ABSTRACT

PHYSICOCHEMICAL AND IMMUNOCHEMICAL PROPERTIES OF THE PROTEINS IN BOVINE SEMEN

by Alan G. Hunter

To characterize the protein antigens from bovine sperm and seminal plasma, 39 ejaculates of semen were obtained from 16 Holstein bulls. Sperm were separated from seminal plasma and washed once or five times by centrifugation. Testicular, epididymal, and vas deferens sperm were obtained from slaughterhouse material. Antibodies to seminal preparations and to bovine blood sera were produced in rabbits by intradermal injections with Freund's adjuvant.

Complement fixation and agar-gel-diffusion data indicated that ejaculated sperm, seminal plasma and blood sera contained common antigens such that if antibody was produced against one, it also reacted with the other two. The cross reactive material on sperm was of seminal plasma origin. Antisera prepared against epididymal sperm did not cross react with seminal plasma or blood sera but reacted with ejaculated and epididymal sperm, showing that sperm per se were antigenic. Ejaculated sperm, epididymal sperm, or seminal plasma reacted with antisera to blood sera which was absorbed with blood sera.

by Alan G. Hunter

A hemolytic factor, which limited the use of complement fixation, was present in some samples of seminal plasma. A hemolytic assay was developed and consisted of mixing 0. 2 ml of sheep red blood cells ($10^6/mm^3$) with 0. 2 ml of seminal plasma, incubating at 37^oC for 30 min, storing for 12 hrs at 2^oC and estimating the per cent hemolysis. The hemolytic factor was non-dialyzable, destroyed at 100^oC within 5 min, inhibited by rabbit or bovine blood sera, precipitated with ammonium sulfate, and was as concentrated in the second through fourth ejaculates as in the first.

Agar-gel-diffusion data showed that ejaculated sperm possessed at least seven antigens when reacted with anti-ejaculated sperm immune sera. At least five sperm antigens were shared with seminal plasma and at least one was shared with blood serum. Absorption of anti-ejaculated sperm sera with seminal plasma and blood serum revealed that sperm from the head and body of the epididymis and ejaculated sperm shared at least three antigens which were not of seminal plasma or blood serum origin. The three sperm specific antigens stained as proteins and one of the three as a glycoprotein.

Immuno-electrophoresis revealed that sperm had at least six antigens one of which was heterogeneous, when reacted with anti-ejaculated sperm sera. The same immune sera absorbed with seminal plasma and blood sera revealed three antigens of sperm origin rather than of seminal plasma origin.

by Alan G. Hunter

Saline extraction, freeze-thawing or mechanical rupture with glass beads released the same number of antigens from bovine sperm. Mechanical rupture gave the largest yield of protein. Moving boundary electrophoresis indicated at least three components were in the saline extract of ruptured sperm. Sedimentation velocity ultracentrifugal analysis of the saline extract of ruptured bovine sperm revealed at least three sedimentation gradients. The two major gradients possessed S_{20} values of 1. 7 and 12. 6. Diffusion coefficients of 4. 2 and 10. 2 Ficks were obtained for two of the three sperm specific antigens by an agar-gel-diffusion method.

The three sperm specific antigens in the saline extract of ruptured sperm were precipitated with ammonium sulfate at 33 and at 50% saturation. Fractionation of the saline soluble sperm proteins with DEAE-cellulose chromatography revealed that a protein fraction was not adsorbed on the anion-exchange cellulose equilibrated with 0. 02 M phosphate buffer (pH 7). This protein fraction was tightly bound to CM-cellulose at pH 6. 0 and moved toward the cathode in agar-gel-electrophoresis. The proteins adsorbed on DEAE-cellulose equilibrated with 0. 02 M phosphate buffer (pH 7) were eluted with 0. 175 M to 0. 225 M sodium chloride.

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INTRODUCTION

Ejaculated semen is a combination of the products of the testes, the products of the excurrent ducts, and the secretions of the adnexal glands along the excurrent ducts.

Bovine semen is composed of two parts. The particulate portion includes spermatozoa and various cellular fragments that have been cast off by the spermatozoa in their maturation. The liquid portion is termed seminal plasma. The major sources of secretions that contribute to the fluid volume of bull semen are the seminal vesicles, the prostate gland and the Cowper's gland.

Very little is known about the protein composition and function in bull semen. Animal proteins function physiologically in many diverse ways. All enzymes are protein and many hormones are protein. All antibodies are protein. Actomyosin--the contractile element of muscle, hemoglobin--the respiratory carrier of oxygen, and ceruloplasmin--a carrier of copper in blood plasma, are all proteins. Ferritin--a storage unit for iron, and rhodopsin, which is essential in the visual process, are proteins. Proteins function in hair, hoof and hide in a structural role. Proteins are involved in cellular membranes, and in chromosomes. Proteins have been implicated in the process of fertilization and probably are involved in embryonic mortality.

If more were known about the protein composition of bull sperm, the process of fertilization might be better understood. The origin of the proteins found in seminal plasma and their effect(s) on fertility are poorly understood.

The complementary structure and specificity of antibody make it a very desirable reagent for the study of many of the physiological reactions of sperm proteins. Using specific antibody, one could study sperm or seminal protein from the time of their formation to their eventual intake into the ovum. However, in order to correlate immunochemical reactions with physiological effects, one must identify and isolate the specific protein reactants in sperm or in seminal plasma.

The purpose of this thesis was to isolate and characterize the protein antigens from bull spermatozoa and seminal plasma.

REVIEW OF LITERATURE

Species Specificity and the Cross Relations of the Antigens of Semen

Landsteiner (1899) was the first researcher to report that sperm were antigenic. An in vivo method was used to demonstrate an immune response to sperm. Bull sperm remained active when injected intraperitoneally into non-immunized guinea pigs but rapidly became immotile when injected into the peritoneal cavity of guinea pigs that previously had been injected parenterally with bull sperm.

Metchnikoff (1899, 1900) confirmed that sperm were antigenic by injecting guinea pigs with semen or macerated testes of humans, bulls, guinea pigs and rabbits. The serum of the immunized guinea pigs agglutinated and immobilized the sperm of the above animals. The conclusion was drawn that the antiserum was species specific in its action.

Farnum (1901) injected rabbits intraperitoneally with semen or testicular material of dogs, bulls and men. The sera of the treated animals contained precipitins which were specific for each antigen. Farnum also reported that the amniotic fluid of one rabbit gave the same reaction as her serum.

Guyer (1922) prepared antisperm sera by injecting fowl repeatedly with rabbit sperm. The prepared antisera was toxic to rabbit and guinea pig sperm.

Hektoen and Manly (1923) injected human semen and swine, bovine, and equine seminal fluids into rabbits. Testing the antisera for precipitins, they concluded that species specific and semen specific antibodies had been induced.

Pommerenke (1928) found rabbit and rat sperm cross-reacted when tested with antisera to rat sperm.

Mudd and Mudd (1929) employed electrophoretic and complement-fixation techniques to show that the sperm of man, guinea pig, ram, and bull induced species specific antibodies in rabbits. A degree of cross reaction between bull and ram sperm and their corresponding antisera was also noted.

Henle (1938) contended that species specificity in sperm was of a dominant rather than of an absolute nature. Strong antigenic cross reactions between bull sperm and ram sperm were observed but less reactively occurred between bull sperm and human sperm. Antisera against sperm reacted best with homologous sperm but distinct cross reactions occurred with heterologous sperm. The cross reactions were stronger in closely related species but they also occurred between more distantly related species.

Henle and Henle (1940) injected guinea pigs with bull sperm and observed that only a few antisera also reacted with guinea pig sperm and then only weakly.

Smith (1949) prepared rodent sperm antisera in goats, sheep,

and rabbits. All prepared antisera cross agglutinated strongly the sperm of other rodents, weakly the sperm of ferret and dog, and not at all those of the bull, goat and fowl. After absorption of these antisera with whole sperm of the species against which they had been prepared, homologous reactions were abolished, but heterologous reactions persisted. Absorption with heterologous sperm of any species, prevented agglutination of sperm of that species only. It was concluded that the sperm of rabbit and guinea pig were more closely related to one another than they were to those of the mouse.

Tissue Specificity and the Cross Reactions of the Antigens of Semen

Pfeiffer (1905) reported that rabbits given injections of bull sperm produced antisera which reacted strongly with semen and slightly, if at all, with extracts of other bovine organs. Absorption with organ extract left only precipitins in the anti-sperm sera specific for bull semen.

Strube (1902) and Hektoen and Manly (1923) injected rabbits with human semen and seminal plasma, respectively, and found precipitins for human blood serum as well as for human semen. The latter investigators showed that absorption of the antisera for semen with human blood serum did not eliminate the seminal precipitins. Distinct reactions remained for seminal plasma as well as for human sperm.

Conversely, Uhlenhuth (1904) determined that an antiserum against human blood serum yielded a precipitate with human sperm.

This was confirmed by Hektoen and Manly (1923).

Landsteiner and van der Scheer (1927-1928), after testing anti-sperm sera with sperm, suspensions of thymic tissue, tracheal epithelium and renal tissue, concluded that sperm were tissue specific.

Henle (1938) referring to the above paper wrote that the problem of tissue-specificity of sperm could not be considered settled until a comparison with a greater number of tissues had been made. Using the complement fixation test, he demonstrated that anti-bull sperm sera reacted with bull sperm but not with bull serum. Antisera against bull serum reacted with bull serum but not with bull sperm. Identical results were obtained with human and rat combinations. The lack of cross reaction between blood and sperm was attributed to the method of obtaining the sperm samples such that the sperm suspensions were practically free of blood.

Lewis (1934) could not distinguish testicular tissue from brain tissue when those antigens were reacted with antibrain sera. Antibrain sera reacted with brain and testes but not with lung, liver, heart, spleen, kidney or ovary. Lewis's studies indicated that brain and testes had common antigens and did not exhibit tissue specificity but rather showed a marked selectivity toward each other.

Lewis (1941) expanded his previous study to show that antisera against alcoholic extracts of brain or testes would also cross react with both organs. Both organs had otherwise complete organ specificity

except for cross reactions with corpus luteum.

Common antigenicity between brain and testis also was reported by Freund, Lipton and Thompson (1953) and Katsh and Bishop (1958). These papers will be reviewed later since they relate to a direct effect on the mature germ cell.

Auto- and Iso-immunization with Semen

Guinea Pig

Metalnikoff (1900) was the first investigator to demonstrate that an animal could produce antibodies against sperm of its own species. Metalnikoff (1900), Adler (1909), Savini and Savini-Castano (1911), Kennedy (1924) and Eiseman and Friedman (1929) all were able to induce "spermatoxins" in the blood with guinea pig semen. Kennedy (1924) further concluded that the guinea pig sperm-immobilizing antibodies produced in the male guinea pigs were more potent than those produced in the immunized females. Henle and Henle (1940) obtained an antibody response in 60 to 77 per cent of the female guinea pigs injected with homologous sperm. Katsh (1957) demonstrated uterine anaphylaxis in guinea pigs sensitized to guinea pig sperm when challenged with guinea pig sperm. Further effects of auto-immunization with guinea pig sperm will be discussed in a later section.

Rabbits

Dittler (1920), Guyer (1922), Pommerenke (1928), and Mudd and Mudd (1929) and Henle (1938) reported the production of anti-rabbit "spermatotoxins" in rabbits. Eiseman and Friedman (1929) and Oslund (1926) were not successful in iso-immunizing rabbits with sperm.

Pommerenke (1928) reported that the serum as well as the vaginal secretions of female rabbits were toxic to rabbit sperm. Repeated intravaginal injections of rabbit sperm into female rabbits induced antibody in the blood serum as well as in the vaginal secretions of the rabbits.

Weil and Finkler (1959) reported that rabbit seminal plasma contained at least one iso-antigenically active component. No iso-antigenicity was observed with rabbit sperm under comparable experimental conditions even though the sperm removed homologous antibody to seminal plasma and were agglutinated by isologous anti-seminal plasma sera.

Edwards (1960) found that repeated insemination of pregnant and non-pregnant rabbits with rabbit semen failed to induce any antibodies. However, intramuscular injections of rabbit semen with adjuvant into five rabbits led to sperm agglutinins in the sera of all five rabbits, and to sperm-immobilizing antibodies, a precipitin against seminal plasma, and complement-fixing antibodies against seminal plasma from a vasectomized buck in two of the rabbits.

Rats

McCartney (1923), Fogelson (1926), Wang (1936) and Henle (1938) injected rat sperm into rats and produced antibodies to the rat sperm. Eiseman and Friedman (1929) and Oslund (1926) were unsuccessful in such attempts.

McCartney (1923) induced iso-antibodies to rat sperm in the vaginal and uterine secretions which immobilized and agglutinated rat sperm.

Man

Fogelson (1926) could not show agglutinins, lysins, or toxins in either the sera or cervical secretions of 17 women classified as having idiopathic cases of sterility.

Wilson (1954) detected sperm agglutinins in the seminal plasma and blood serum of two sterile men whose sperm agglutinated after ejaculation. Later, Wilson (1956) reported a third case which agreed with the above findings. Weil, Kotsevalov, and Wilson (1956) tested two specimens of human serum containing the above human sperm agglutinating antibody and found the antibody was not reactive in complement fixation tests with seminal plasma or with sperm, but a zone of precipitation with seminal plasma as antigen was produced in the agar diffusion test.

Rumke and Hellinga (1959) examined the serums of 2,015 male partners of sterile couples for sperm agglutinins. Approximately three per cent of the above population had auto-antibodies to human semen. Using agar gel diffusion methods, these authors found that the lipoproteins of seminal plasma were responsible for the auto-antibody formation.

Birds

McCartney (1923) injected rooster sperm suspensions subcutaneously into egg laying hens. The rate of egg production was not influenced but infertile eggs were laid for 12 to 67 days. Lamoreaux (1940) also injected homologous sperm into laying hens and induced high titers of sperm antibodies.

Cattle

Kiddy, et al. (1959) intravenously injected bull semen into heifers. The sera of the heifers did not contain specific antibodies against washed bull sperm by either agglutination or complement-fixation tests. However, anaphylactic-like reactions were shown after injection, indicating that an immunological response had been obtained. According to Menge, et al. (1962), after the above experiments were reported, agglutinating antibodies against washed sperm were demonstrated in some of those sera by a gelatin-agglutination method.

The Antigenic Composition of Semen

Rabbit

Smith (1949) injected goats with rabbit seminal plasma or rabbit sperm. The induced antisera to seminal plasma reacted with seminal plasma but did not agglutinate rabbit sperm. However, the anti-spermatozoal sera reacted with both sperm and seminal plasma.

Contrary to the above findings, Weil and Finkler (1958) found both guinea pig anti-rabbit seminal plasma and anti-rabbit sperm immune sera strongly agglutinated sperm from fresh semen and also thrice washed rabbit sperm. The antigens of sperm and seminal plasma were so closely related that no differentiation could be made between them. The suggestion was made that the effective antigens found in seminal plasma and in semen originated in the seminal vesicle. Five components were demonstrated in rabbit seminal plasma by agar-gel-diffusion.

Weil and Finkler (1959) reported isologous immune serum to rabbit semen showed only one precipitation band in agar-gel-diffusion. A reaction of identity was formed with the strongest line obtained with heterologous immune serum and was removed by absorption of the immune serum with either seminal plasma or washed sperm.

Weil (1960) concluded that rabbit sperm from the epididymis lacked the antigenic material present on ejaculated spermatozoa. Ejaculated sperm had antigens in common with the seminal plasma.

Complement fixation results provided indirect evidence that antigenic material was taken up by the sperm from seminal plasma.

Although Weil (1960) presented no evidence showing that testicular sperm were antigenic, the data of Henle (1938), Henle et al. (1938), Voisin et al. (1951) and Freund et al. (1955) demonstrated that testicular sperm were not devoid of antigenic material. Pernot (1956) and Pernot et al. (1958) demonstrated that testicular antigenicity was still present in ejaculated sperm.

Using fluorescein-conjugated anti-rabbit seminal plasma immune sera, Weil and Rodenburg (1962) reported that rabbit ejaculated sperm showed a delicate coating with antigen. The coat was lacking in epididymal rabbit sperm. Within the male genital tract of the rabbit, only the seminal vesicle yielded fluorescent staining with anti-sperm coating antigen immune globulin conjugate. The epithelial cells lining the lumen and those of the glands within the muscularis, contained numerous fine fluorescing granules and were therefore believed to be the site of production of the sperm coating antigen.

Guinea Pig

Pernot (1956) reported that guinea pig sperm and seminal plasma had strong and seemingly preponderant antigens in common. Beck, Edwards and Young (1962), utilizing immuno-fluorescence have shown that one of the antigens involved in the production of iso-antibodies in the guinea pig was located on the sperm tail.

Cattle

Henle, Henle and Chamber (1938), specifically absorbed anti-bull sperm immune sera and demonstrated head specific and tail specific antigens. Both antigens were heat labile. A species specific heat stable antigen was found to be common to both heads and tails of bull sperm. Antibodies against the head and tail specific antigens led to two different types of agglutination. Using unabsorbed anti-sperm immune sera allowed fine strings of sperm to form immediately with attachment from head to head or tail to tail. After absorption of the serum with heads, only antibodies against the tails were left and these caused an immediate agglutination of the tails with all the heads at the outside of the agglutinated mass of sperm. When the serum was absorbed with tails, the heads clumped together forming long rows with the tails still vigorously motile. These investigators also found that one of the head antigens was not active in the native cell. It became active only after breaking the cell. Antibodies against this substance were not found in antisera against native bull sperm but were formed when "vibrated" sperm or heads were injected into rabbits.

Pernot and Szumowski (1958) in an immuno-electrophoretic study of bull seminal plasma found several constituents whose mobilities were similar to blood serum constituents but only an albumin and sometimes a beta globulin were antigenically identical with those of serum.

Rao and Sadri (1960), using agar-gel-diffusion found that buffalo

seminal plasma contained 16 antigens and that sperm contained seven, of which four were common to the seminal plasma. Prostatic secretion had four antigens common to semen and blood serum. Seminal vesicle secretion and ampullar secretion had five and three antigens, respectively, in common with semen. Seminal vesicle secretion had two antigens in common with sperm and two in common with ampullar secretion. Six antigens of buffalo blood serum were common to seminal plasma.

Gordon and Hunter (1961) analyzed agar-gel-diffusion data and reported that bull sperm contained at least seven antigens, of which at least five were common to seminal plasma.

Human

Weil, Kotsevalov and Wilson (1956) using complement fixation and agar-gel-diffusion concluded that all of the human sperm antigens were also present in the seminal plasma and that the two materials could not be distinguished immunologically. Azoospermic ejaculates of men contained the full complement of antigen thus suggesting that the human sperm antigens originated in the adnexal glands of the genital tract rather than in the testis.

Weil and Rodenburg (1960) showed that sperm from human spermatoceles lacked the antigenic material present on ejaculated sperm, which these latter cells shared with seminal plasma. This provided

direct evidence that human sperm acquired antigenic materials when they were mixed with the contents of the adnexal glands of the genital tract.

Rao and Sadri (1960), using agar-gel-diffusion, characterized human semen as having at least 16 antigens, of which seven were attributed to sperm and four to prostatic secretion. Seven antigens in human semen were common to blood serum. All four antigens of prostatic secretion were common to blood. Rao, Sheth and Sadri (1961) demonstrated that of the seven human sperm antigens, four were common to seminal plasma and three were specific to sperm.

