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PHYSICOCHEMICAL AND ELECTRO-PHORETIC METHODS.

The Ohio State University, Ph.D., 1974
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BOVINE SEMEN ISO-ANTIGENS: DETECTION, DETERMINATION OF ORIGIN AND PARTIAL CHARACTERIZATION BY IMMUNOLOGICAL, PHYSICO-CHEMICAL AND ELECTRO-PHORETIC METHODS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Kirkland Earle Mellad, B.Sc., M.Sc.

The Ohio State University
1974

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INTRODUCTION

The ability of animals to form antibodies against spermatozoa was first demonstrated in 1899. The question of whether the immunization of females against spermatozoa would affect the breeding performance was soon raised, but no satisfactory answer was forthcoming. Many experiments were carried out on rats, rabbits, guinea pigs, and even, perhaps prematurely, on women. Although positive results were sometimes claimed, they sometimes proved difficult to substantiate, since more extensive and carefully controlled investigations failed to confirm earlier findings. The great number of conflicts in reports has certainly stimulated researchers' interests over the years, thus leading to more detailed investigations in this field of immuno-reproduction.

Immunization is an acceptable process in most parts of the world, so if immunological control of fertility becomes feasible it is likely that this method will be an extremely popular one. There are quite a number of limitations in the immunological induction of infertility, the foremost of which seems to be the side effects produced by adjuvants. The use of adjuvants in order to enhance the immunological response is very popular, but there are reports which indicate that the Freund type of adjuvant induces proliferative lesions in the brain, liver and kidneys of mice, guinea pigs and hamsters, in addi-
tion to inducing the development of unsightly abscesses. Much more investigation needs to be done in this area of research if fertility control by immunological means is to become a reality.

Over the past years, evidence has been accumulating that semen or washed spermatozoa can prevent fertilization or induce embryonic death. Data from rabbits have indicated that iso-immunization with semen or testicular material decreased the rate of embryo survival as well as fertilization rate. There is evidence that iso-antibodies against semen might lower conception rate and reduce embryo survival in cattle. Auto-antibodies have been found occasionally in the serum of men and these have been positively correlated with the fertility status in many cases. Natural sperm agglutinins have been found in women with poor fertility, suggesting that these females might have been naturally iso-immunized with semen antigens during coitus.

Spermatozoa are suspended in a fluid medium from the time of their formation to the time of their exit in the ejaculate. Several studies have shown that the spermatozoa, in addition to having specific antigens, acquire some additional ones from the various areas of the reproductive tract. These antigens serve as a coat for the sperm, and hence they are designated sperm-coating antigens, with most of them arising from the seminal vesicles. The importance of the sperm coating antigens from the accessory sex glands in the induction of infertility has not been elucidated, since most studies indicate that the antigens which evoke antibody production for the lowering of fertility are of testicular origin. Most studies relating to the origin of semen antigens have been conducted through hetero-immunization. It is obvious that many of the antigens
detected by hetero-immunization are species specific, and thus probably of little practical importance in terms of intra-species incompatibility effects. Studies on these hetero-antigens have been conducted in many species, including the bovine, and partial characterization of some of these antigens has been achieved.

In cattle there is much to be learned about iso-antigens of the male reproductive tract which might be implicated immunologically in the reproductive process. If one is hoping to lower or improve fertility in an individual by immunological means, a proper knowledge of the iso-antigens is absolutely necessary. The aims of this investigation are to determine (1) the degree of iso-antigenicity of bovine semen antigens, (2) the origin of bovine iso-antigens in the male reproductive tract, (3) the degree of cross-reactivity between these iso-antigens and substances in other bovine secretions, (4) and to partially characterize these antigens in terms of their physical and chemical nature. It is hoped that the information obtained from this investigation will help to explain the mechanism or mechanisms involved in the immunological induction of infertility in man, cattle and other animals. A more complete understanding of semen iso-antigens and the corresponding antibody response should provide a basis for the development of techniques or methods which can be utilized for restoring fertility, or inducing sterility as a means of population control.
REVIEW OF LITERATURE

Semen Antigens and Fertility

Evidence has been accumulating in recent years that antibodies against semen or washed spermatozoa can prevent fertilization, or may cause embryonic death. Weil and Rodenburg (1960) and Weil and Finkler (1958) reported that antigenic material is taken up by the spermatozoa from the seminal plasma. There is also evidence that spermatozoa possess antigenic properties originating in the spermatozoa themselves as indicated by Freund, Thompson and Lipton (1955). Pernot and Szumowski (1958), demonstrated that spermatozoa and seminal plasma shared common antigenic constituents.

