts. This then allows the use of immunological techniques in forensic science for the identification of human seminal stains.

SUMMARY AND CONCLUSIONS

I. The basic research into the immunological and electrophoretic properties of semen produced the following results and conclusions:

1. Electrophoresis of human semen or seminal plasma revealed between 7 and 10 zones of migration, depending on the technique used. There are indications that human semen shares, with serum, common components and at the same time contains its own specific proteins.

2. Electrophoresis of washed human spermatozoa, using cellulose acetate, produced at least 2 zones of migration. When disc electrophoresis was employed the resulting patterm of protein separation could not be distinguished from that of semen or seminal plasma electrophoresis. This is good evidence that proteins being removed from these sperm are either very similar to or identical with the proteins present in the seminal plasma. A great deal more research is needed to fully characterize the proteins involved with human sperm.

3. Immunological properties of human semen were investigated by immunological double diffusion and immunoelectrophoresis. The latter technique demonstrated at least 6 antigenic components in semen. This was resolved

into 4 antigens common with human serum and 4 antigens specific to human semen. This clearly demonstrates that semen is composed of both serum components and components specific to semen. The use of immunoelectrophoresis increased the number of semen components that could be separated to at least 10 and further subdivided them into 5 common to serum and 5 specific to semen. The double diffusion test was sensitive to dilutions of 1:1000 of semen. There is limited evidence to indicate a common substance between semen and saliva.

II. The characterization of proteins in human seminal stains produced the following results and conclusions:

1. The serum adsorbed AHSp was found to be organ specific for human semen and was considered to be species specific as shown by the literature.

2. Human semen in three graded amounts (.1 ml, .05 ml, and .03 ml) was pipetted onto pieces of cloth. They were subjected to various experimental conditions. These conditions were normal stains dried at room temperature, stains subjected to dry heat at 42^{o} C and 62^{o} C for 30 minutes, stains subjected to moist heat at 68^{o} C for 30 minutes and stains washed for 5 minutes in soapy water. It was possible to extract the seminal, proteins and obtain a precipitin reaction with the three different antisera.

3. All stains could be identified as human semen because of their reaction with adsorbed AHSp.

4. This research definitely demonstrates that proteins present in human seminal stains remain immunologically reactive over a great variety of environmental conditions.

5. In conclusion, it is believed that the results presented in this thesis demonstrate that the immunological double diffusion technique, along with proper controls, can be used to identify human seminal stains. A lack of a positive reaction is a good indication that the stain is not human semen.

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APPENDIX

1. Immunodouble Diffusion Buffer

Phosphate, ionicity 0.15,[1] pH 7.4

Na_2HPO_4	12.8	gm.
$NaH_2PO_4 \cdot H_2O$	2.62	gm.
H_2O	1000	ml.

2. Protein Stain

Amidoschwarz

Amidoschwarz	0.5	gm.
12% Acetic Acid	225	ml.
1.6% Sodium Acetate	225	ml.
Glycerol	50	ml.

[1]Ionic strength has been determined by electric conductivity in comparison with isotonic sodium chloride by Crowle (1961).