Blood Groups and Sperm Antigens

Landsteiner and Levine (1926) and Yamakami (1926) reported that sperm of humans of appropriate blood type specifically absorbed antibodies to the A and B antigens of human erythrocytes.

Docton et al. (1952) presented evidence that bovine iso-immune sera to bovine erythrocytes also reacted with bovine sperm. Bovine sperm antibodies produced in sheep lysed the erythrocytes of certain cattle in addition to agglutinating bovine sperm. The authors theorized that antigens recognizable in bovine erythrocytes had similar or identical counterparts in sperm.

Menge et al. (1962) failed to duplicate the Docton et al. work. They reported that antisera to erythrocytes did not possess sperm

agglutinins. Similarly, antisera to bull semen or washed sperm had few or no specific agglutinins against erythrocytes.

Gullbring (1957) claimed serological detection of A antigen on some sperm and B antigen on other sperm from each of several men with AB blood type.

Shahani and Southam (1962) demonstrated the presence of blood group antigen A or B on human sperm by using specific immuno-fluorescent antisera. The evidence indicated that sperm carry a single ABO blood group antigen on their surface.

Naturally Occurring Antigen-Antibody-Like Systems

Natural hetero-agglutinins for the sperm and blood cells of many species of animals were found in the blood serum of lobsters and other invertebrates by Tyler and Metz (1945), Tyler and Scheer (1945) and Tyler (1946). Those studies revealed that the broad range of hetero-agglutinating activity was due to distinct hetero-agglutinins in the sera and each hetero-agglutinin acted on a separate whole class of animals.

Weil and Finkler (1959) found that normal rabbit sera non-specifically fixed complement with rabbit sperm. Edwards (1960) confirmed this non-specificity and found complement was fixed when normal rabbit sera was mixed with rabbit semen, washed or epididymal sperm or the non-dialyzable fraction of seminal plasma obtained from semen. Seminal plasma obtained from a vasectomized buck did not fix complement

with normal serum. Edwards (1960) raised the question of whether the complement fixing activity of normal rabbit serum added to rabbit sperm was due to a natural antibody.

Antagglutins (Lindahl and Killstrom, 1954) were defined as materials that could inhibit the spontaneous agglutination of washed sperm of various animals. In the bull, antagglutin was produced in the prostate gland. Lindahl (1960) reported that antagglutin did not counteract agglutination of sperm by specific antisera. It seemed to operate through another non-serologic type of mechanism.

Lillie (1913) observed, in experiments with sea urchins, that the sea water from above a suspension of the unfertilized eggs was capable of agglutinating the sperm of the homologous species. He termed the agglutinating substance, fertilizin. Tyler (1961) in a review paper reported that fertilizin made up practically the entire gelatinous coat of the egg, that the egg surface layer of the egg proper also contained it, that it was a glyco-protein of about 300, 000 molecular weight, and may occur in an agglutinating or non-agglutinating form. The complementing receptor substance on the surface of the sperm, with which fertilizin combined in the agglutination reaction, has been termed antifertilizin.

A theory has been devised by Tyler (1961) to account for fertilization. The primary points of the theory were that specific adherence of egg and sperm occurred by virtue of binding by their respective receptor substances, fertilizin and antifertilizin, and that penetration

of the sperm into the egg was a pinocytotic engulfment of the sperm by the egg.

Semen Antibodies and Male Infertility

De Leslie (1901) rendered male mice sterile for intervals of 16 to 20 days by injections of anti-sperm serum obtained in guinea pigs.

Guyer (1922) prepared anti-rabbit sperm immune sera in fowl. Intravenous injection of the anti-sperm sera at four to five week intervals into three male rabbits, induced partial or complete sterility. In one rabbit, sterility was partial or temporary, in another, it was complete without evidence of testicular injury, and in the third, marked degeneration of the testes was found.

McCartney (1923) injected rat sperm into male rats and noted a tendency toward destruction of the sperm and a temporary atrophy of the testes.

Kennedy (1924) reported that male guinea pigs were sterilized by injections of guinea pig sperm. Degenerative changes occurred in testicles of some of the injected males. Autologous injections were most effective in inducing sterility in the male.

Oslund (1926) injected male rats and rabbits with homologous sperm and found a variable degree of degeneration of the testes in three rats and in neither of the two rabbits used. Although temporary aspermatogenesis was found in some cases, the conclusion was made that it was not the result of specific antibody build up.

Quick (1926) injected homologous sperm or testicular homogenate into rats and interpreted the degenerative changes he found in the testicles as due to cyst formation in the epididymides and/or displacement of the testes from their normal scrotal environment.

Voisin, Delaunay, and Barber (1951) reported testicular damage in guinea pigs injected with homologous testicular homogenate in Freund adjuvant. They attributed the spermatogenic lesion to a nonspecific stress factor.

Freund, et al. (1953) demonstrated that autologous or homologous testicular material injected with adjuvant induced selective destruction of the spermatogenic tissue in guinea pigs. They later (1954) reported impairment of spermatogenesis in rats injected with homologous testes plus adjuvant.

Freund, Thompson and Lipton (1955) found that heterologous testicular material or sperm from bull, rabbit, hamster or sheep did not induce aspermatogenesis in the guinea pig, even though adjuvants were used.

Voisin and Delaunay (1955) extended and confirmed their previous findings that aspermatogenesis could be induced, but now concluded that the aspermatogenesis was due to an immunologic mechanism.

Katsh and Bishop (1958) and Katsh (1958a, b, c, 1959a, b, 1960) confirmed the results of Freund et al. (1953) and further demonstrated that guinea pig brain in adjuvant as well as human semen also induced

some testicular destruction in the guinea pig. Where spermatogenic tissue destruction was obtained by immunologic means, no impairment of the ability of the testes to secrete androgens was seen since the accessory organs were completely unaffected. The destruction of the spermatogenic tissue was not due to the injection of androgen present in the homogenate since injections of sperm produced the same results and injections of aspermatogenic testes (harvested from guinea pigs previously sensitized with sperm) did not affect the spermatogenic tissue in the recipient animals' testicles.

Katsh (1960) in summarizing his previous work, reported that serological studies revealed complement-fixing, agglutinating, precipitating, and sperm-immobilizing antibodies were found in the sera of animals with testicular lesions, but the titers bore no relationship to the degree of germinal damage. Moreover, high antibody titers were found in the sera of rabbits injected with homologous testes or sperm but, no spermatogenic tissue destruction was observed. Using the Schultz-Dale test (for anaphylaxis in vitro) and employing the ileum as the indicator organ, Katsh (1958a, b, c, 1959b, 1960) found the strength and duration of contraction of the isolated ileum of the sensitized animal was a reliable indicator of the degree of testicular damage. This demonstrated that fixed or tissue antibodies were involved rather than circulating antibodies in the testicular destruction.

Katsh (1960) proposed the following scheme to account for the mechanism of destruction of spermatogenic tissue. The antigen-hapten complex is ingested by macrophagic cells at the site of injection and transported to all organs of the body. In those organs that contain antibody-forming cells (e. g., spleen, lymph nodes, Peyer's Patches of the ileum) the arrival of antigen induced antibody production in that organ. Upon excision of the ileum, for example, and challenging the ileum with a provoking dose of antigen in vitro, antigen interaction with the antibody containing cells in that organ caused the release of a factor which stimulated the ileum to contract in anaphylaxis. However, in the testis, the arrival of antigen permitted interaction with antibody-containing cells in that organ and its lymph nodes. The testicle does not contain a large amount of lymphoid tissue and, at first the damage would be rather small. However, as a result of cytolysis of some of the cells, more antigen was released and, thus the animal perpetuated the attack on the testicle by releasing more antigen to react with more antibody. The reaction could go to completion, with the elimination of all spermatogenic tissue excepting the spermatogonia. In time, after all antigen had been eliminated from the testicles, and after anti-spermatogenic antibody had subsided, regeneration of the spermatogenic tissue could occur.

Baum et al. (1961) injected guinea pigs intradermally with homologous sperm or testis in Freund's adjuvant and developed the following

manifestations of hypersensitivity. Histamine was released from the lung after treatment with antigen in vitro. Schultz-Dale reactions occurred occasionally. Antibodies capable of passive sensitization in vitro and antibodies capable of immobilizing sperm in presence of complement were found. Skin reactions with an immediate and a delayed component were also observed. Although living sperm acted as an effective antigen, its activity increased after freezing and thawing. The antigen in sperm was stable at 56°C but appreciable inactivation, increasing with time, occurred at 100°C. Fixation of antibody on the acrosomal portion of sperm was demonstrated by means of fluorescein tagged anti-globulin serum.

Bishop, Narbaitz and Lessof (1961) demonstrated that neonatal guinea pigs injected with homologous testicular homogenate combined with adjuvant underwent aspermatogenesis after they reached maturity. Sensitization thus occurred early and there was an early release of circulating antibody. Seminiferous differentiation and spermatogenesis were not inhibited or prevented but testicular destruction followed later. Histologically normal testes, removed from sensitized animals, induced aspermatogenesis and the release of circulating antibody in mature recipients, but damaged testes from sensitized animals failed to do so. No granulomatous lesions or perivascular leucocytic infiltration characterized the early stages of germinal destruction. One of the first abnormal findings was the filling of the epididymal ducts with cellular

debris sloughed from the germinal epithelium. The antigenic material was limited to the adult testis, containing spermatids and spermatozoa which gave the staining reactions of polysaccharides. Neonatal testes failed to induce aspermatogenesis when injected into adult guinea pigs.

Semen Antibodies and Female Infertility

Darwin's (1871) statement that "the diminution of fertility may be explained in some cases by the profligacy of the women" might be interpreted today to mean a sensitization against the male reproductive products.

Savini and Savini-Castano (1911) attempted to induce sterility in female rabbits and guinea pigs with sperm injections. Fifty-seven rabbits were injected five to six times at weekly intervals subcutaneously or intraperitoneally with sperm and one week later were allowed an opportunity to mate for ten to fifteen days with males. No young were obtained from that exposure but many fertile matings resulted from subsequent copulations.

Venema (1916) reported two experiments in which sterility resulted in female rabbits that were injected with a testis saline suspension. Dittler (1920) injected female rabbits with semen twice at four-day intervals and found an induced sterility which lasted only a few weeks.

In 1921 an editorial in The Journal of the American Medical Association (1921) asked, "If spermatozoa invade the female tissues and

cause formation of specific antibodies which are capable of preventing fertilization, may not such a process participate in the problem of sterility? May not the traditional sterility of the prostitute depend sometimes on such a process . . . ."

McCartney (1923a, b) injected rat sperm or testis extract into female rats and observed that sterility of two to twenty-seven weeks duration resulted. When pregnancy occurred, the experimental rats had smaller litters than the controls. Upon injection of pregnant rats with sperm, five out of thirteen animals aborted. The infertility was presumably due to the presence of spermatoxins in the vaginal and uterine secretions, since these fluids immobilized and agglutinated sperm.

Kennedy (1924) reported female guinea pigs could be sterilized by injection of guinea pig sperm.

Pommerenke (1928) after intravenously injecting rabbit sperm or testes into female rabbits found that the longevity of sperm deposited in the genital tract during mating was shortened. Infertility for six to twenty-five weeks was induced as the result of such injections. He was unable to obtain conclusive evidence that sterility was induced by intravaginal injections of sperm although circulating antibodies to sperm were demonstrated.

Fogelson (1926) demonstrated that injections of rat, guinea pig or human sperm into female rats resulted in sterility of from six to

twenty-nine weeks duration.

Guyer and Clause (1933) reported that seven injections of bull epididymis containing sperm into twenty female rats at five day intervals retarded fertility decidedly in most of the females. The litters of those that resumed fertility were smaller. Injections of washed ejaculated bull sperm and testicular nucleoproteins also retarded fertility in rats.

Baskin (1937) was awarded U. S. Patent Number 2, 103, 240 for a nonspecific spermatoxic vaccine. The object of the invention was "the production from material obtained from the lower animals, of a determinant for human sperm and semen and usable as a vaccine or antigen in vaccination of human female to produce spermatoxic conditions in her blood and secretion."

Eastman, Guttmacher, and Stewart (1939) reported that dog, horse and ram testicular material was not effective in altering fertility in the female rat. Rat sperm injections given to female rats reduced the number of females which became pregnant. However, they concluded that sperm injection reduced fertility only slightly.

Parsons and Hyde (1940) from their research concluded that there was no evidence that pregnancy was prevented or delayed by treating rats or rabbits with sperm. However, the intravaginal injection of immune sera to rabbit sperm temporarily arrested fertility in rabbits.

Henle et al. (1940) failed to induce temporary sterility in mice by passive immunization with spermatozoal antisera. Henle and Henle

(1940) injected female guinea pigs intraperitoneally with homologous sperm and obtained an antibody response but observed no decrease in fertility.

Brunner (1941) immunized female rabbits with a bull sperm phospholipid suspended in sheep serum and found no effect on fertility but a small percentage of the offspring showed hermaphroditism.

Katsh (1958b, c, 1960) injected female guinea pigs with homologous and heterologous testicular homogenate or sperm in saline with incomplete adjuvant or with complete adjuvant. Each of these treatments induced a sensitization, as indicated by the anaphylactic contraction of the isolated ileum and uterus, which lasted only to about the fortieth day after injection.

Katsh (1959a, 1960) showed that a large proportion of female guinea pigs were rendered infertile by homologous testicular homogenate or sperm in adjuvant. Isojima, Graham and Graham (1959) confirmed this work.

Kiddy et al. (1958) found that fertility was not affected in heifers given intravenous injections of blood from a bull to which they were later bred. Nor did the above authors (1959a) find that fertility was affected by intravenous injections of bull semen or by intra-uterine injections of bull blood prior to insemination. The sera of the heifers immunized with semen did not show antibodies against washed sperm by either agglutination tests or complement-fixation tests. However,

anaphylactic-like reactions were observed after injection with semen, indicating that an immunological response had been induced.

Kiddy, Stone, and Casida (1959b) also demonstrated that the treatment of washed rabbit sperm with antibodies produced against them, had anti-fertility effect. A significant increase in fertilization failure and in embryonic death resulted when semen used in artificial insemination was treated with antibodies produced in cattle against pooled rabbit semen. The average embryo survival percentage for 58 females inseminated with immune sera treated semen was 38. 2 compared to 53. 1 for 59 females inseminated with normal bovine serum treated semen. The loss of whole litters was much more common in the immune sera group (23 females out of 58) than it was in the normal sera group (3 females out of 59).

Menge et al. (1962a) produced immune sera against bull semen in cattle and against washed bull sperm in rabbits. An anti-fertility effect (fertilization failure or possibly early embryonic death) was observed upon treating the bull semen with the antisera prior to insemination of heifers. Absorption of the rabbit anti-bull sperm sera and the cattle anti-rabbit semen sera with erythrocytes of bulls and male rabbits, respectively, failed to remove the sperm agglutinins or the anti-fertility effect.

Fragmentation of Sperm

As a preliminary to protein analysis of the sperm, one must separate the sperm from seminal plasma. Centrifugation is the most popular method but according to Mann (1954) it may produce an injury to the sperm cell which, even if not apparent upon ordinary microscopic examination, nevertheless may result in a leakage of certain proteins from the sperm into the surrounding medium. Washing of the sperm may cause a loss of lipoprotein from the "lipid capsule" around the sperm cell. For that reason, Mann (1954) concluded that one must view with suspicion the results of protein analyses of sperm.

Miescher (1896) severed fish sperm heads from the tails by centrifuging in distilled water. The heads being heavier, broke off and settled to the bottom while the tails and midpieces were plasmolyzed and remained in the supernatant fluid.

Mammalian spermatozoa, in contrast to those of fish, can not be plasmolyzed and their heads do not come off in water or acid (Mann, 1954).

Henle et al. (1938) made use of a magnetostriction oscillator to fragment bull sperm into heads and tails. Examination of the fragmented sperm with specific antibody to whole bull sperm revealed a marked increase in titer as compared with that found with whole sperm. The increased titer was apparently due to some change in the heads since neither tails nor supernatant fluid gave such an increase. A new antigen

was believed to have been unmasked on the surface of the head during the sonic treatment.

Henle et al. (1938) also reported that other mechanical procedures such as washing the sperm several times, vigorous shaking, or grinding, also increased the titer of the heterologous antiserum. In such suspensions, a considerable number of tail-less heads were observed.

Zittle and O'Dell (1941) exposed bull epididymal sperm to ultrasonic waves and separated heads from middle pieces and tails. Slow centrifugation of the disintegrated sperm suspension resulted in heads settling out first. The middle pieces formed a pellet at faster speeds and most of the fragmented tails were found in the supernatant fluid.

Mann (1954) reported disruption of ram sperm by shaking them with fine glass beads in a mechanical disintegrator of the Mickle type. Such treatment led to fragmentation of the middle pieces and tails, but not of the heads. Centrifugation yielded a yellow colored, opalescent fluid which was thought to represent the sperm cytoplasm.

Protein Studies on Seminal Plasma

Human

Goldblatt (1935) reported that human seminal plasma contained proteoses, considerable amounts of albumin, a small quantity of nucleoprotein and traces of globulin and mucin. No peptones, protamine or histone were found. Trichloroacetic acid failed to precipitate a large

proportion of the protein like material in the seminal plasma.

Huggins, Scott and Heinen (1942) and Gray and Huggins (1942) found from a trace up to 18 per cent of human seminal plasma proteins were heat-coagulable at pH 7 and at pH 5. 5. Approximately 60 per cent of the proteins were of a proteose nature and were dialyzable. Electrophoretically, the undialyzable proteins migrated similar to the alpha, beta and gamma globulins and the albumin of human blood serum.

Ross, Moore and Miller (1942) and Ross (1946) concluded that although human seminal plasma contained proteoses, blood globulins and glycoproteins in quantity, there was very little serum albumin. Ross (1946) calculated that the glycoprotein component had a sedimentation velocity of 7 (S_{20}, w units) while the low molecular weight proteins did not sediment.

Using gel electrophoresis, Hermann et al. (1958) detected ten fractions in human seminal plasma. Hermann (1959) reported the immuno-electrophoretic separation of eight fractions in fresh seminal plasma and of ten fractions after allowing the seminal plasma to stand for 32 to 48 hours. Components were observed with mobilities corresponding to serum pre-albumin, albumin, and to alpha, beta and gamma globulin.

Rabock (1961) usually detected six components in seminal plasma by paper electrophoresis. On the basis of comparison of electrophoretic diagrams of normal ejaculates with ejaculates from men with obstructive

azoospermia, the view was expressed that the testes do not distinctly influence the qualitative or quantitative protein spectrum in human seminal plasma.

Cattle

Larson and Salisbury (1954) found that non-dialyzable protein accounted for approximately 90 per cent of the total nitrogen present in bovine seminal plasma. Seventy-six per cent of these proteins were heat coagulable. Seminal plasma proteins, after exhaustive dialysis against water, yielded an extremely low test for carbohydrate and fat. The per cent nitrogen on a fat and moisture free basis was 14.4. Tiselius type electrophoresis revealed the presence of at least eleven components. However, three components accounted for the majority of the proteins. Comparative electrophoretic analyses of bovine blood serum and seminal plasma indicated little relationship between the relative proportions of the proteins in the two systems. The majority of the protein constituents of seminal plasma exhibited electrophoretic mobilities similar to those of the alpha globulins of blood.

Larson, Gray and Salisbury (1954) performed ultracentrifugal analyses on the proteins of bovine seminal plasma. At least five components were found. The majority of the proteins appeared in three components having $S_{20, w}$ values of about three, five and seven.