The occurrence of natural iso-immunization with seminal antigens has been suggested as one possible cause of female infertility in mammals. The ability of antibodies against semen to cause increased levels of fertilization failure and embryonic death suggests that this might be one of the causes of infertility in cattle. A report by Franklin and Dukes (1964) indicated that a high percentage of women with unexplained infertility possessed sperm agglutinating antibody. On the basis of this finding it was hypothesized that it is possible for absorption of antigenic components to occur from the reproductive tract during coitus rendering the women immunized.
The antigenicity of bull spermatozoa was confirmed by Metchnikoff (1899) who tested for agglutinins and sperm immobilizing antibodies. Henle, Henle and Chambers (1938) working with bull spermatozoa, demonstrated head-specific and tail-specific antigens by means of complement fixation and slide agglutination. Whether or not spermatozoa were themselves antigenic was questionable in the light of the work of Weil and Finkler (1958) who found that both guinea pig anti-rabbit seminal plasma and anti-rabbit sperm immune sera strongly agglutinated spermatozoa from fresh semen as well as three times washed rabbit-spermatozoa. The antigens of rabbit spermatozoa and seminal plasma were so closely related that they could not be differentiated by these investigators.

In 1959, Isojima and Stepus discovered that the mature testis and sperm of guinea pigs contained at least three tissue-specific antigens, but these same antigens were not present in the immature testis of 2 week old guinea pigs. Work done by Isojima and Li (1968) revealed that at 30 days of age, Sprague-Dawley rats had one testes specific antigen which was not present in other organs, even though no sperm was present microscopically. Hunter and Hafs (1964) reported that ejaculated spermatozoa possessed at least seven antigens. At least five of these were shared with seminal plasma and at least one with blood serum. Although there is as yet no uniform view about the origin, the proportion, and distribution of seminal antigens in any species, it seems likely from the results of many authors, including Matousek (1964) and Weil and Rodenburg (1960), that both spermatozoa and seminal plasma have specific antigens.

Beck and his colleagues (1962) used the immunofluorescent antibody technique to determine the iso-antigenicity of mammalian spermatozoa.
They reported that serum from guinea pigs immunized with homologous spermatozoa stained the surface of homologous sperm tails, in addition to the acrosomal staining. Immunization of rabbits with homologous spermatozoa, however, failed to produce any tail staining. They suggested that the tail staining of the guinea pig spermatozoa was due to an induced iso-antibody. They claimed that tail agglutination is considered to be specific for induced iso-antibodies against spermatozoa; the tail of the spermatozoa seems to be the site of the agglutinating antigen. Menge and Christian (1971) were able to determine the presence of at least three auto-immunogens in bull spermatozoa. This was accomplished by the use of the bull immune sera in immunodiffusion and immunoelectrophoresis.