Szumowski (1956) distinguished seven components in bovine seminal plasma by paper electrophoresis. Szumowski (1959) estimated,

by agar-gel-electrophoresis of bull seminal plasma, that the average relative concentration of albumin was about 2. 7 per cent while the alpha globulin accounted for about 74 per cent of the total protein.

Vesselinovitch (1958) used starch-gel-electrophoresis to demonstrate 16 seminal plasma proteins. No qualitative relationships were found between seminal plasma and blood serum proteins.

Discussions of the enzymes found in seminal plasma and/or in sperm may be found in the reviews by Mann (1954) and Salisbury and Vandemark (1961).

Protein Studies on Sperm

Keratin-like Protein

Miescher (1878) reported that a sulfur rich substance containing more than four per cent sulfur was present in the heads of bull sperm.

Anatomical data which indicated that human sperm heads possessed an extraordinary degree of elasticity was reported by Moench (1929). He was able to hook the head of a sperm with a microsurgical needle and stretch it considerably.

Green (1940) reported that the heads of ram sperm were encased by a membrane, a part of which defied digestion with trypsin or pepsin and was resistant to 0. 01 N alkali and acid treatments. The sperm membrane contained 19. 3 per cent nitrogen and was high in cystine, histidine and arginine but lacked lysine. Green hypothesized that the membrane

around the sperm was a keratin-like protein.

Zittle and O'Dell (1941) partially solubilized the sperm membrane of bull sperm with thioglycolic acid and completely solubilized it in four per cent trimethylbenzlammonium hydroxide. The conclusion was that the membrane was composed of a keratin-like protein.

Thomas and Mayer (1949) treated boar sperm with 1 N sodium hydroxide and found the portion of apparently undissolved sperm consisted of "ghost" sperm heads. The shapes of these resembled the sperm membranes.

Histone-like Protein

Miescher (1897) was the first to report on the chemistry of the sperm nucleus. He was able to extract nucleoprotamine and nucleohistone from fish sperm with distilled water or dilute salt solutions. Attempts at extraction of the same material from mammalian sperm failed.

Dallam and Thomas (1953) were not able to extract nucleoprotamine or nucleohistone with water or neutral salt solutions from the sperm of the bull, man, dog, boar or ram. However, after homogenization, a histone-like protein was extracted from the sperm heads with 1 N sodium hydroxide. The histone-like protein precipitated from the sodium hydroxide solution when diluted with four volumes of water. Once the histone-like protein was precipitated from solution, it would

not redissolve in a 1 N sodium hydroxide solution. Chemical analyses showed 20. 4 per cent nitrogen and 27. 8 per cent arginine. The precipitated protein was found to contain nucleic acid.

Berry and Mayer (1960) isolated a basic protein from bovine sperm by homogenizing at high speeds with microglass beads, extracting with dilute acid and raising the alkalinity to pH 11 which caused precipitation of the protein. Chromatographic and electrophoretic studies confirmed the histone-like character of the basic proteins of both mature and immature sperm.

Lipoprotein

Dallam and Thomas (1953) isolated a lipoprotein complex from bull, boar, ram, dog and human sperm. It was extracted with 0. 1 N sodium hydroxide, precipitated with five per cent acetic acid and redissolved and reprecipitated in the same way three times. Electrophoresis of the lipoprotein of bull sperm revealed only one component if the nucleic acid soluble at pH 7 was removed before extraction of the lipoprotein. The total lipid of the bull sperm head (6. 5 per cent) was accounted for, within experimental error, by the lipid content (31. 1 per cent) of the lipoprotein. The latter comprised 19. 6 per cent of the whole sperm head. Immunological tests supported the belief that the lipoprotein was on the surface of the sperm head since antiserum, specific for the lipoprotein, agglutinated intact sperm heads. Microscopic appearance of the sperm heads

after removal of the lipoprotein indicated that it formed a membrane-like structure around the head.

Berry and Mayer (1957, 1959) isolated a fraction from lyophilized bull sperm which was soluble in 0.01 N sodium hydroxide and precipitated with acetic acid (pH 6). About 42 per cent of the dry weight of bull sperm was constituted by this protein. It was shown to be a lipoprotein complex containing phospholipid, protein, cholesterol, glucosamine, and glucuronic acid.

MATERIALS AND METHODS

Antigen Preparation

Bovine Semen

Thirty-nine first ejaculates of semen were collected from 16 Holstein bulls in routine service at Michigan Artificial Breeder's Cooperative. Two or three false mounts were imposed during a period of sexual preparation previous to each ejaculation. Seminal volume was recorded and the concentration and motility of the sperm were determined for each ejaculate. Samples of the whole semen were stored at -20°C until used and the remainder was used immediately to obtain the seminal plasma and ejaculated sperm as described below.

Seminal Plasma

Fresh semen was centrifuged within 5 min of collection at 6,620 X g for 5 min. The supernatant fluid was removed and centrifuged at 10,300 X g for 5 min. A sample of the seminal plasma was frozen until used and the remainder was lyophilized.

Ejaculated Sperm

The sperm separated from the seminal plasma above were suspended in 0.005 M phosphate buffered saline (pH 7.4) and washed once or five times. The washed sperm were diluted to 100 X 10^7 sperm/ml in phosphate buffered saline if they were to be frozen or in distilled

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water equal to the original volume of semen if they were to be lyophilized. Centrifugations after each wash were at 10, 300 X g for a 5 min interval.

Ejaculated Sperm Heads and Tails

One hundred and forty-four billion sperm from 22 ml of bull semen were washed five times with distilled water. Centrifugation after each wash was run at 7, 716 X g for 10 min. The final sperm precipitate was suspended in 25 ml of distilled water and homogenized for 5 min with four teaspoons of size 100 Scotchlite Superbrite glass beads in a Servall Omnimixer at a rheostat setting of 70 with the container submerged in an ice water bath. The sample was observed with a microscope for breakage of the tails from the heads of the sperm. The heads were precipitated by centrifuging at 276 X g for 10 min. The supernatant fluid containing the tails was removed and centrifuged at 7, 716 X g for 10 min to precipitate the tails. The tails were resuspended in the supernatant fluid to contain 20 X 10^7 tails/ml and stored at -20°C. The remaining tail supernatant fluid contained protein and was lyophilized.

The sperm heads that formed a pellet at 276 X g were washed three times with three 25-ml changes of distilled water, centrifuging at 300 X g each time. The final precipitate was resuspended in barbital sodium chloride buffer (pH 7. 4), adjusted to contain 20 X 10^7 heads/ml by centrifugation and stored at -20°C.

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Ejaculated Soluble Sperm Proteins

One g of lyophilized sperm that had been washed five times was suspended in 40 ml of phosphate buffered saline (pH 7.4). Three teaspoons of Scotchlite Superbrite glass beads, size 100 (Minnesota Mining and Manufacturing Co.), were added and the mixture was homogenized for 15 min at a rheostat setting of 80 in a Servall Omnimixer with the container submerged in an ice water bath. The homogenate was allowed to extract at 4°C for 24 hr. It was then centrifuged at 10, 300 X g for 10 min. The supernatant fluid was removed and Biuret protein content was determined according to the method of Gornall et al. (1949). The supernatant fluid was adjusted to 2% protein by dialyzing the protein inside a cellulose tubing against 20% polyethylene glycol in 0.85% saline.

Ejaculated Sperm Lipoprotein Complex

The sperm residue which remained after extraction of the soluble proteins from 2 g of lyophilized sperm was washed five times with distilled water. The fifth wash was free from chloride ions when tested with 0.5 M silver nitrate. The washed sperm residue was suspended in 40 ml of 0.01 N sodium hydroxide with a Teflon and glass tissue homogenizer. The suspension was stirred for 40 min and then centrifuged at 10, 300 X g for 15 min. The supernatant fluid was pipetted off and the pH was lowered to 5.8 by addition of a 5% solution of glacial acetic acid. The lipoprotein precipitate which formed was removed by centrifugation at 10, 300 X g, washed twice with distilled water and stored at -20°C until used.

Testicular Sperm

Bull testes were obtained from the abattoir. Approximately four testes were cut into sections and the inner yellowish tissue scraped away from the tunica albuginea and the mediastinum testes. The yellow tissue was stored at -20°C until used. The liquid contents of the beaker were poured off after thawing. The testicular material was placed in a Waring Blendor with 100 ml of phosphate buffered saline (pH 7.4) and blended at the low speed setting for 1 min. The mash was poured into a beaker and allowed to extract at 4°C overnight. Due to the thickness of the mash, 130 ml of it was diluted with 130 ml of phosphate buffered saline before it was centrifuged at 10,300 X g for 10 min. The precipitate was resuspended in phosphate buffered saline and was filtered twice through four layers of cheesecloth. The material passing through was centrifuged at 10,300 X g for 12 min. The precipitate was resuspended in phosphate buffered saline and the sperm concentration determined. The concentration was adjusted to 4×10^7 sperm/ml and stored at -20°C until used.

Epididymal Sperm

Bull epididymides were obtained from the abattoir and the head and body of the epididymis was separated from the tail portion and stored at -20°C until used. Approximately ten epididymides were thawed and diced into sections about one cm thick. These were placed in a Waring Blendor with 100 ml of barbital sodium chloride buffer (pH 7.4) and were

blended for 2 min at the high speed setting. The mash was strained through two layers of cheesecloth twice to remove large particles and finally through six layers of cheesecloth. The remaining suspension was made to 50 ml and washed four times with phosphate buffered saline (pH 7.4). Centrifugations between washes were at 10,300 X g for 10 min intervals. The final suspension was diluted to contain 8×10^7 sperm/ml and stored at $-20^{\circ}C$ until used.

Vas Deferens Sperm

Bull reproductive tracts were obtained from the abattoir. Approximately a 6 in length of vas deferens was removed close to the tail of the epididymis. Each 6 in. length of vas deferens was flushed with 1 ml of 0.85% saline. The material flushed from twelve vas deferens were combined and adjusted to contain 8×10^7 sperm/ml. It was stored at -20° C.

Blood Sera

Blood was collected from six bulls by vena puncture from the jugular vein and allowed to clot at room temperature. The clot was loosened from the sides of the beaker and allowed to retract overnight at $5^{\circ}C$ to express the serum. The serum was removed with a pipette and centrifuged at 276 X g to remove any residual red blood cells. The serum was stored at $5^{\circ}C$ until used.

Antisera Preparation

Rabbit Anti-bovine Ejaculated Sperm

A stock solution of five times washed bull sperm was thawed. It contained 125 X 10^7 sperm/ml and was centrifuged at 10, 300 X g for 10 min. The precipitate was resuspended in the supernatant fluid such that approximately 519 X 10^7 sperm were contained per milliliter. An emulsion consisting of 0. 5 ml of this sperm suspension and 0. 5 ml of Freund's complete adjuvant (Bacto-Adjuvant, Difco Labs.) was injected intradermally into five sites in the scapular region of each rabbit. One wk later, each rabbit was injected in the same manner but with an emulsion consisting of 0. 5 ml of the sperm suspension and 0. 5 ml of Freund's incomplete adjuvant. Two wk after the last injections, each rabbit was bled by cardiac puncture. The blood was allowed to clot. The serum was pipetted off, centrifuged at 275 X g to remove residual red blood cells and stored at -20°C until used.

Four rabbits were immunized with bull ejaculated sperm. Freund's complete adjuvant consisted of a mixture of 1. 5 ml Arlacel A (mannide mono-oleate), 8. 5 ml Bayol F (paraffin oil) and five mg of Mycobacterium butyricum. Incomplete adjuvant did not contain the bacteria.

Rabbit Anti-bovine Seminal Plasma

A stock solution of bovine seminal plasma was thawed and adjusted to 2% Biuret protein with phosphate buffered saline. An emulsion consisting

of 0. 5 ml of the 2% protein seminal plasma and 0. 5 ml of Freund's complete adjuvant was injected intradermally into five sites in the scapular region of each rabbit. One wk later, each rabbit was injected in the same manner, but with an emulsion of 0. 5 ml seminal plasma and 0. 5 Freund's incomplete adjuvant. Two wk after the last injection, each rabbit was bled by cardiac puncture. The blood was allowed to clot and the serum was pipetted off, centrifuged at 276 X g to remove red blood cells and stored at -20°C until used.

Four rabbits were immunized with bull seminal plasma.

Rabbit Anti-bovine Blood Sera

A stock solution of bovine blood serum was thawed and adjusted to 2% Biuret protein with phosphate buffered saline. Four rabbits were immunized similar to the procedure described under the preparation of anti-seminal plasma.

Rabbit Anti-bovine Semen

A stock solution of bull semen was thawed. An emulsion of the undiluted semen and Freund's adjuvant was made and used as described for the preparation of anti-seminal plasma. Four rabbits were immunized with bull semen.

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Rabbit Anti-bovine Epididymal Sperm

A stock solution of bull epididymal sperm was thawed. The suspension was centrifuged at 10, 300 X g for 10 min and the precipitate was resuspended in a half volume of supernatant fluid. The sperm concentration was then 16 X 10^7 sperm/ml. An emulsion of the epididymal sperm and Freund's adjuvant was made and used as described previously for the preparation of antisera to bovine ejaculated sperm. Three rabbits were immunized with bovine epididymal sperm.

Hemolytic Activity of Bovine Seminal Plasma

Preparation of Bovine Seminal Plasma

Seminal plasma used for this study was prepared as described previously except that it was never lyophilized and usually it was used without being frozen. In addition, four bulls were ejaculated four times each within a period of 1 hr to evaluate the effect of repetitive ejaculation on the hemolytic activity of the seminal plasma.

Preparation of Sheep Red Blood Cells

Alsever's solution was prepared by dissolving 24. 60 g glucose, 9. 60 g sodium citrate (dihydrate) and 5. 04 g sodium chloride in 1, 200 ml of distilled water. The pH was adjusted to 6. 1 with citric acid and the solution was sterilized by passing it through an ultrafine sintered glass filter. Sheep blood was aseptically drawn into an equal volume of

the sterile Alsever's solution and allowed to age at 4°C for 1 wk prior to use.

Hemolytic Assay

Washed sheep red blood cells were adjusted to 10^6 cells/mm^3 with barbital-sodium chloride buffer. The hemolytic activity of bovine seminal plasma was assayed by mixing 0. 2 ml of the adjusted sheep red blood cells with 0. 2 ml of the sample to be tested. The mixture was incubated in a water bath at 37°C for 30 min. It was then stored for 12 hr at 2°C to facilitate estimation of the degree of hemolysis that had taken place.

Dialysis Treatment

Pooled samples of proven hemolytic bovine seminal plasma were placed in cellulose tubings with pore sizes large enough to allow compounds smaller than 10, 000 molecular weight to escape. The samples were dialyzed against barbital-sodium chloride buffer (pH 7. 4) for 24 hr before being tested for hemolytic activity.

Heat Treatment

Pooled samples of proven hemolytic bovine seminal plasma were heated at 56°C for 30 min, 65°C for 15 min or at 100°C for 5 min. Unheated and heated samples were tested for hemolytic activity.

Bovine Seminal Vesicle and Bulbo-urethra Preparation

The seminal vesicles and bulbo-urethral glands were taken from a bull at slaughter. The fluid was manually expressed from the seminal vesicle and stored at -20°C until used. The glandular portions of the seminal vesicle and bulbo-urethra were diced separately with scissors and placed in a Waring Blendor with one volume of 0.85% saline. Each sample was blended for 5 min at the high setting.

The blended samples were centrifuged at 10,300 X g for 10 min and the supernatant fluids were tested for hemolytic activity.

Bovine Blood Sera

The preparation of bull blood sera was described earlier. Serial dilutions of blood sera were added to bovine seminal plasma in a 1:1 proportion. The solutions were then tested for hemolytic activity.

Ammonium Sulfate Fractionation of Hemolytic Seminal Plasma

Salting out of the proteins in hemolytic samples of bovine seminal plasma with ammonium sulfate was performed at 25, 33, 50, and 100% saturation. All precipitates were resolubilized in 0.85% saline and tested for hemolytic activity.

In Vitro Reactions of Antisera and Antigens

Complement Fixation

The complement fixation technique was used to detect antigenic activity. The complement fixation test is based upon the fact that when an antigen reacts with its antibody, a blood component called complement is fixed by the antigen-antibody complex and can no longer react with other antigen-antibody systems. As an indicator of the presence and relative amount of uncombined (unfixed) complement, erythrocytes sensitized with antibodies to erythrocytes are used. Lysis of the erythrocytes constitutes evidence of unfixed complement; conversely lack of hemolysis indicates that complement has completely reacted with the test antigen and antibody.

Equal volumes (0. 2 ml) of dilutions of antigen, antiserum and complement were mixed and incubated at 37°C for 40 min. Then, 0.2 ml of sensitized sheep red blood cells (1×10^4 cells/mm^3) was added and the tubes were shaken and incubated at 37°C for 30 min. Two 100% units of guinea pig complement and four units of sheep erythrocyte hemolysin were always employed. The tubes were stored at 5°C overnight before the per cent hemolysis was estimated. Antisera were always heated at 56°C for 30 min to inactivate inherent complement. The degree of lysis in a tube was estimated visually as 0, 25, 50, 75, or 100%.

The appendix contains the directions for determining the titer of

complement, for sensitization of sheep red blood cells, and for preparing the barbital-sodium chloride buffer (pH 7.4) used as a diluent in the complement fixation test. The various controls which were simultaneously performed are also described in the appendix.

Agar-gel-diffusion

The double diffusion plate technique of Ouchterlony (1958) was used to observe the antigenic spectrum of bovine semen. The test is based upon the fact that when an antigenic solution is placed in a well in an agar containing dish and antibody to that antigen in another well, the two components diffuse outward and at some point in the agar, they react to form an antigen-antibody complex which precipitates as a band in the agar. The number of lines or bands is probably equivalent to the minimum number of antigen-antibody systems present. According to Korngold and Van Leeuwen (1957) lines formed with the same antiserum and different antigens which merge in the plates indicate identity of antigens or very close relationships. The plates for this technique were prepared as follows:

1. Eight strips of Whatman No. 1 filter paper (1 5/8 in X 1/2 in) were folded so that one end was lying flat in the bottom of the male half of a 90 mm petri dish and the other end hung over the side. The eight strips were arranged evenly around the periphery of the petri dish. A 26 ga stainless steel wire, 24 in long, was placed inside the male half

of the petri dish to hold the paper strips against the sides and the bottom.

2. Thirty ml of melted 0.85% Oxoid Ionagar No. 2 (Consolidated Labs.) dissolved in 0.005 M phosphate buffered 0.85% saline (pH 7.4) was poured into each plate. Merthiolate (0.01%) was incorporated into the above agar to retard bacterial growth.

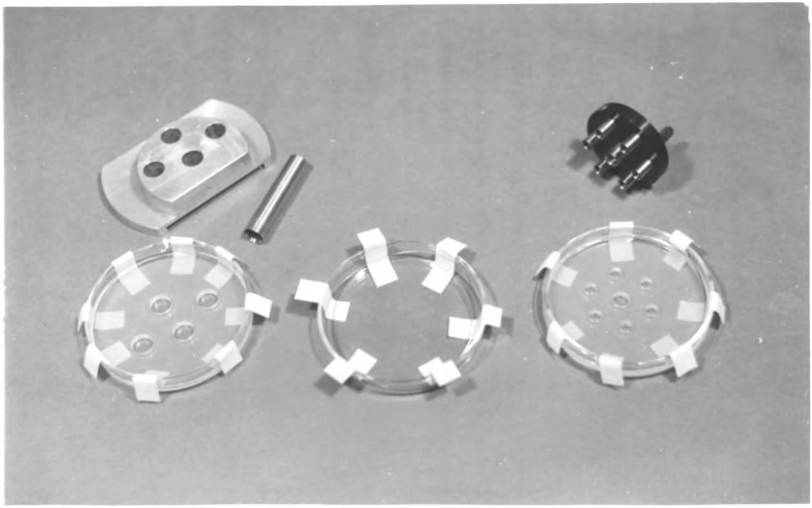
3. Upon solidification of the agar, wells were cut in an "H" pattern (Fox, 1959) with a template and tube cutter. Seven-well patterns were cut with a Feinberg No. 1801 agar cutter (Consolidated Labs.). The cut-out pieces of agar were removed with a needle. A few drops of agar were added to each well to seal the bottom. Figure 1 presents the component parts used in the agar-gel-diffusion method.

4. The wells were filled with appropriate antigens and antisera and the plates were stored at 4°C for 30 days before being discontinued.

5. The plates were photographed against a dark background with the angularly transmitted light to record the progress of precipitin line formation.

6. The intact agar layer was removed from discontinued plates and washed with 0.85% saline for 1 wk. The agar layer was then placed on a 3 1/4 X 4 in glass slide. Excess agar was trimmed from the periphery and the remaining agar sheet was placed upside down on two sheets of Whatman No. 1 filter paper to dry overnight. The filter paper was removed from the dried agar film with water and the film was blotted dry.

Fig. 1 Agar-gel-diffusion Materials. Left side of diagram contains template and tube cutter for diamond or "H" pattern. Right side of diagram contains Feinberg No. 1801 agar cutter and 7 hole pattern. Center of diagram contains petri dish with paper wicks and wire support embedded in agar.



7. The dried plates were stained for protein with Amido Black 10 B, for glycoprotein with alpha-naphthol-paraphenylenediamine, or were double stained for protein and lipid with Azocarmine B and Sudan Black B (Crowle, 1961). The appendix contains the details on each staining procedure.

The Bjorklund (1952) modification of Ouchterlony's double diffusion technique was also used. Using this method, specific absorption was performed by adding inhibiting antigen to the antiserum well 24 hr before the well was to be filled with antiserum. The other wells were filled at the time the antiserum was added. Theoretically, the combination of antigen and antisera in the well should result in a precipitate in the well, allowing only unprecipitated antibodies to diffuse from the well and react with antigen diffusing from another well.

Absorption was also performed in test tubes. Undiluted antisera were added to varying concentrations of antigen. The mixture was incubated at 37^{o}C for 30 min. This absorbed antisera was added directly to the wells in the double diffusion plates.

The precipitin reactions of bull sperm were also analyzed by the diffusion-in-gel method of Allison and Humphrey (1960). Various concentrations of bull sperm proteins were placed in a narrow trough cut in agar at a right angle to a trough containing antisera to ejaculated bull sperm. The angle formed by the radially precipitating complex of antigen and antibody was measured by projecting a photograph of a plate

on graph paper and the tangent of the angle was calculated. Allison and Humphrey demonstrated that the relationship of the tangent of the angle to the diffusion coefficients of the reactants was as follows:

$$\tan \theta = (Dg/Db)^{1/2},$$

where Dg = the diffusion coefficient of the antigen,

and Db = the diffusion coefficient of the antibody.

Immuno-electrophoresis

The immuno-electrophoresis technique of Wieme (1961) was used to give increased resolution of the antigenic spectrum of bull semen. This technique involved an electrophoretic separation of proteins on the basis of mobility differences with subsequent double diffusion of antigen and antibody in agar to form precipitin arcs. The purpose of this immuno-electrophoresis was to spatially separate the antigens before reacting them with antibody. The plates for this technique were prepared as follows:

1. A level horizontal agar base was made by pouring 0.85% Oxoid Ionagar No. 2 (Consolidated Labs.) dissolved in sodium barbital buffer (pH 8.4, μ = 0.05) into a pan. Upon solidification of the agar, microscope slides (75 X 50 X 1 mm or 75 X 25 X 1 mm) were placed on the agar base and covered with the previously described agar to a thickness of 2 mm. After gelation of the second layer, the slides were placed in a moist container and stored at 4°C until used.

2. The electrophoresis tank was filled with identical agar to that used for the slides. The melted agar was poured into the two lateral chambers, each consisting of two compartments, until agar reacted a level above the opening connecting both compartments. Upon gelation, the two inner compartments were completely filled with agar such that the agar layer formed a meniscus at the inner edge. The filled tank was stored at $4^{\circ}C$ after gelation. Before using, half the agar layer in the outer two compartments was removed so that the two platinum electrodes were free. Barbital buffer (pH 8.4, $\mu = 0.1$) was placed around the electrodes and petroleum ether (b.p. 25° - $70^{\circ}C$) was placed in the center part of the tank.

3. In preparation for electrophoresis, the agar covered slides were cut from their storage container. A four mm slit was made in the agar approximately at the center of the plate. The slit was dried by inserting a rectangular piece of filter paper into it. The volume of liquid to be removed from the slit was subjectively regulated by the length of the filter paper. The material to be electrophoresed was immediately placed in the slit with a ten-lambda pipette so that the slit would not refill with buffer.

4. The agar covered slide was then placed between the two agar compartments of the electrophoresis tank under the petroleum ether. The slide was inverted so that agar on the slide was in contact with agar in the tank. An electrical potential of 150 volts was applied for 25 min.

The amperage with the smaller slides never exceeded 40 ma while 80 ma was the maximum with the larger slides.

5. After electrophoresis the slides were treated in one of two ways. They were either fixed immediately in a mixture of 70 ml ethyl alcohol, 5 ml acetic acid and 25 ml distilled water or were prepared for immuno-diffusion. For immuno-diffusion, troughs were cut at right angles to the sample slit. The troughs were 60 X 5 mm and were located approximately 7 mm from the sample slit. The bottom of each trough was sealed with a few drops of agar. The troughs were filled with antisera and precipitin arcs were developed at $4^{o}C$ in a moist chamber.

6. Maximum arc development occurred by 14 days. The plates were photographed, washed, dried and stained as previously described for Ouchterlony plates.

Extraction of Soluble Proteins from Bovine Sperm

Experiments were carried out to determine the optimum method of obtaining saline soluble proteins from bull spermatozoa.

A stock solution of five times washed bull spermatozoa (50×10^{7} cells/ml) in 0.005 M phosphate buffered saline (pH 7.4) was thawed and used throughout the following experiments. Protein values were determined by the Folin-Ciocalteu reaction method of Sutherland et al. (1949).

Freeze-Thawing

The above stock sperm suspension was thawed and 7. 5 ml was placed in a 6 in test tube. A sample of 1. 5 ml was removed for time 0, the pretreatment control. The test tube with the remaining 6 ml of sperm suspension was immersed in a dry ice alcohol bath (-79°C) for 3 min and then thawed in 30°C water. This freeze-thawing was repeated eight times. A 1. 5 ml sample was removed for the time 0, post treatment control. Three other tubes were each filled with 1. 5 ml of the freeze thawed sperm and allowed to extract at 4°C for one, two, or three days. At the end of each time period, the sperm sample was centrifuged at 10, 300 X g for 15 min and the supernatant fluid removed and frozen for later protein determination and analysis by agar-gel-diffusion.

Saline Extraction

Four tubes were filled with 1. 5 ml amounts of the stock sperm suspension. The supernatant fluid was removed from one tube by centrifugation at 10, 300 X g for 15 min. This was the 0 time control. The other three tubes were placed at 4°C for one, two, or three days after which the supernatant fluids were removed by centrifugation at 10, 300 X g for 15 min. All supernatant fluids were frozen for later protein determination and analysis by agar-gel-diffusion.

Mechanical Rupture

The micro attachment (0 M - 2, 000) of a Servall Omnimixer was filled with 3 ml of the stock bull sperm suspension and 2 cc of size 100, Scotchlite Superbrite glass beads (Minnesota Mining and Manufacturing Co.). The mixture was homogenized in an ice water bath for 15 min at a rheostat setting of 80. The material was removed and placed in a test tube. The micro attachment was refilled with the stock suspension of sperm and glass beads and homogenized as before. The two homogenized samples were pooled and four, 1.5 ml sub-samples were made. The supernatant fluid was removed from one sub-sample by centrifugation for the 0 time post treatment control. The other three sub-samples were placed at $4^{\circ}C$ for one, two, or three days, after which the supernatant fluids were removed by centrifugation at 10, 300 X g for 15 min. All supernatant fluids were frozen for later protein determination and analysis by agar-gel-diffusion.

Physical Chemical Characterization of the Soluble Proteins of Bull Sperm

Electrophoresis

Soluble bull sperm proteins and a sperm lipoprotein whose isolation from the sperm cells was described previously were analyzed by moving boundary electrophoresis in an American Instrument Company portable electrophoresis apparatus. This instrument was equipped with

a cylindrical lens and rotating slit. All runs were made in barbital buffer (pH 8.6, $\mu = 0.1$) at 1.5°C. The amperage was maintained at 15 ma. All protein solutions were dialyzed against the barbital buffer for 48 hr at 4°C before electrophoresis. The electrophoretic pattern was usually photographed at 30 min intervals. The proteins were usually electrophoresed in the 6 ml cell for 120 min but occasionally the 3 ml cell was used.

The conductivity of the protein and buffer was determined with a standard conductivity bridge and conductivity cell.

After a run was completed, the Polaroid negatives were placed in an enlarger and enlarged 3.39 times. The distance that a component traveled from a fixed landmark was recorded and corrected for the initial boundary displacement from that landmark. After making enlarging corrections, the distance the component had travelled in a specified period of time was plotted on graph paper. Usually four points were used to plot and verify the slope of the straight line. The slope of the straight line was the velocity in mm/min. The mobility of a protein component was computed from the following formula:

$$\mu = \frac{(v)(q)(c)}{(I)(R)},$$

where v = velocity in cm/sec,

q = cross sectional area of cell in cm^2,

c = conductivity cell constant

I = amperage in amps,

R = resistance in ohms,

and μ = mobility in 1×10^{-5} cm^2 $volt^{-1}$ sec^{-1}.

Ultracentrifugation

The soluble bull sperm proteins whose isolation from the sperm cell was described previously, were analyzed by ultracentrifugation in a Spinco Model E ultracentrifuge, with a synthetic boundary cell. The sperm soluble protein solution was dialyzed against 0.02 M sodium phosphate buffer (pH 7) for 24 hr at $4^{\circ}C$ prior to ultracentrifugation.

The ultracentrifuge was operated at the top speed of 59,780 rev/min at $20^{\circ}C$. Photographic plates of the schlieron patterns were used to record the distance each boundary moved with time. The angle of the slit was 70°. Photographs were taken at 4, 8, 12, 16, 20, 24, 28, 32, and 64 min.

After the run was completed, the distance from the boundary of each component to the axis of rotation was calculated and its logarithm plotted against time in min. This plot resulted in a straight line for each component. The slope of each straight line was calculated and put into the following formula to find the sedimentation coefficient:

$$S_{obs} = \frac{2.303 \, d \log X / dt}{(60)(w^2)},$$

or

$$S_{obs} = \frac{(slope)(2.303/60)}{(\pi)\frac{(59,780)}{60}}$$

where S_{obs} = the observed sedimentation coefficient in 1×10^{-13} sec.

Fractionation of the Soluble Proteins of Bull Sperm

The purpose of the attempt to fractionate the sperm proteins was to simplify the antigenic system so that one or two specific proteins could be studied more fully. Three methods were investigated: salting-out with ammonium sulfate, preparative electrophoresis in a Tiselius unit and column chromatography with diethylaminoethyl (DEAE) and with carboxymethyl (CM) cellulose.

Ammonium Sulfate Fractionation

Salting out with ammonium sulfate was performed at 25, 33, 50, and 100% saturation. The fractions were reprecipitated twice and the final precipitate was suspended in 0.005 M phosphate buffered saline (pH 7.4) or in barbital buffer (pH 8.4 and $\mu = 0.05$).

Preparative Electrophoresis

Electrophoresis was conducted at 1.5°C in an American Instrument Company portable electrophoresis apparatus equipped with a cylindrical lens and rotating slit. All runs were made in barbital buffer (pH 8.6 and $\mu = 0.1$). The amperage was maintained at 15 ma through each run. The soluble proteins from bull sperm described previously were adjusted to 2% protein and dialyzed against barbital buffer for 48 hr at 4°C before

being electrophoresed. At the end of 140 min, when the protein was migrating out from the arm of the 6 ml electrophoresis cell toward the electrodes, the run was stopped. The bottom plane of the cell was sheared to the right. An 18 ga, 10 in. needle was inserted through the glass capillary fitting into the left arm of the cell. Approximately one-fourth inch of needle extended into the top of the arm of the cell and 1 ml of material was slowly removed. This should have contained the fastest moving proteins.

The same procedure was followed to obtain a 1 ml sample from the top of the right arm of the cell. This should have contained the slowest moving proteins.

The center section of the cell was then sheared to the left and the buffer vessels were removed from the cell and the top of the cell was emptied of buffer. The center of the cell was then sheared back and the contents of the ascending arm removed. The contents of the descending arm were then removed. The bottom of the cell was then re-aligned and its contents removed.

All samples were stored at -20°C until used.

Column Chromatography

Seventeen grams of DEAE cellulose (Cellex D, Bio-Rad Labs.) or CM cellulose were suspended in 500 ml of 0.02 M sodium phosphate buffer (pH 7.0). The DEAE cellulose was allowed to settle for 30 min.

The adsorbent remaining in suspension was siphoned off and discarded. A 2 cm column was mounted in a vertical position with its outlet closed in a 4°C room. A Whatman No. 41 filter paper disc was placed on the sintered glass disc at the base of the column and the column was filled to one-third capacity with starting buffer. The DEAE slurry was poured into the buffer and when a 2 to 5 cm layer had accumulated, the outlet was opened and a slow stream of buffer was allowed to flow out. The slurry was added to the column until a bed of 30 cm accumulated. This packed column was washed with 500 ml of starting buffer.

Usually one-fourth gram of soluble sperm proteins contained in 4 ml of initial starting buffer was dialyzed for 24 hr against the starting buffer. The dialyzed protein was placed on the column and the column washed with 200 ml of starting 0.02 M sodium phosphate buffer (pH 7). The column was either eluted stepwise or with a linear gradient.

Two hundred ml quantities of 16 different concentrations of sodium chloride (see Appendix) dissolved in the starting buffer were used to stepwise elute the proteins from the column. Twenty-five ml fractions were collected with a fraction collector.

Linear gradient elution was carried out by placing the initial buffer in a cylinder connected to the column and placing 0.7 M sodium chloride in a second cylinder identical to the first. By means of interconnecting tubing at the bottom of the cylinders and equal hydrastatic heads, a linear gradient was obtained provided the contents of the

mixing cylinder were stirred constantly. Five ml fractions were collected with a fraction collector. All fractions were examined for absorbency at 280 mμ.

Fractionation of the Proteins in Bovine Seminal Plasma

The purpose of the attempt to fractionate bull seminal plasma was to reduce the number of antigens present in any one sample. One method was investigated: namely column chromatography with DEAE cellulose.

Column Chromatography

The procedure was identical to that described previously for chromatography of bull sperm proteins. However, stepwise elution was carried out in a 15 X 4.5 cm column.

RESULTS AND DISCUSSION

Characteristics of the Hemolytic Factor in Bovine Seminal Plasma

While using bovine seminal plasma as an antigen in the complement fixation test, it was observed that certain samples of bovine seminal plasma would hemolyze the sheep red blood cells employed in the test. A search of the literature revealed that Millar (1956) had observed this phenomenon earlier. He associated the hemolytic activity of bovine seminal plasma with reduced fertility. In view of that finding, a further investigation of the nature of the hemolytic factor was undertaken.

Dialysis

Dialysis of three hemolytic samples of bovine seminal plasma did not result in any decrease in hemolytic activity of the seminal plasma (Table 1). The buffer which surrounded the dialysis tubing showed no hemolytic activity. This result was in contrast to Millar's (1956), who observed that the saline surrounding the dialysis tubing became hemolytic to the same degree as the seminal plasma. Millar dialyzed his samples for 48 hr while the samples in the present experiment were dialyzed for only 24 hr. The finding that the hemolytic factor can not be dialyzed agreed with that reported by Mitscherlich and Paufler (1960).

Table 1. Effect of dialysis on hemolytic activity[a] of bovine seminal plasma.[b]

Treatment	Dilution of bovine seminal plasma							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
No dialysis	4	4	4	4	4	4	2	0
Dialysis	4	4	4	4	4	4	3	0

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

[b]Average of 3 bulls.

Heat

The effect of heat on hemolytic activity is recorded in Table 2. Heating a pooled sample of hemolytic bovine seminal plasma to 56°C for 30 min had no effect on the 100% hemolytic endpoint. The conditions for inactivation of blood complement, which is involved in many hemolyzing systems, are to heat at 56°C for 30 min. Since no inactivation of hemolytic activity was found after heating the seminal plasma to 56°C for 30 min, the hemolytic system probably did not involve complement. Heating the hemolytic seminal plasma at 65°C for 15 min decreased the hemolytic titer slightly while 100°C for 5 min completely inactivated the hemolytic factor. Mitscherlich and Paufler (1960) reported nearly complete inactivation of the hemolytic factor by heating at 70°C for 15 min.

Table 2. Effect of heat on hemolytic activity[a] of bovine seminal plasma.

Treatment	Dilution of bovine seminal plasma							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Unheated	4	4	4	4	4	4	3	1
56°C for 30 min	4	4	4	4	4	4	2	1
65°C for 15 min	4	4	4	4	4	3	2	1
100°C for 5 min	0	0	0	0	0	0	0	0

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

Origin of the Hemolytic Factor

Saline extracts of bovine bulbo-urethral gland or seminal vesicles did not produce hemolysis in the test system (Table 3). However, strong hemolytic activity was observed when the seminal vesicular fluid was tested. This finding suggested that the seminal vesicle was the place where the hemolytic factor was formed.

Table 3. Hemolytic activity[a] of various bovine organs and secretions.

	Dilution of extract or secretion							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Extract of bulbo-urethra	0	0	0	0	0	0	0	0
Extract of seminal vesicle	0	0	0	0	0	0	0	0
Seminal vesicle fluid	4	4	4	4	4	4	4	2
Seminal plasma	4	4	4	4	4	4	2	0

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

Effect of Blood Serum on Hemolytic Activity

A possible explanation as to why the saline extract of the seminal vesicle failed to cause hemolysis was that blood serum present in the extract was inhibiting the hemolytic activity.

The addition of increasing concentrations of bull blood serum to hemolytic seminal plasma progressively decreased the degree of hemolysis observed in the test system (Table 4). Evidently, the inhibition with blood serum was non-specific since rabbit blood serum also decreased the observed 100% hemolytic endpoint of hemolytic bovine

seminal plasma (Table 5). Millar (1956) also observed the non-specific inhibition of the hemolytic factor by blood serum.

Table 4. Inhibitory effect of bull blood sera on hemolytic activity[a] of bovine seminal plasma.