Origin of hetero- and iso-antigens

There are many conflicting reports regarding the source and origin of sperm specific and miscellaneous antigens. The mixing and interaction of many components from various areas of the male reproductive tract, as well as different methods for processing and analysis, undoubtedly accounts for some of this disagreement. Sperm are suspended in a fluid medium from the time of their formation in the testes to the time of their exit from the male in the seminal ejaculate. Crabo (1965), Nicander (1965) and Satchell et al. (1969) reported that the composition of this fluid varies from region to region in the male reproductive system due to regional secretion and or absorption. The fluids originate in the seminiferous tubules, rete testis, epididymis, vas deferens and within the major accessory sex glands which contribute the bulk of the volume to the ejaculate. Seminal plasma is a composite of all these secretory
fluids. The time that any secretion is in contact with the sperm depends upon the transit time through the ductular system, the ratio of secretion to absorption for specific secreted products and whether specific secretion can be adsorbed onto the surface of the sperm. Hunter in (1969) reported that rabbit sperm were coated with two antigens originating in the testis, two from the epididymis and eight from above the level of the vas deferens. Barker and Amann (1970) showed that differences in the antigenic spectrum of sperm also arise during epididymal maturation. Baum (1959) and Katz (1960) reported that the appearance of antigen in the guinea pig sperm occurred at late spermatid stage. This is consistent with reports from Barth and Russell (1964) concerning mouse spermatozoa.

Johnson and Hunter (1972), using the fluorescent antibody technique, also reported that the sperm-specific antigens of rabbits became detectable at the spermatid stage of spermatogenesis. Johnson and Setchell (1968) showed, by absorbing antisera to ram rete testis fluid with blood sera, that only antigens present in the blood serum were constituents of rete testis fluid. This report is in agreement with work done by Johnson and Hunter (1972) with the rete testis fluid of rabbits. These authors also demonstrated that these antigens, associated primarily with the acrosome and the cytoplasmic droplet, and to a lesser extent with the midpiece and tail, were also associated in an amorphous mass which disappeared after transport from the testis to the caput epididymis. Johnson and Hunter also reported that the gradual loss of fluorescence by cytoplasmic droplets during passage through the epididymis was paralleled by the appearance of similarly detectable antigens in the epithelia of the epididymis, especially in the cauda epididymis. The phenomenon was more
pronounced in sexually rested bucks than in sexually active bucks, suggesting loss of antigens from the spermatozoa and absorption by the epididymal epithelia.

Tissue-specific and cross-reacting antigens have been described for the prostate by Barnes et al. (1965), Rao and Badri (1960), Yantorni et al. (1966), and for the seminal vesicles by Weil (1961), and Weil and Finkler (1958). Unfortunately, not many investigators have been able to explain satisfactorily the origin and reciprocal sharing of antigens from the spermatozoa and the seminal plasma. Barker and Amann (1969) used immunodiffusion and immunoelectrophoretic analyses to determine the heterogeneity of antigens in bull spermatozoa and reproductive fluids and to characterize antisera against these antigens. They produced antisera against bull seminal plasma, seminal vesicle fluid, cauda epididymal plasma, blood serum, washed ejaculated spermatozoa and sperm fractions in rabbits. Their immunologic tests revealed four sperm-specific antigens. One antigen was located in the sperm head, one in the sperm tail and two others were common to both head and tail. They also indicated that the major antigens of spermatozoa were localized in the cell membrane and acrosome. These authors also reported the presence of sperm antigens in the cauda epididymal plasma and seminal plasma, suggesting that these antigens may have been released by physiologically normal spermatozoa or they may represent end products of sperm dissolution within the epididymis and vas deferens. Their reports also indicated that cauda epididymal plasma and seminal vesicle fluid shared at least two antigens not present in the blood serum.

The origin of the antigens in semen has received considerable
attention. However, the exact origin of antigens that induce antibodies which cause infertility has not been studied in any great detail. With this thought in mind, Menge and Protzman (1967) undertook a study to determine the origin of the antigens in rabbit semen that induce the antibodies which prevent spermatozoa from fertilizing ova in rabbits. These authors reported that cattle and guinea pig antisera against rabbit testis, epididymal spermatozoa and ejaculated semen had high titers of sperm agglutinins and prevented fertilization of ova in rabbits bred with semen treated with these sera. Although cattle antisera produced against seminal plasma from vasectomized rabbits, 9 day old rabbit conceptuses and rabbit brain, and guinea pig antiserum to seminal plasma containing epididymal secretions exhibited relatively high titers of sperm agglutinins, none of these sera prevented spermatozoa from fertilizing ova. The antifertility antibodies were removed or inactivated by absorptions with testis material, epididymal spermatozoa and semen, but not by absorption with seminal plasma. This indicates clearly that in the rabbit the source of semen antigens that elicit antibodies capable of preventing fertilization is the testis. Menge (1968) claimed that the semen antigens which are capable of inducing infertility iso-antibodies were probably an integral part of the sperm cell, as suggested by results in rabbits in which washed epididymal spermatozoa were capable of inducing iso-antibodies and infertility. Menge and Protzman (1967), using the agar gel-diffusion and immunoelectrophoretic techniques revealed that ejaculated rabbit semen contained at least nine antigens. Seven were common to seminal plasma, two to blood serum and two appeared to be spermatozoa specific, originating in the testis.
Spermatozoa-coating antigens