Treatment	Dilution of bovine seminal plasma							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
BSP +1:10 bull blood serum	-	2	2	2	0	0	0	0
BSP +1:100 bull blood serum	-	3	3	3	3	2	0	0
BSP	4	4	4	4	4	4	3	1

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

Table 5. Inhibitory effect of rabbit blood sera on hemolytic activity[a] of bovine seminal plasma.

Treatment	Dilution of bovine seminal plasma							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
BSP +1:2.5 rabbit blood serum	2	0	0	0	0	0	0	0
BSP + 1:5 rabbit blood serum	3	1	0	0	0	0	0	0
BSP	4	4	4	4	4	0	0	0

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

Ammonium Sulfate Fractionation of the Hemolytic Factor

The addition of ammonium sulfate to hemolytic bovine seminal plasma produced precipitates at 25, 33, 50 and 66% saturation. Little hemolytic activity was found in the resolubilized 25 and 33% fractions (Table 6). However, the 50 and 66% fractions produced hemolysis equivalent to that found with the seminal plasma which was not treated. Millar (1956) also noted that the hemolytic factor could be precipitated with ammonium sulfate. This data might be interpreted as suggesting that the hemolytic factor was a protein, or it could mean that the hemolytic factor was a prosthetic group attached to some protein.

Table 6. Hemolytic activity[a] of $(NH_4)_2SO_4$ precipitated fractions from bovine seminal plasma.[b]

Fraction	Dilution of fraction							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
25% $(NH_4)_2SO_4$ fraction	2	0	0	0	0	0	0	0
33% $(NH_4)_2SO_4$ fraction	2	1	0	0	0	0	0	0
50% $(NH_4)_2SO_4$ fraction	4	4	4	4	3	2	1	0
66% $(NH_4)_2SO_4$ fraction	4	4	4	4	4	3	2	0

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

[b]Average of 3 bulls.

Effect of Consecutive Ejaculation

The seminal plasma from each of four bulls collected four times within a period of 1 hr showed hemolytic activity (Table 7). The first three ejaculates from each bull produced the same 100% hemolytic end-point while the fourth ejaculate from each bull produced slightly more hemolysis. The bulls used for this part of the experiment were in routine once-a-week use at the local breeding cooperative and had normal fertility. Millar (1956) reported that the seminal plasma of healthy, fertile bulls in full use, was not hemolytic immediately after collection. However, he reported a tendency for the first ejaculate to be hemolytic if the bull was not used to its "full mating capacity," but that subsequent ejaculates from that bull on the same day were free of the hemolytic factor. The data presented in Table 7 contradict Millar's statements.

Table 7. Effect of consecutive ejaculation on hemolytic activity[a] of bovine seminal plasma.[b]

	Dilution of bovine seminal plasma							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Ejaculate 1	4	4	4	4	3	3	2	1
Ejaculate 2	4	4	4	4	3	3	2	1
Ejaculate 3	4	4	4	4	3	3	2	0
Ejaculate 4	4	4	4	4	4	3	2	1

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

[b]Average of 4 bulls.

Romaniuk (1961) found the highest concentrations of the hemolytic factor were in the first, second and third ejaculates.

Antigenicity and Cross-reactions of Sperm, Seminal Plasma and Blood Sera

Complement Fixation Data

The complement fixation test was used to determine antibody response. Bovine seminal plasma reacted with antisera to bovine ejaculated sperm, seminal plasma or blood sera (Table 8) but did not react with control serum. The strongest reaction occurred between bovine seminal plasma and its homologous antisera. The second strongest reaction occurred with the antisera to blood sera. Antibodies against bovine seminal plasma were also present in the anti-sperm immune sera. The control sera from pretreatment rabbits did not react with bovine seminal plasma. The immune reactions of the three antisera with bovine seminal plasma constituted evidence that sperm, seminal plasma and blood sera had common antigens which induced antibodies that reacted with seminal plasma.

Bovine blood sera also reacted with antisera to bovine ejaculated sperm, seminal plasma or blood sera (Table 9) but did not react with control serum. The strongest reaction occurred between bovine blood sera and is homologous antisera. The second strongest reaction occurred with the antisera to seminal plasma. Antibodies against bovine

Table 8. Hemolysis[a] resulting from the reaction of various rabbit immune and control sera (diluted 1/10) with bovine seminal plasma.

Sera	Dilution of bovine seminal plasma ($\times 10^{-2}$)								
	1:16	1:20	1:32	1:40	1:64	1:80	1:128	1:168	1:256
Anti-bull sperm	2	2	2	2	2	2	3	3	3
Anti-bull seminal plasma	0	0	0	0	0	1	3	3	4
Anti-bull blood sera	0	0	0	0	2	2	3	4	4
Control	4	4	4	4	4	4	4	4	4

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

Table 9. Hemolysis[a] resulting from the reaction of various rabbit immune and control sera (diluted 1/10) with bovine blood serum

Antisera	Dilution of bovine blood sera						
	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Anti-bull sperm	1	1	1	2	3	3	4
Anti-bull seminal plasma	0	0	0	2	3	4	4
Anti-bull blood serum	0	0	0	1	2	3	4
Control	4	4	4	4	4	4	4

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

blood sera were also present in the anti-sperm immune sera. The immune reactions of the three antisera with bovine blood sera constituted evidence that sperm, seminal plasma and blood sera had common antigens which induced antibodies that reacted with bovine blood sera.

Bovine ejaculated sperm (five times washed) also reacted with antisera to bovine ejaculated sperm, seminal plasma or blood sera (Table 10) but did not react with control serum. The strongest reaction occurred between bovine ejaculated sperm and its homologous antisera. The second strongest reaction occurred with the antisera to seminal plasma although blood sera gave the same 100% hemolytic endpoint. The immune reactions of the three antisera with bovine ejaculated sperm constituted evidence that sperm, seminal plasma and blood sera had common antigens which induced antibodies that reacted with bovine ejaculated sperm.

An analysis of the data presented in Tables 8, 9 and 10 indicated that bovine ejaculated sperm, seminal plasma and blood sera contained some common antigens such that if antibody was produced against one, it also reacted with the other two.

Weil et al. (1958, 1959) observed that human and rabbit ejaculated sperm were coated with a seminal plasma protein. From the data so far presented, it appeared that bovine sperm were also coated with a seminal plasma protein. The coating antigen apparently was stereochemically similar to certain blood proteins, in view of the cross

Table 10. Hemolysis[a] resulting from the reaction of various rabbit immune and control sera (diluted 1/10) with bovine ejaculated spermatozoa.

Sera	Sperm/ml x 10^9								
	800	400	200	100	50	25	12.5	6.25	3.13
Anti-bull sperm	0	0	0	0	0	3	3	3	4
Anti-bull seminal plasma	0	0	0	0	1	3	4	4	4
Anti-bull blood sera	0	0	0	0	2	4	4	4	4
Control	4	4	4	4	4	4	4	4	4

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

reactions with antisera to blood sera. A possible alternative to the coating explanation was that a protein leaked from the sperm cell and contaminated the seminal plasma. Thus, the cross reactions between antisera to sperm and seminal plasma could be caused by antibodies produced to the sperm proteins which leaked from the cell. This protein again would have to stereochemically resemble some constituent of blood serum in order to account for its cross reaction with antisera to blood sera.

Sperm from bovine vas deferens reacted with antisera to bovine ejaculated sperm, seminal plasma or blood sera (Table 11) but did not react with control serum. It was interesting to note that vas deferens sperm reacted to the same extent with all three antisera. Evidently, if the sperm were coated with a protein from the seminal plasma, the protein had to come in contact with the sperm before the contributions of major accessory sex glands came in contact with the sperm. The prostate, seminal vesicles and Cowper's are the major glands whose secretions constitute the bulk of the volume of the seminal plasma.

Table 11. Hemolysis[a] resulting from the reaction of various rabbit immune and control sera (diluted 1/10) with bovine spermatozoa from the vas deferens.

Sera	Sperm/ml x 10^9								
	800	400	200	100	50	25	12.5	6.25	3.13
Anti-bull sperm	0	0	0	0	1	2	3	4	4
Anti-bull seminal plasma	0	0	0	0	1	2	4	4	4
Anti-bull blood sera	0	0	0	0	1	2	4	4	4
Control	3	4	4	4	4	4	4	4	4

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

Weil et al. (1962) working with rabbit semen, demonstrated that the sperm coating antigen came from the seminal vesicle. If bull sperm has a coating antigen, at least part of it has to arise lower in the genital tract than the coating antigen of rabbit sperm.

Sperm from the head and tail of the bovine epididymis reacted with antisera to bovine ejaculated sperm, seminal plasma or blood sera (Table 12) but did not react with control serum. The strongest reaction occurred between epididymal sperm and the antisera to the ejaculated sperm. The second strongest reaction occurred with the antisera to the seminal plasma. By comparing similar reactions in Tables 11 and 12, it was determined that when any of the three antisera

Table 12. Hemolysis[a] resulting from the reaction of various rabbit immune and control sera (diluted 1/10) with bovine epididymal spermatozoa.

Sera	Sperm/ml x 10^9								
	800	400	200	100	50	25	12.5	6.25	3.13
Anti-bull sperm	0	0	0	1	2	3	4	4	4
Anti-bull seminal plasma	0	0	0	2	2	3	4	4	4
Anti-bull blood sera	0	0	1	2	3	3	4	4	4
Control	3	3	3	4	4	4	4	4	4

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

were reacted with vas deferens sperm, a stronger reaction occurred than that recorded with epididymal sperm. This might mean that the common material which is giving the cross-reactions was in greater concentration in the vas deferens as compared with the epididymis, or that epididymal sperm were less permeable than vas deferens sperm.

Sperm from the testis also reacted with antisera to bovine ejaculated sperm, seminal plasma, and blood sera (Table 13) and also to some extent with the control serum. Evidently, there was some non-specific complement absorbing substance in the mash from which the testicular sperm were isolated. The testicular sperm preparation was the least desirable as a test antigen because of the high ratio of testicular somatic cells to sperm. In other words, the main reactant may not have been sperm in the testis preparation.

Absorption is the usual immunological technique for removing specific antibodies from a complex mixture. If the various antisera were absorbed with bovine seminal plasma only antibodies toward components other than those found in bovine seminal plasma would be left. This was attempted but as was discussed in the previous section, high concentrations of bovine seminal plasma hemolyzed sheep red blood cells. Thus it was not possible to specifically absorb the various antisera with bovine seminal plasma since the concentration of seminal plasma needed to remove antibodies to seminal plasma hemolyzed the sheep red blood cells.

Table 13. Hemolysis[a] resulting from the reaction of various rabbit immune and control sera (diluted 1/10) with bovine testicular spermatozoa.

Sera	Sperm/ml x 10^9								
	400	200	100	50	25	12.5	6.25	3.13	1.56
Anti-bull sperm	0	0	0	0	0	0	1	2	3
Anti-bull seminal plasma	0	0	0	0	0	0	1	2	3
Anti-bull blood sera	0	0	0	0	0	1	3	3	4
Control	2	2	3	3	3	4	4	4	4

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

The problem of "non-specific" hemolysis after absorption was not encountered when the three antisera were absorbed with bovine blood sera. The absorption of bovine blood serum antibodies from the antisera to ejaculated sperm, seminal plasma or blood sera was complete as no reaction occurred between blood sera and the three absorbed antisera or with control sera (Table 14). However, bull epididymal sperm reacted with all three absorbed antisera (Table 15). Control serum reacted with the higher concentration of sperm but its reaction could easily be distinguished from the positive reaction found with the three antisera.

Table 14. Hemolysis[a] resulting from the reaction of various rabbit immune and control sera (diluted 1/10) absorbed with bull blood sera when tested with bull blood sera.

Sera	Dilution of bull blood sera						
	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Anti-bull sperm	4	4	4	4	4	4	4
Anti-bull seminal plasma	4	4	4	4	4	4	4
Anti-bull blood sera	4	4	4	4	4	4	4
Control	4	4	4	4	4	4	4

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

Table 15. Hemolysis[a] resulting from the reaction of various rabbit immune and control sera (diluted 1/10) absorbed with bull blood sera when tested with bull epididymal spermatozoa.

Sera	Sperm/ml x 10^9								
	800	400	200	100	50	25	12.5	6.25	3.13
Anti-bull sperm	0	0	1	2	3	4	4	4	4
Anti-bull seminal plasma	0	0	1	2	2	3	3	3	4
Anti-bull blood sera	0	0	1	2	3	4	4	4	4
Control	2	3	3	4	4	4	4	4	4

[a]Hemolysis ratings of 0, 1, 2, 3, and 4 are equivalent to 0, 25, 50, 75, and 100% hemolysis, respectively.

The complement fixation data so far presented did not indicate if sperm cells themselves were antigenic and if the cross reactions between sperm, seminal plasma and blood sera were the result of leakage from the cell or coating of the sperm with seminal plasma.

The antisera prepared against bull epididymal sperm, did not cross react with seminal plasma or bull blood serum (Table 16). It did react with bovine epididymal sperm and ejaculated sperm. The strongest reaction occurred between the antisera and its homologous antigen. Since the ejaculated sperm reacted weaker than epididymal sperm, ejaculated sperm probably lost some antigenic material either as the result of maturation or by washing. However, the sperm cells per se were antigenic since seminal plasma and blood sera failed to react with antisera to the epididymal sperm. This experiment answered the question of whether the cross reacting antigen found through earlier experiments came from sperm or from the seminal plasma. The cross reacting antigen must come from seminal plasma since if it leaked from the sperm into the seminal plasma, the seminal plasma would have reacted with the antisera to epididymal sperm. No reaction of seminal plasma with the antisera to epididymal sperm was observed.

Henle (1938) reported that rabbit anti-bovine epididymal sperm reacted with sperm but not with blood serum. The results presented in Table 16 agree with his findings. However, Henle (1938) reported that rabbit anti-bovine blood serum did not react with epididymal bovine

sperm. The data presented in Table 12 conflicted with that finding. The reason for this difference may lie in the fact that Henle used no adjuvant to promote antibody response. Furthermore, when rabbit anti-bovine blood sera was absorbed with blood sera, antibodies were still present which reacted with epididymal sperm.

Table 16. Hemolysis[a] resulting from the reaction of rabbit antisera (diluted 1/10) to bull epididymal spermatozoa with bovine seminal plasma, blood sera, ejaculated spermatozoa and epididymal spermatozoa.

Antigen	Dilution of antigen								
	1:0	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Bovine seminal plasma[b]	4	4	4	4	4	4	4	4	4
Bovine blood sera[c]	4	4	4	4	4	4	4	4	4
Bovine ejac. sperm[d]	0	0	1	2	3	4	4	4	4
Bovine epidid. sperm[e]	0	0	0	0	1	2	3	4	4

[a]Hemolytic ratings of 0, 1, 2, 3, and 4 represent approximately 0, 25, 50, 75, and 100% hemolysis, respectively.

[b]1:0 dilution equals a 1:1,600 dilution of bovine seminal plasma.

[c]1:0 dilution equals a 1:50 dilution of bovine blood sera.

[d]1:0 dilution contains 800×10^9 sperm/ml.

[e]1:0 dilution contains 800×10^9 sperm/ml.

Agar-gel Diffusion Data

The agar gel double diffusion method of Ouchterlony (1958) was used to determine antibody response and to assess the antigenic spectrum of bovine semen. The following points must be taken into account in interpreting immuno-diffusion results. The number of lines formed with diluted antigens and antibodies are usually less than the number formed with concentrated reactants. If two different proteins have the same equivalence point for precipitation, the two precipitin lines may merge and appear as one line. If an antigen is not soluble in the buffer used for dissolving the agar, it can not be detected by immuno-diffusion. Therefore, the number of precipitin lines only indicates the minimum number of antigens in the system.

The antigenic complexity of bovine semen was indicated by the studies employing the Ouchterlony (1958) double diffusion technique. The reaction of rabbit anti-bovine ejaculated sperm immune sera resulted in the formation of at least seven lines with ejaculated sperm, at least seven lines with bovine seminal plasma and at least one line with bovine blood sera (Figs. 2 and 3). Five times washed ejaculated sperm had at least five antigens in common with seminal plasma as determined by the number of chevrons connecting sperm lines with seminal plasma lines (Fig. 2). No lines could be distinguished as being specific for ejaculated sperm due to the heavy wide precipitin band that formed with seminal plasma and the antisera to sperm.

Fig. 2. Sperm and Seminal Plasma Cross-reactions.

Center well	(lower)	Rabbit anti-bull ejaculated sperm
Well 1	(left)	Once washed ejaculated bull sperm
Well 2	(upper)	Bovine seminal plasma
Well 3	(right)	Five times washed ejaculated bull sperm

Fig. 3. Sperm, Seminal Plasma and Blood Sera Cross-reactions.

Center well	(lower)	Rabbit anti-bull ejaculated sperm
Well 1	(left)	Bovine seminal plasma
Well 2	(upper)	Bovine blood sera
Well 3	(right)	Five times washed ejaculated bull sperm

Fig. 4. Antigenic Spectrum of Bovine Washed Ejaculated Sperm.

Center well	Rabbit anti-bull ejaculated sperm
Well 1*	Supernatant fluid of 5X washed ejaculated bull sperm
Well 2	20×10^7 washed 5X, ejaculated bull sperm
Well 3	40×10^7 washed 5X, ejaculated bull sperm
Well 4	60×10^7 washed 5X, ejaculated bull sperm
Well 5	80×10^7 washed 5X, ejaculated bull sperm
Well 6	100×10^7 washed 5X, ejaculated bull sperm

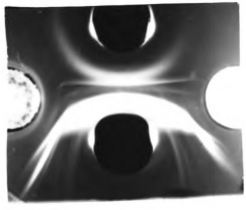
Fig. 5. Cross-reactions of Antisera to Bovine Sperm.

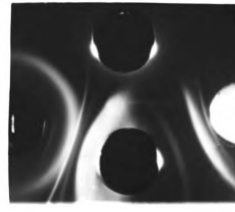
Center well	Rabbit anti-bull ejaculated sperm
Well 1*	Washed 1X, ejaculated bull sperm
Well 2	Bovine blood sera
Well 3	Washed 5X, epididymal bull sperm
Well 4	Washed 5X, ejaculated bull sperm
Well 5	Testicular bull sperm
Well 6	Bovine seminal plasma

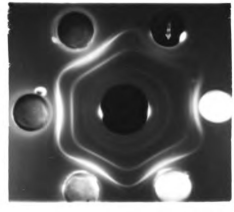
Fig. 6. Demonstration of Specific Antigens in Sperm.

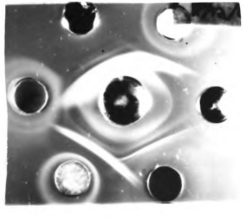
Center well	Rabbit anti-bull ejaculated sperm absorbed with seminal plasma and blood sera
Wells 1*-6	(See Fig. 5)

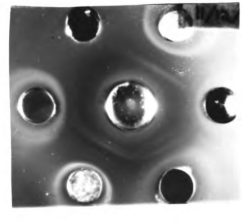
*Wells numbered clockwise from Well 1 at one o'clock.











The sharpness of an individual line is a function of the concentration of each reactant. Figure 4 illustrates this point. Various concentrations of five times washed ejaculated bull sperm were placed in the peripheral wells and rabbit anti-bovine ejaculated sperm immune sera was placed in the center well. The sperm concentration of 60 X 10^7 sperm/ml gave the best resolution of the seven sperm antigens. This plate also demonstrated that antigenic material had to diffuse from the sperm cells after they were placed in the wells since the supernatant fluid prepared from the same stock sperm solution as that used for filling the wells with sperm, produced a line pattern which was characteristic of that found with the dilute antigen concentrations. This is shown in Well 1 of Fig. 4.

Experiments were carried out employing bovine testicular sperm, epididymal sperm, ejaculated sperm, blood sera and seminal plasma as antigens and unabsorbed or absorbed rabbit anti-bull sperm immune sera. The antisera were absorbed with bovine blood sera and seminal plasma to determine if the sperm contained antigens which were not of seminal plasma or blood serum origin. Figure 5 presents the data obtained with unabsorbed rabbit anti-bull ejaculated sperm immune sera when reacted with various antigens. Although the total number of lines appearing between individual reactants is less than that shown in Fig. 2 and 3, it is still significant to note that all the antigens tested shared an

antigen common to bovine blood sera. Figure 6 presents the data obtained with the absorbed antisera and the same antigens. The absorption of antibodies to bovine blood sera and seminal plasma was complete since no lines for those antigens appeared. However, ejaculated five times washed bull sperm still formed three lines which formed identities with the three lines from epididymal sperm. This constituted evidence that bovine sperm from the head and body of the epididymis and ejaculated sperm shared at least three antigens which were not of seminal plasma or blood sera origin. Once washed sperm showed only one sperm specific line. This finding might be interpreted to mean that washing changes the permeability of the sperm cell and allows its internal contents to diffuse from the cell. Testicular sperm also showed only one sperm specific line. More lines might have been observed if the concentration of sperm in the testicular sample had been higher.

The three sperm specific lines stained as proteins rather than as lipoproteins when reacted with the Azocarmine-Sudan Black double stain. Reacting the three sperm specific lines with alpha-naphthol-paraphenylenediamine caused one of the three lines to stain as a glycoprotein.

Rao and Sadri (1960) working with buffalo semen and the antisera to it, demonstrated three sperm specific antigens in ejaculated and epididymal sperm. The data obtained with bovine sperm and antisera

to bovine sperm agreed with their finding. The finding of at least seven antigens in bovine sperm also paralleled their finding of seven sperm antigens in the buffalo.

Experiments were carried out employing bovine testicular sperm, epididymal sperm, ejaculated sperm, blood sera and seminal plasma as antigens and unabsorbed or absorbed rabbit anti-bovine seminal plasma. The antisera was absorbed with either bovine blood sera or ejaculated bull sperm to determine if seminal plasma contained antigens which were not of sperm or blood sera origin. Figure 7 presents the data obtained with unabsorbed rabbit anti-bovine seminal plasma when reacted with various antigens. The complexity of the antigenic spectrum was attested to by the number of lines present and their coalescence. It is significant to note that all the tested antigens reacted with the antisera to produce precipitin lines. Figure 8 presents the data obtained with the antisera absorbed with ejaculated five times washed bovine sperm and reacted with the same antigens. The absorption of antibodies to five times washed ejaculated sperm was complete since no lines formed for that antigen. However, seminal plasma still formed at least five lines. Once washed ejaculated sperm formed at least two lines, bovine blood sera at least two and epididymal sperm at least four. The significant thing about this plate was that the epididymal sperm preparation formed at least two strong precipitin lines which were not common to blood sera. This constituted evidence that the head and body of the epididymis

Fig. 7. Cross-reactions of Antisera to Bovine Seminal Plasma

Center well	Rabbit anti-bull seminal plasma
Well 1*	Washed 1X, ejaculated bull sperm
Well 2	Bovine blood sera
Well 3	Washed 5X, epididymal bull sperm
Well 4	Washed 5X, ejaculated bull sperm
Well 5	Testicular bull sperm
Well 6	Bovine seminal plasma

Fig. 8. Effect of Absorption with Sperm on Antibodies in Antisera to Seminal Plasma.

Center well	Rabbit anti-bull seminal plasma absorbed with bovine ejaculated sperm
Wells 1* - 6	As in Fig. 7

Fig. 9. Effect of Absorption with Blood Sera on Antibodies in Antisera to Seminal Plasma.

Center well	Rabbit anti-bull seminal plasma absorbed with bovine blood sera.
Wells 1* - 6	As in Fig. 7

Fig. 10. Cross-reactions of Antisera to Bovine Blood Sera.

Center well	Rabbit anti-bovine blood sera
Wells 1* - 6	As in Fig. 7

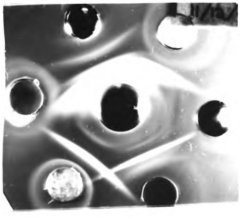
Fig. 11. Effect of Absorption with Sperm on Antibodies in Antisera to Blood Sera.

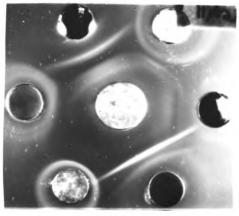
Center well	Rabbit anti-bovine blood sera absorbed with bovine blood sera
Wells 1* - 6	As in Fig. 7

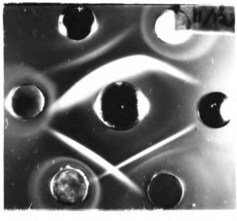
Fig. 12. Effect of Absorption with Seminal Plasma on Antibodies in Antisera to Blood Sera.

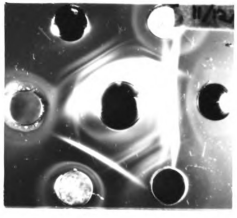
Center well	Rabbit anti-bovine blood sera absorbed with bovine seminal plasma
Wells 1* - 6	As in Fig. 7

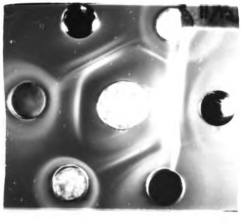
*Wells numbered clockwise from Well 1 at one o'clock.

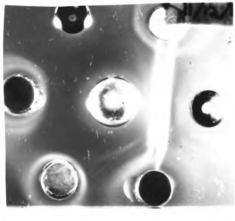












contributed at least two protein antigens to the seminal plasma. Figure 9 presents the data obtained with the seminal plasma antisera absorbed with bovine blood sera and reacted with the same previously described antigens. The absorption of antibodies to bovine blood sera was complete as no lines for that antigen formed. However, seminal plasma still formed at least five lines, once washed ejaculated sperm formed at least three, epididymal sperm formed at least four, and five times washed ejaculated sperm formed at least two lines. The significant thing about this plate was that the precipitin lines formed with five times washed sperm cross and did not coalesce with the precipitin lines formed with the epididymal sperm suspension. This was evidence of a non-identity type of reaction and strengthened the hypothesis that the head and body of the epididymis contributed at least two protein antigens to the seminal plasma which were not identical to blood components.

Experiments were carried out employing bovine testicular sperm, epididymal sperm, ejaculated sperm, blood sera and seminal plasma as antigens and unabsorbed or absorbed rabbit anti-bull blood sera. The antisera was absorbed with either five times washed ejaculated bull sperm or seminal plasma to determine if bovine blood sera contained antigens not common to those of ejaculated sperm or seminal plasma. The antisera was also absorbed with bovine blood sera to determine if it could still react with sperm and seminal plasma. Figure 10 presents the data obtained with unabsorbed antisera when reacted with various antigens.

A number of lines formed between each antigen and the antisera to bovine blood sera. The total number of lines formed indicated that the antisera to blood sera was more complex than the antisera to sperm or seminal plasma. Each reactant, with the exception of testicular sperm and five times washed ejaculated sperm, formed at least five lines with the antisera to blood sera. The washed bull sperm formed at least three lines while testicular sperm formed at least two lines. Figure 11 presents the data obtained with the blood sera antisera absorbed with five times washed bull sperm and reacted with the previously described antigens. The absorption of antibodies to five times washed bovine ejaculated sperm was complete as no lines formed for that antigen. The other antigens continued to form precipitin lines. The complexity of the system was not simplified with the exception that absorption with ejaculated sperm removed the heavy diffuse precipitin band characteristic of seminal plasma reactions. Figure 12 presents the data obtained with the blood sera antisera absorbed with bovine seminal plasma and reacted with the previously described antigens. The absorption of antibodies to bovine seminal plasma was complete as no lines formed for that antigen. In addition, no lines were formed with testicular sperm or ejaculated sperm. The three epididymal sperm lines, formed identities with blood sera in the system. Although the plates are not shown, absorption of the antisera to blood sera with seminal plasma and blood sera eliminated any line from forming when reacted with the same antigens described

earlier. The data obtained by absorbing rabbit anti-bovine blood sera with blood sera and reacting it with the previously described antigens is presented in Fig. 13. Although the absorption was incomplete as shown by the formation of one line with bovine blood sera, at least four lines formed with bovine seminal plasma, at least two with ejaculated bull sperm and at least four with the epididymal sperm preparation. One of the epididymal lines formed an identity with the unabsorbed blood sera line. This data supported the complement fixation data presented earlier which showed that rabbit anti-bovine blood sera absorbed with blood sera continued to react with seminal plasma, ejaculated sperm and epididymal sperm. Antibodies against bovine blood sera were completely absorbed from the antisera used for the complement fixation test.

Control sera from non-immunized rabbits formed no precipitin lines when tested with all previously described antigens. This fact constituted evidence that the lines formed with the various antisera were actual antigen-antibody precipitin reactions rather than the result of non-specific precipitation.

Evidence that not all the soluble proteins could diffuse from the sperm cell is found in Fig. 14. In this plate, at least six lines formed with ejaculated bull sperm and at least three with a commercial preparation of calf thymus histone (Nutritional Biochemicals Corp.) after reaction with antisera to ejaculated sperm. No identities between histone lines and sperm lines were observed. This may mean that the sperm

Fig. 13. Effect of Absorption with Blood Sera on Antibodies in Antisera to Blood Sera.

Center well	Rabbit anti-bovine blood sera absorbed with bovine blood sera
Well 1	Washed 1X, ejaculated bull sperm
Well 2	Bovine blood sera
Well 3	Washed 5X, epididymal bull sperm
Well 4	Washed 5X, ejaculated bull sperm
Well 5	Testicular bull sperm
Well 6	Bovine seminal plasma

Fig. 14. Non-identity of Sperm Precipitin Lines with Histone Lines.

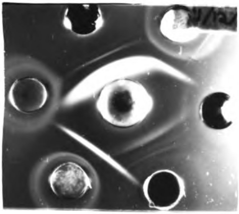
Center well	(lower)	Rabbit anti-bovine ejaculated bull sperm
Well 1	(left)	Salmon protamine nucleate
Well 2	(upper)	Washed 5X, ejaculated bull sperm
Well 3	(right)	Calf thymus histone

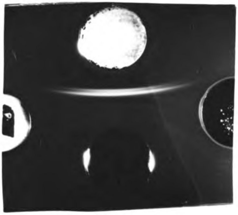
Fig. 15. Comparison of Head and Tail Antigens with Whole Sperm Antigens.

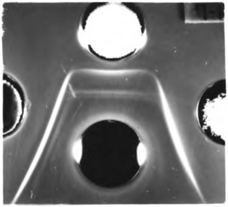
Center well	(lower)	Rabbit anti-bovine ejaculated sperm
Well 1	(left)	Bull sperm head preparation
Well 2	(upper)	Washed 5X, ejaculated bull sperm
Well 3	(right)	Bull sperm tail preparation

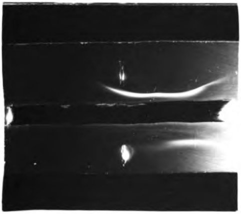
Fig. 16. Immuno-electrophoretic Analysis of the Reaction of Sperm and Seminal Plasma with Anti-sperm Sera. Bovine seminal plasma was placed in the upper slit and bovine saline soluble sperm proteins in the lower slit. The anode (+) is to the right. The center trough was filled with rabbit anti-bovine ejaculated sperm.

Fig. 17. Immuno-electrophoretic Analysis of the Reaction of Sperm and Seminal Plasma with Anti-sperm Sera Absorbed with Seminal Plasma. Bovine seminal plasma was placed in the upper slit and bovine saline soluble sperm proteins in the lower slit. The anode (+) is to the right. The center trough was filled with rabbit anti-bovine ejaculated sperm absorbed with bovine seminal plasma and blood sera.









cell was degraded during immunization and antibodies formed against its internal histones. However, when intact sperm cells were used in the immuno-diffusion tests, insufficient, if any, histone diffused from the cell. An alternative explanation would be that the histone preparation was contaminated with blood sera proteins but this explanation could account for only one of the three histone lines since bovine blood sera reacted with antisera to ejaculated sperm to form only one line. A third explanation is that the immunological specificity of sperm histones was different from that of thymus histone.

Experiments were performed to determine if the head of the sperm contained soluble antigens which were different from those of the tail. No antigenic distinction could be made between head or tail suspensions by means of the Ouchterlony double diffusion technique (Fig. 15). The antisera used was rabbit anti-bull ejaculated sperm immune sera. Comparison of the sharpness of certain head lines with the sharpness of the comparable tail lines led to the conclusion that even though the total number of heads in Well 1 equalled the number of tails in Well 3 there were differential concentrations of antigens in the two wells.

Immuno-electrophoresis

Experiments employing immuno-electrophoresis were performed to ascertain if more than seven soluble antigens were present in bovine sperm.

The lower half of Fig. 16 presents the results obtained when the saline soluble constituents of bovine sperm were electrophoresed and reacted with rabbit anti-bull sperm. Only six arcs could be distinguished for bull sperm. However, one of the arcs is a long arc of only moderate curvature and this suggested that it had been formed by an antigen which was electrophoretically heterogeneous and consisted of a population of molecules with smoothly graduated differences in electrophoretic mobility (Crowle, 1961). The upper half of Fig. 16 shows the immuno-electrophoretic pattern obtained for seminal plasma. The lower half of Fig. 17 presents the results obtained when the saline soluble constituents of bovine sperm were electrophoresed and reacted with antisera to ejaculated sperm absorbed with blood sera and seminal plasma. Three sperm specific arcs were distinguishable although only two show up on the photograph. The immuno-electrophoresis data constituted evidence that ejaculated sperm have at least three antigens which were not of seminal plasma origin.

Extraction of Bull Sperm Soluble Proteins

Experiments were carried out to find out whether freeze-thawing, extraction with saline or mechanical rupture with glass beads would release the largest amount of antigen from the sperm into the surrounding medium. Another purpose of this experiment was to observe if any new antigens were released from the sperm by the above treatments.

The sperm free supernatant fluid from the five times washed ejaculated sperm sample which had extracted in saline increased by approximately 0.2 mg protein/ml after 3 days storage (Table 17). The supernatant fluid from the sperm sample which had been freeze-thawed eight times showed an increase of approximately 0.2 mg protein/ml after 3 days storage. The supernatant fluid from the sperm sample which had been subjected to glass bead bombardment in a Servall Omnimixer showed an increase of 1.1 mg protein/ml after 3 days storage. These data indicated that mechanical rupture of bovine ejaculated sperm in an Omnimixer with glass beads was superior to freeze-thawing or extraction in saline as a method of extracting soluble proteins from sperm.

Table 17. Effect of extraction procedure and time on amount of protein[a] present in supernatant fluids of sperm suspensions.[b]

	Time after treatment			
	0 hr	24 hr	48 hr	72 hr
Saline extraction	1.8	1.9	--	2.0
Freeze-thawing	1.5	1.8	1.6	1.7
Mechanical rupture	1.8	2.7	2.7	2.9

[a]Protein values are in mg/ml.

[b]Sperm concentration 50 X 10^7 cells/ml.

Figure 18 presents the data obtained by reacting the various supernatant fluids obtained from the previously described extraction treatments with rabbit anti-bull sperm immune sera. Comparison of the number of lines formed with each supernatant fluid with the number of lines formed with the untreated control sperm suspension, led to the conclusion that no new antigens had been released from the sperm cell by any treatment. The three treatments were identical as far as their ability to qualitatively extract the proteins from the sperm cell.

Physical Chemical Characteristics of the Soluble Proteins of Bull Sperm

Electrophoresis

Moving boundary electrophoretic analysis in a Tiselius cell was carried out on the soluble proteins of bovine ejaculated sperm. The purpose of this study was to determine the electrophoretic mobilities of the proteins from bovine ejaculated sperm.

Figure 19 displays the electrophoretic pattern of the saline extractable bovine sperm proteins. The electrophoretic pattern indicated the heterogeneous nature of bovine sperm proteins and the presence of two major components and one minor one. The mobilities of the electrophoretic components and their relative concentrations are presented in Tables 18 and 19. Barbital buffer (pH 8.6, $\mu = 0.1$) was used throughout this study since it was known to yield good electrophoretic separation of components.

Fig. 18. Reaction of Anti-sperm Sera with Soluble Sperm Proteins.

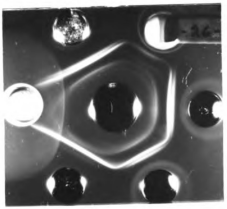
Center well	Rabbit anti-bovine ejaculated sperm
Well 1*	Five times washed, bovine ejaculated sperm
Well 2	Freeze-thawed sperm supernatant fluid
Well 3	Saline extraction sperm free fluid
Well 4	Mechanical ruptured sperm supernatant fluid
Well 5	Calf thymus histone
Well 6	Mechanical ruptured sperm supernatant fluid

*Wells numbered clockwise from one o'clock.

Fig. 19. Electrophoretic Pattern of Saline Soluble Sperm Proteins. The buffer was sodium barbital at pH 8.6 and $\mu = 0.1$. The field strength was 6.6 volts per cm^2.

Fig. 20. Electrophoretic Pattern of Water Soluble Sperm Proteins. The buffer was sodium barbital at pH 8.6 and $\mu = 0.1$. The field strength was 6.1 volts per cm^2.

Fig. 21. Electrophoretic Pattern of Sperm Lipoprotein Complex. The buffer was sodium barbital at pH 8.6 and $\mu = 0.1$. The field strength was 17.0 volts per cm^2.



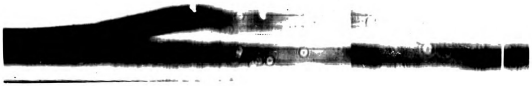


Table 18. Electrophoretic mobilities[a] of various bovine soluble sperm protein samples.

Treatment	Mobility of component				
	Component No.				
	1	2	3	4	5
Saline extraction	2.0	-	3.8	-	5.1
Distilled water extraction	-	3.3	-	4.3	5.4
0.01 N NaOH extraction after removal of saline soluble proteins	-	3.6	3.9	4.5	4.9

[a]Mobilities in 1 X 10^{-5} cm^2 $volt^{-1}$ sec^{-1}.

Table 19. Relative proportion of various soluble bovine sperm proteins.

Treatment	Per cent of component				
	Component No.				
	1	2	3	4	5
Saline extraction	20	-	50	-	30
Distilled water extraction	-	25	-	27	48
0.01 N NaOH extraction of sperm residue after removal of saline soluble proteins	-	1	23	74	2

Figure 20 displays the electrophoretic pattern of the distilled water extractable bovine sperm proteins. The electrophoretic pattern indicated the heterogeneous nature of bovine sperm proteins and the presence of three components. The mobilities of the electrophoretic components and their relative concentrations are presented in Tables 18 and 19.