The seminal vesicles of man and rabbit produce a highly antigenic material which becomes firmly attached to the surface of the seminal spermatozoa. For that reason, the designation of spermatozoa-coating antigen has been suggested for this substance by Weil and Rodenburg (1962). Although the sperm-coating antigen is species- and organ-specific, an antigenic relationship with lactoferrin has been reported by Heckman and Rumke (1969). Kirton and Hafs (1965) hypothesized that the removal of the sperm-coating antigen from the spermatozoa was involved in capacitation. Weil and Stefanovic (1969) reported that the decapacitation factor is found in the epididymis, whereas sperm-coating antigen is the product of the seminal vesicles. These investigators also demonstrated that the spermatozoa of the rabbit retain this coating of antigen on their travel through the female tract up to the fallopian tube, thus contradicting the hypothesis of Kirton and Hafs.

Roberts and Boettcher (1969) identified the sperm-coating antigen of human spermatozoa as an iron binding protein present in the seminal plasma. It was shown to have an electrophoretic mobility similar to transferrin, and different from lactoferrin, whereas it shared immunological characteristics with lactoferrin rather than transferrin. With these features in mind, these investigators proposed the name scaferin for this compound.

Hunter and Nornes (1969) suggested that a sperm-coating antigen of seminal plasma origin possessed the biological activity to block fertilization in rabbits. This antigen was identified as a glycoprotein of approximately 170,000 molecular weight, and it showed an electro-
phoretic mobility similar to serum β-globulin. This sperm-coating antigen was absent from the inactive supernatant fluid fraction of seminal plasma after 4 hr of ultracentrifugation at 105,000 g. Hunter (1969), showed that ejaculated rabbit spermatozoa possessed twelve seminal plasma antigens which coated the spermatozoa during their passage through the reproductive tract. Two of these sperm-coating antigens were reported to originate in the testis, two in the epididymis and eight above the level of the vas deferens. This author hypothesized that these sperm-coating antigens that are of seminal plasma origin might block or coat the antigenic reactive sites on the surface of the spermatozoa and normally prevent immunologically induced aspermatogenesis in the male or immunologically induced infertility in the females.

Auto-antibodies and fertility

It is now well established that some infertile males possess auto-antibodies against spermatozoa. As was originally described by Wilson (1954), these auto-antibodies agglutinate and sometimes immobilize the otherwise normal spermatozoa in the ejaculate. The agglutinated spermatozoa can no longer penetrate the cervical mucus so that the patient is infertile. Rumke in 1954 and Rumke and Hellinga (1959) reported that sperm agglutinins were present in the seminal fluid as well as in blood plasma, and that they were specific for spermatozoa. They indicated that most sera agglutinated the spermatozoa by their tails or by tails and heads, while some sera only agglutinated the heads. These investigators also reported that there was, in general, a parallel between serum titers and the inability of the patient's spermatozoa to invade the cervical mucus.
They indicated that there were a few exceptions to the rule, since some patients had strong auto-agglutination with rather low serum titers, and occasionally a patient had only partial and slow starting agglutination in spite of a high serum titer.