Comparison of Figures 19 and 20 led to the conclusion that the slowest moving component was not soluble in distilled water. This component had a mobility of 2.0, which was similar to that of the gamma globulin of blood sera. Solubilization in distilled water would not be expected if this protein was a gamma globulin. It was interesting to note that the mobility of 3.8 for one of the saline extractable proteins was identical to the average mobility of two distilled water extracted proteins (3.3 and 4.3). This could have been caused by a protein-protein interaction such that distilled water extraction broke the bonds by which these two components interacted. An alternative explanation was that two separate new proteins were present in the distilled water extraction sample.

The sperm residue that remained after extraction of the saline soluble proteins was solubilized in 0.01 N sodium hydroxide. A lipoprotein complex was prepared from the sodium hydroxide extract according to directions given by Berry and Mayer (1959). Figure 21 displays the electrophoretic pattern of the bovine sperm lipoprotein

complex. The electrophoretic pattern indicated the heterogeneous nature of the fraction. One major component and three minor components were found. The major component had a mobility similar to that of the alpha globulins of blood serum.

Ultracentrifugation

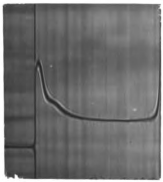
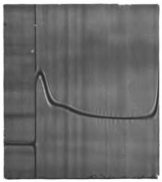
A sedimentation velocity ultracentrifugal analysis was performed to further characterize the saline soluble proteins of bovine ejaculated sperm. The results (Fig. 22 and Table 20) revealed that the saline soluble proteins of bull sperm produced two major sedimentation gradients. In addition to the apparent heterogeneity of the gradients, a degree of polymorphic behavior was observed as determined by the shape of each gradient. The S_{20} value of 1.7 was indicative of a relatively low molecular weight compound or a compound which geometrically would resemble a long rod. The S_{20} value 12.6 was indicative

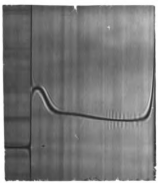
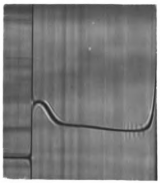
Table 20. Ultracentrifugal analysis of bovine saline soluble sperm proteins.

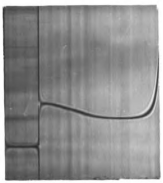
Sedimentation gradient no.	Svedberg units[a]
1	1.7
2	12.6

[a]Svedberg units equal 1×10^{-13} sec.

Fig. 22. Ultracentrifugal Patterns of Saline Soluble Sperm Proteins. The pictures were taken 4, 8, 12, 16, 20, 24, 28, 32 and 64 min after the rotor had attained the operating speed of 59, 780 rev/min.

of a larger molecular weight compound or a compound which geometrically would resemble a globulin. Polymorphic aberrations were observed and were interpreted to be caused by different polymorphic species in relatively low concentrations. Polymorphism is a characteristic of proteins that complex with each other, such as mucoproteins.

Diffusion

The Allison-Humphrey-diffusion-in-gel method was used to determine the diffusion coefficient for the sperm proteins that were of sperm origin rather than of seminal plasma origin. The saline soluble preparation of ruptured sperm was reacted with antisera that had been absorbed with blood sera and seminal plasma. The tangents of the angles between the antigen well and the precipitin lines (Fig. 23) were calculated to be 1.0566 and 1.6364. Using these values and the reported diffusion coefficient of 3.8×10^{-7} cm^2 sec^{-1} for rabbit gamma globulin, diffusion coefficients of 4.2 and 10.2×10^{-7} cm^2 sec^{-1} were calculated for the two sperm specific antigens.

Fractionation of the Soluble Proteins of Bull Sperm

Ammonium Sulfate Fractionation

The saline soluble proteins of bovine sperm were not salted-out at 25% saturation with ammonium sulfate. A precipitate formed when the protein solution was made 33% saturated with ammonium sulfate.

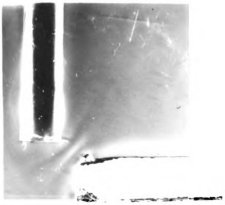
Fig. 23. Determination of the Diffusion Coefficient of the Sperm Specific Antigens.

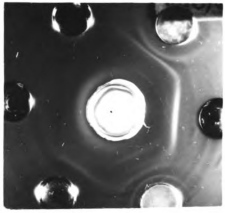
Vertical well	Five times washed bovine ejaculated sperm
Horizontal well	Rabbit anti-bovine ejaculated sperm sera absorbed with bovine seminal plasma and blood sera

Fig. 24. Reaction of Anti-sperm Sera Absorbed with Seminal Plasma to Soluble Sperm Proteins Fractionated by Ammonium Sulfate

Center well	Rabbit anti-bovine ejaculated sperm absorbed with bovine seminal plasma
Well 1*	Once washed, bovine ejaculated sperm
Well 2	Bovine sperm protein, insoluble 33% saturated $(NH_4)_2 SO_4$ fraction
Well 3	Five times washed, bovine ejaculated sperm
Well 4	Bovine sperm protein, insoluble 33 to 50% saturated $(NH_4)_2 SO_4$ fraction
Well 5	Bovine seminal plasma
Well 6	Bovine blood sera

* Well numbered clockwise from one o'clock.





The supernatant fluid from the 33% saturation yielded a precipitate at 50% saturation. This precipitate constituted the majority of the precipitable material from soluble sperm proteins. The supernatant fluid left after removal of the 50% ammonium sulfate precipitated protein, yielded a very small precipitate, upon being made to 100% saturation.

The data obtained when these ammonium sulfate fractions were dissolved in phosphate buffered saline (pH 7. 4) and reacted in agar-gel-diffusion is displayed in Fig. 24. The antisera was rabbit anti-bull ejaculated sperm immune sera absorbed with seminal plasma. The unfractionated sample of five times washed bull sperm formed three sperm specific lines which merged with the three lines formed against the 33% ammonium sulfate fraction of bovine sperm and with the two lines formed against the 50% ammonium sulfate fraction of bovine sperm. A definite concentration of one of the sperm specific antigens is shown by the density of the lines formed with the 33% fraction. The precipitate obtained at 100% saturation did not form a line with the absorbed antisera. Either the 100% fraction did not contain any sperm specific antigens or the concentration of protein was too low (0. 1%) to give a precipitin line.

Preparative Electrophoresis

The 1 ml samples taken of the fastest and slowest moving proteins after electrophoretic separation in a Tiselius electrophoresis cell did

not contain sufficient total protein to be tested by immuno-diffusion even though the proteins were concentrated by dialysis against polyethylene glycol. The protein concentration was less than 0. 1%.

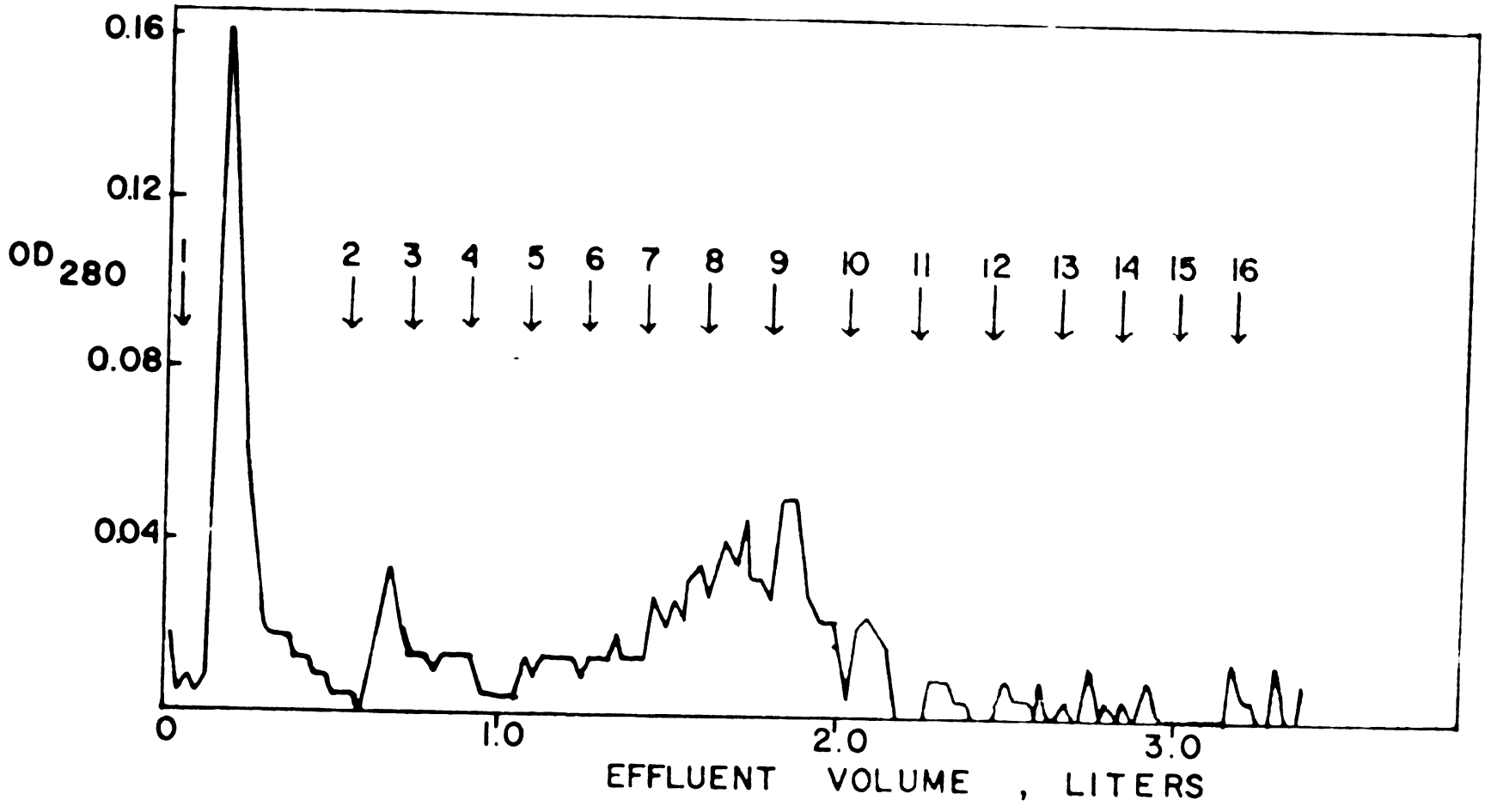
The samples taken from the ascending arm, descending arm and bottom of the electrophoresis cell, all formed three sperm specific lines when tested with rabbit anti-bull ejaculated sperm absorbed with blood sera and seminal plasma. The preparative electrophoresis method applied here was thus inadequate for the separation of individual sperm specific antigens. This could have been due to the relatively small differences in mobilities between the fastest sperm proteins and the slowest sperm proteins.

Column Chromatography

A preliminary study of separation of the soluble proteins of bull sperm on modified cellulose ion-exchange adsorbents was undertaken.

Figure 25 presents the chromatographic data obtained for stepwise elution from a DEAE column of the soluble proteins of bovine sperm. The first peak represented material which was not adsorbed on with the anion-exchange cellulose. This material when subjected to agar-gel-electrophoresis migrated toward the cathode indicating that it probably had a net positive charge at pH 7. Only 100 mg of soluble protein was placed on this column, and because 25 ml samples were collected, the observed OD_{280} values were very low. Despite this problem, the major portion

Fig. 25. Stepwise Elution Diagram of Saline Soluble Sperm Proteins from a DEAE-Cellulose Column. Twenty-five milliliter fractions were collected at a flow rate of 750 ml/hr. The number and vertical arrow show the point of change of the eluting buffer added to the column. The composition of buffers 1 — 16 is contained in the appendix.


0.16
0.12
OD 280
0.08
0.04
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
0
1.0
2.0
3.0
EFFLUENT VOLUME , LITERS

of the protein was eluted from the column by buffers with a molarity in regard to sodium chloride of between 0.175 M to 0. 225 M. This corresponds to buffers 7, 8, and 9 in Fig. 25. The protein eluted by buffers 8 and 9 after concentration, was subjected to agar-gel electrophoresis. No banding of components was observed. The material migrated as a broad diffuse zone of equal intensity toward the anode with the material eluted with buffer 9 migrating slightly further toward the anode than did the material eluted with buffer 8.

Figure 26 presents the chromatographic data obtained by gradient elution of soluble bull sperm proteins from a DEAE cellulose column. The first peak represented material which was not adsorbed on the anion-exchange cellulose. This material, when subjected to agar-gel electrophoresis, migrated toward the cathode indicating that it probably had a net positive charge at pH 7. Two hundred mg of soluble protein were placed on this column and 5 ml samples were taken and this procedure resulted in more acceptable OD_{280} values. Although the gradient elution produced a rapid change in conductivity and hence represented a rapid change in molarity of the eluting buffer, a sharp peak representing protein was eluted at approximately 0. 2 M sodium chloride. Little, if any, protein was eluted from the column when the concentration of sodium chloride was increased from 0. 35 M to 0. 70 M.

Figure 27 presents the chromatographic data obtained by gradient elution and stepwise elution from a CM cellulose column containing 100

Fig. 26. Gradient Elution Diagram of Saline Soluble Sperm Proteins from a DEAE-Cellulose Column. Five milliliter fractions were collected at a flow rate of 30 ml/hr. A linear gradient of NaCl was started at 300 ml. Final molarity shown is 0.35 M NaCl but up to 0.7 M NaCl failed to elute further protein. -o-o-o- equals conductivity measured with a Wheatstone Bridge on effluent.

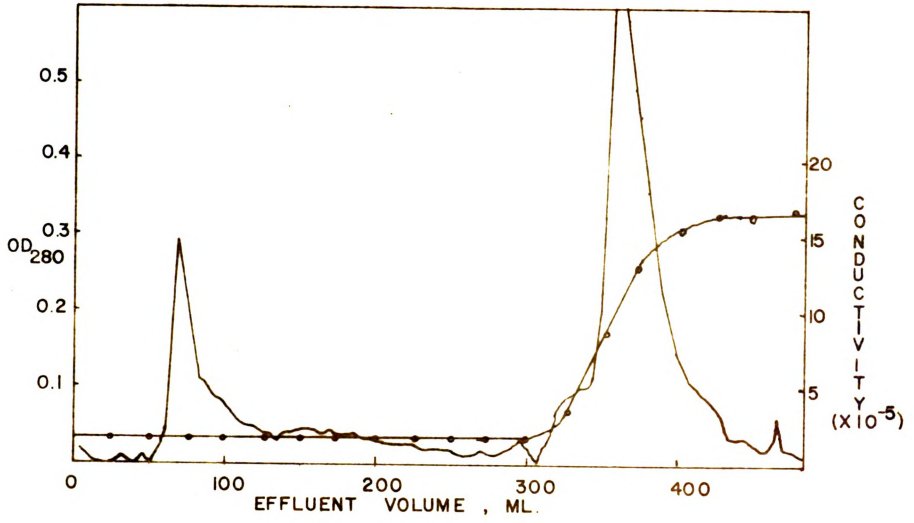
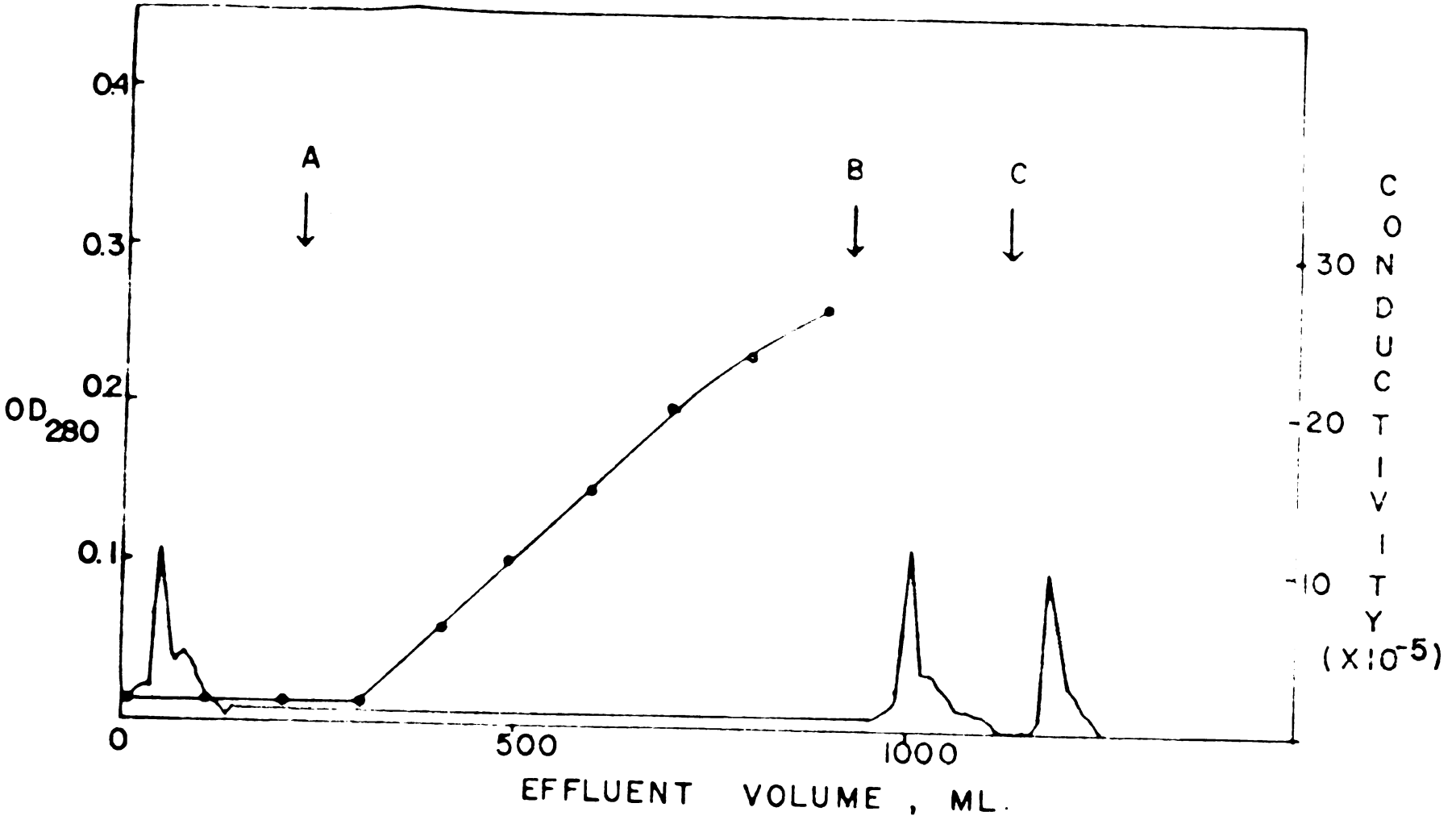

0.5
0.4
0.3
OD_{280}
0.2
0.1
0
100
200
300
400
EFFLUENT VOLUME , ML.
20
15
10
5
CONDUCTIVITY
(X 10^{-5})

Fig. 27. Gradient and Stepwise Elution Diagram of Saline Soluble Sperm Proteins Not Adsorbing to DEAE-Cellulose. The resin is CM-cellulose and five milliliter fractions were collected at a flow rate of 30 ml/hr. A linear gradient of NaCl from 0 to 0.7 M in 0.005 M Na_3PO_4, pH 6.0, was started (A) at 225 ml and stopped at 925 ml. At point B, the buffer was changed to 0.1 M Na_3PO_4, pH 7.6 and at point C, the buffer was changed to 0.5 M K_3PO_4, pH 11. -o-o-o- equals conductivity measured with a Wheatstone Bridge on effluent.


0.4
0.3
0.2
0.1
0
OD280
A
B
C
500
1000
EFFLUENT VOLUME , ML.
30
20
10
CONDUCTIVITY
(X10-5)

mg of sperm protein material which was not adsorbed on DEAE cellulose columns. A small initial breakthrough peak representing material not adsorbed on the cation-exchange cellulose was observed. A linear gradient of sodium chloride from 0 to 0.7 M in 0.005 M sodium phosphate buffer at pH 6.0 did not elute any protein from the column. This was interpreted to mean that the protein was tightly bound to the column. Earlier, the fact that this protein solution had a net positive charge at pH 7.0 had been established by agar-gel-electrophoresis and by DEAE chromatography. The CM chromatographic data provided evidence that this protein was positively charged at pH 6.0. A stepwise elution with 0.1 M sodium phosphate buffer at pH 7.6 resulted in some protein being released from the column. Further protein was eluted from the column with 0.5 M potassium phosphate at pH 11.

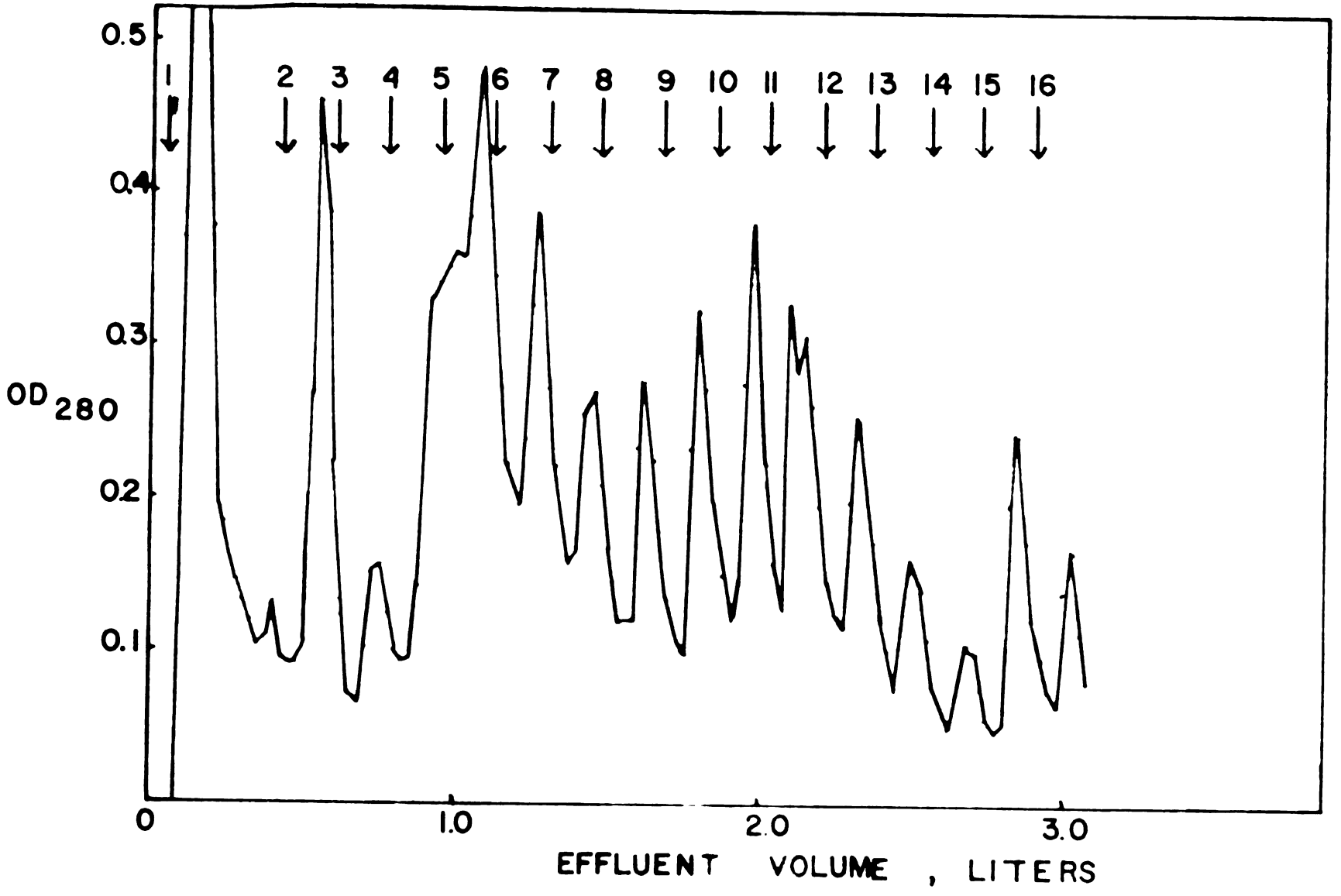
Fractionation of the Protein Antigens of Bovine Seminal Plasma

Column Chromatography

A preliminary study of the separation of the protein antigens of bovine seminal plasma on modified cellulose ion-exchange adsorbents was undertaken.

Figure 28 displays the chromatographic data obtained for stepwise elution from a DEAE column of the proteins of bovine seminal plasma. One g of bovine seminal plasma proteins was placed on the

Fig. 28. Stepwise Elution Diagram of Bovine Seminal Plasma Proteins from a DEAE-Cellulose Column. Twenty-five milliliter fractions were collected at a flow rate of 750 ml/hr. The number and vertical arrow show the point of change of the eluting buffer added to the column. The composition of buffers 1 → 16 is contained in the appendix.


0.5
0.4
0.3
0.2
0.1
OD 280
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
0
1.0
2.0
3.0
EFFLUENT VOLUME , LITERS

column and 25 ml fractions were collected of the material eluted from the column. The 16 buffers eluted 16 protein fractions from the column. The material in the two tubes with the highest protein concentration from each peak (Fig. 28) was concentrated and reacted in agar-gel-diffusion with rabbit anti-bull semen (Fig. 29). No evidence that a single antigenic protein had been separated by the step-wise elution of bovine seminal plasma was found by analyzing the agar-gel-diffusion plates. However, the antigenic complexity of bovine seminal plasma was simplified as shown by the fewer number of lines that formed with certain step-wise elution fractions of bovine seminal plasma.

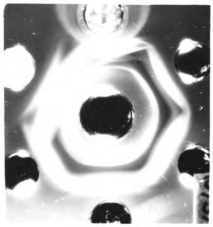
Fig. 29. Reaction of Anti-semen Sera with Seminal Plasma Antigens Fractionated by Anion Exchange Chromatography.

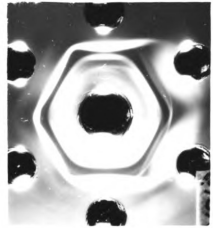
a.	Center well	Rabbit anti-bovine semen
	Well 1*	Buffer 2 effluent
	Well 2	Buffer 3 effluent
	Well 3	Buffer 4 effluent
	Well 4	Buffer 5 effluent
	Well 5	Buffer 6 effluent
	Well 6	Buffer 1 effluent

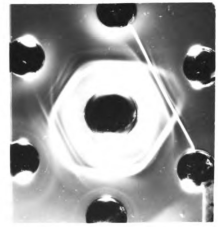
b.	Center well	Rabbit anti-bovine semen
	Well 1*	Buffer 8 effluent
	Well 2	Buffer 9 effluent
	Well 3	Buffer 10 effluent
	Well 4	Buffer 11 effluent
	Well 5	Buffer 12 effluent
	Well 6	Buffer 7 effluent

c	Center well	Rabbit anti-bovine semen
	Well 1*	Buffer 14 effluent
	Well 2	Buffer 15 effluent
	Well 3	Buffer 16 effluent
	Well 4	Tail suspension of bull semen
	Well 5	Bovine ejaculated bull sperm
	Well 6	Buffer 13 effluent

* Wells numbered clockwise from one o'clock.







SUMMARY

A study of the antigenicity and cross reactions of bovine seminal constituents was undertaken employing complement fixation, and agar-gel-diffusion. Bovine ejaculated sperm, seminal plasma and blood sera were found to contain common antigens such that if antibody was produced against one, it also reacted with the other two. The material responsible for the cross reactions of seminal plasma, blood sera and sperm with antisera to ejaculated sperm was of seminal plasma origin rather than being the result of leakage from the sperm cell. Sperm cells per se were shown to be antigenic when injected intradermally with Freund's adjuvant into rabbits. In addition, ejaculated sperm cells were shown to be coated with seminal plasma proteins. At least some of the seminal plasma coating antigens were found on epididymal sperm and were therefore deposited before the contributions of the major accessory sex glands came in contact with the sperm. Absorption with bovine blood sera of antisera prepared against ejaculated sperm, seminal plasma or blood sera, failed to remove all antibodies with the ability to react with epididymal sperm. Antisera prepared against epididymal sperm did not cross react with seminal plasma or blood sera but reacted with ejaculated and epididymal sperm.

A hemolytic factor was present in some samples of seminal plasma. The hemolytic factor was found to be non-dialyzable. Heating seminal

plasma at 56°, 70°, and 100°C decreased the hemolytic factor's activity with complete inactivation occurring at 100°C within 5 min. Seminal vesicular fluid was strongly hemolytic, suggesting that this was the origin of the hemolytic factor. The hemolytic factor was inhibited by normal bovine or rabbit blood serum. Half saturation with ammonium sulfate precipitated the hemolytic factor along with seminal plasma proteins. The collection of four consecutive ejaculates revealed that the concentration of the hemolytic factor in the second, third and fourth ejaculates was at least as great as that found in the first ejaculates. The presence of the hemolytic factor prevented the use of seminal plasma as an absorbent of antibodies in antisera used in the complement fixation test.

A study of the antigenic spectrum of bovine semen was undertaken employing agar-gel-diffusion and immuno-electrophoresis. Ejaculated sperm were shown to possess at least seven antigens when reacted with anti-ejaculated sperm immune sera. At least five of the sperm antigens were shared with seminal plasma and at least one was shared with blood serum. Absorption of anti-sperm immune serum with seminal plasma and blood serum revealed that sperm from the head and body of the epididymis and ejaculated sperm share at least three antigens which were not of seminal plasma or blood serum origin. The three sperm specific antigens stained as proteins and one of the three stained as a glycoprotein. Evidence, in the form of precipitin lines with calf thymus histone which

did not interact with precipitin lines of ejaculated sperm, demonstrated not all the soluble antigens could diffuse from the sperm cell during agar-gel-diffusion. No antigenic distinction could be made between head and tail suspensions of sperm with the Ouchterlony double diffusion technique. Immuno-electrophoresis revealed that sperm had at least six antigens when reacted with anti-sperm immune sera. One of these antigens was electrophoretically heterogeneous. Immuno-electrophoretic analysis using anti-sperm sera absorbed with seminal plasma and blood sera revealed three antigens of sperm origin rather than of seminal plasma origin. In agreement with the complement fixation data, the agar-gel-diffusion data showed that anti-bovine blood sera absorbed with blood sera continued to react with seminal plasma, ejaculated and epididymal sperm. The head and body of the epididymis contributed at least two protein antigens to the seminal plasma which were not found among blood sera proteins.

Saline extraction, freeze-thawing or mechanical rupture with glass beads released the same number of antigens from bovine sperm but mechanical rupture gave the largest yield of protein. Moving boundary electrophoresis in a Tiselius cell indicated the presence of at least three protein components in the saline extract of ruptured sperm. Sedimentation velocity ultracentrifugal analysis of the saline extract of ruptured bovine sperm revealed at least three sedimentation gradients. The two major gradients possessed S_{20} values of 1.7 and 12.6. Diffusion

coefficients of 4. 2 and 10. 2 Ficks were obtained for two of the three sperm specific antigens by an agar-gel-diffusion method.

Fractionation of the proteins in the saline extract of ruptured sperm with ammonium sulfate revealed that the three sperm specific antigens were precipitated at 33% and 60% saturation.

Fractionation of saline soluble sperm proteins with DEAE-cellulose chromatography revealed that a protein fraction was not adsorbed on the anion-exchange cellulose equilibrated with 0. 02 M phosphate buffer (pH 7). This protein fraction was tightly bound to CM-cellulose at pH 6. 0 and moved toward the cathode in agar-gel-electrophoresis. The proteins adsorbed on DEAE cellulose equilibrated with 0. 02 M phosphate buffer (pH 7) were eluted in the range of 0. 175 M to 0. 225 M sodium chloride.

Step-wise elution of seminal plasma antigens from a DEAE-cellulose column with sodium chloride in 0. 02 M phosphate buffer (pH 7) resulted in 16 protein peaks, each composed of several antigens.

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APPENDIX

I. Selected Stains for Immunodiffusion (Crowle, 1961)

A. Protein Stain

0.1 g amidoschwarz 10 B

45 ml 12% acetic acid

45 ml 1.6% sodium acetate

10 ml glycerol

Differentiate in 2% acetic acid

B. Double Stain for Protein and Lipid

50 mg azocarmine B

2 ml acetic acid

100 ml 60% ethanol saturated with Sudan black B

Stain pre-dried agar for 25 minutes. Differentiate with 50% ethanol containing 1% acetic acid.

C. Glycoprotein and Polysaccharide Stain

1. Solutions

   a. 1 g periodic acid

      1.64 g anhydrous sodium acetate

      100 ml 50% ethanol

   b. 144 mg alpha naphthol

      100 ml distilled water

      Dissolve with heat and then cool.

c. 108 mg para-phenylenediamine

100 ml distilled water

Prepare immediately before using.

d. 10% hydrogen peroxide

2. Procedure

Soak pre-dried agar for 15 min in "a."

Wash for 10 min in running water and for 5 min in distilled water.

Soak for 5-10 min in solutions of b:c:d 5:5:1, freshly mixed.

Wash for 10 min in running water and finally in distilled water.

II. Complement Fixation Test

A. Preparation of Barbital-Sodium Chloride Diluent

1. Weigh out the following ingredients:

a. 85.0 g NaCl

b. 5.75 g 5, 5-diethyl barbituric acid

c. 3.75 g sodium 5, 5-diethyl barbituric

2. Dissolve the acid in 500 ml hot double distilled water.

3. Add the other ingredients.

4. Cool and make up to 2000 ml with double distilled water.

5. Each day dilute accurately 1 part up to 5 with water.

6. The pH of the diluted buffer should be 7.3 to 7.4.

B. Titration of Hemolysin

1. Prepare sheep cells.

2. Prepare a 1:20 stock solution of hemolysin by mixing.

   2 cc of 50% glycerinized hemolysin

   9 cc of buffer

   9 cc of neutral glycerine

3. Restore guinea pig complement according to manufacturer's directions.

4. Make a 1:20 stock solution of complement by placing 0.5 cc in 9.5 cc buffer.

5. Arrange 8 test tubes and mark 1 - - - 8.

6. Add 0.5 ml buffer to tubes 2 through 8.

7. Place 0.8 ml buffer in tube 1.

8. Add 0.2 ml of 1:20 hemolysin to tube 1, giving 1 ml of 1:100 solution.

9. Transfer 0.5 ml from tube 1 to tube 2.

10. Transfer 0.5 ml from tube 2 to tube 3.

11. Continue transfers to tube 8 and discard 0.5 ml from tube 8 after mixing.

12. Add 0.1 ml portions of unsensitized sheep cells to all tubes with concentration of 1,000,000 cells per cu mm.

13. Add 0.2 ml of 1:20 guinea pig complement to all tubes.

14. Incubate 30 min at 37^{o} - 38^{o}C.

    The highest dilution of hemolysin giving complete lysis is taken as one unit.

    Use 4 units for sensitization.

B. Sensitization of Sheep Red Blood Cells

1. Prepare a 1:1600 dilution of hemolysin as follows:

0. 1 ml of 1:20 solution of hemolysin diluted to 80 ml with buffer.

2. Mix the 1:1600 hemolysin with an equal amount of sheep cells ($1,000,000/mm^3$) by pouring the hemolysin slowly into cell suspension with constant agitation.

3. Incubate at 37° - $38^{\circ}C$ for 10 min.

4. Keep in ice water.

C. Titration of Complement

1. Set up 7 test tubes and mark 1 - 7.

2. Place 0. 2 ml sensitized cells in each tube.

3. Make up 1:20 complement by adding 0. 05 ml restored complement to 0. 95 ml buffer.

4. In tube 1, place 0. 025 ml of 1:20 complement.

5. In tube 2, place 0. 050 ml of 1:20 complement.

6. In tube 3, place 0. 075 ml of 1:20 complement.

7. In tube 4, place 0. 100 ml of 1:20 complement.

8. In tube 5, place 0. 125 ml of 1:20 complement.

9. In tube 6, place 0. 150 ml of 1:20 complement.

10. In tube 7, place 0. 200 ml of 1:20 complement.

11. Add 0. 575 ml buffer to tube 1.

12. Add 0. 550 ml buffer to tube 2.

13. Add 0. 525 ml buffer to tube 3.

14. Add 0. 500 ml buffer to tube 4.

15. Add 0. 475 ml buffer to tube 5.

16. Add 0. 450 ml buffer to tube 6.

17. Add 0.400 ml buffer to tube 7.

18. Incubate at 37°- 38°C for 30 min.

19. Divide dilution factor (df), in this case 20, by volume (V_L) which produced lysis:

$$\frac{df}{V_L} = \text{units/ml of restored complement.}$$

D. Complement-Fixation Test

1. Dilute rabbit antiserum (1:10).

2. Inactivate at 56°C for 30 min.

3. Dilute complement with buffer so that 0.1 ml contains 1 unit.

4. Set up and mark tubes.

5. To each tube add 0.2 ml inactivated antiserum.

6. Add 0.2 ml diluted complement.

7. Mix.

8. Add 0.2 ml antigen (determine dilutions beforehand).

9. Mix and incubate 40 min at 37°C.

10. Add 0.2 ml sensitized sheep cells.

11. Incubate with occasional mixing for 30 min at 37°C.

E. Controls That Must Be Run with Complement Fixation Test

1. Antigen + complement + buffer

2. Antigen + buffer

3. Antigen + antisera + buffer

4. Antigen + complement + buffer

5. Antisera + buffer

6. Complement + buffer

7. Buffer

III. 0.005 M Phosphate Buffered Saline (pH 7.4)

Na_2HPO_4 ..... 1.4198 g

KH_2PO_4 ..... 0.3233 g

NaCl ..... 21.0375 g

HOH ..... 2475 ml

IV. Preparation of Buffers for Stepwise Elution of Chromatography Columns

Buffer No.	Phosphate (M)	NaCl (M)	NaCl g/l	NaCl g/200 ml
1.	0.02	-	-	-
2.	0.02	0.050	2.9225	0.5845
3.	0.02	0.075	4.3838	0.8768
4.	0.02	0.100	5.8450	1.1690
5.	0.02	0.125	7.3063	1.4612
6.	0.02	0.150	8.7675	1.7535
7.	0.02	0.175	10.2288	2.0457
8.	0.02	0.200	11.6900	2.3380
9.	0.02	0.225	13.1513	2.6302
10.	0.02	0.250	14.6125	2.9225
11.	0.02	0.275	16.0738	3.2147

Preparation of Buffers for Stepwise Elution of Chromatography Columns (Continued)

Buffer No.	Phosphate (M)	NaCl (M)	NaCl g/l	NaCl g/200 ml
12.	0.02	0.300	17.5350	3.5070
13.	0.02	0.325	18.9963	3.7992
14.	0.02	0.350	20.4575	4.0915
15.	0.02	0.500	29.2250	5.8450
16.	0.02	1.000	58.4500	11.6900