The finding of serum agglutinins in the serum of patients with obstructed efferent ducts has also been reported by Phadke and Padukone (1964). They discovered sperm agglutinins in the serum of 8 out of 25 men whose vas had been ligated as a family limitation measure two to twenty years previously. In cases of obstruction many pathologists including Rumke (1965) have observed extravasation of sperm into the interstitium of the epididymis accompanied by infiltration of mononuclears, and even spermatozoa in the lymph vessels. Rumke and Helllinga (1959) reported that of 4 vasoligated Jewish men who were examined, one seemed to have sperm agglutinins of the tail type in the serum in a high titer and this seems to support their conclusion that obstruction may result in the formation of antibodies. These investigators discovered that in one of their patients with strong auto-agglutination in the ejaculate, one testis was atrophied after the funiculus was cut through by accident during herniorrhaphy. In this instance, resorption of testicular tissue and spermatozoa was probably the cause of the formation of antibodies.

Numerous experiments, some performed as early as the turn of the century, have shown that spermatozoal or testicular substances can evoke antibodies to spermatozoa. Shulman et al. (1968) reported that an auto-antigen was present in the rabbit seminal vesicle, coagulating gland, bulbourethral gland and seminal plasma. The presence of this antigen was
revealed by auto-immunization of rabbits. Mancini et al. (1965) reported that patients with prostatic carcinoma, immunized with their own testicular tissue incorporated in Freund’s complete adjuvant, developed antibodies specifically reacting with spermatozoa. Thus, patients who resorb their own spermatozoa may be expected to respond with iso-antibody against spermatozoa. Menge and his co-workers (1971) reported that intratesticular injections of 3 bulls with semen or mature testis material with Freund’s complete adjuvant resulted in immediate lesioning and aspermato genesis of the injected testis and eventual aspermatogenesis of the contralateral testis in two of the bulls.

One might ask why three-quarters of the males with obstructions to the reproductive tract do not develop sperm agglutinins in spite of presumed excessive resorption. Phadke (1964), in an effort to answer this question, reported that sperm extravasation in the epididymis was rare in such cases and that a variable mechanism of sperm phagocytosis could account for differences in auto-antibody response. Other possible explanations for the variable sperm agglutination formation in cases of obstruction were given by Rumke (1968), and they are as follows:

1. Resorption of sperm antigens may also induce immune tolerance.
2. An adjuvant inflammatory effect may be necessary to initiate antibody formation.
3. Other kinds of antibodies could block the formation of sperm agglutinating antibodies.
4. Weakly antigenic stimuli may be effective only with individuals genetically more prone to produce antibodies than others.

Other possible explanations for sperm agglutinin formation are suggested
from the experimental work of Weil and his co-workers (1965). These authors found that spermatozoa receive a coating of an antigen derived from the seminal vesicles. Since it was believed that the substance was organ specific it could be postulated that an auto-immune syndrome of the seminal vesicles might exist and that in this case auto-antibodies against the coating antigen would manifest themselves as sperm-specific agglutinins.

**Effects of iso-antibodies on fertility**

Infertility in the female has been induced in several mammalian species by immunization with homologous semen, spermatozoa and testis. In mice, McLaren (1964) reported that induced infertility was due to inhibition of fertilization. Menge (1958), working with rats, reported that iso-immunization with semen and testis decreased the rate of embryo survival as well as the fertilization rate. This investigator also indicated that immunization with homologous conceptus material lowered embryo survival, whereas immunization with seminal plasma had no effect on fertility. Menge, Stone, Tyler and Casida (1962) reported that fertilization was inhibited in cattle by treating semen with anti-semen sera before insemination. Kiddy *et al.* (1959) reported a similar anti-fertilizing effect in rabbits that were iso-immunized with semen. It seems obvious from these reports that iso-antibodies against semen can cause early embryo death as well as inhibition of fertilization.

Menge and his colleagues (1972), reported the incidence of high mortality rates for embryos that were transferred into the reproductive
tracts of female rabbits iso-immunized with semen. These investigators claimed that embryo mortality induced by immune reactions against semen may occur in the oviduct, and definitely occurs in the uterus before and after implantation. They concluded that the antifertility effect of these iso-antibodies appeared to be acting directly on the embryo and not on the mechanisms of implantation. Menge et al. (1972) also indicated that the iso-immunization of the female rabbits with semen exerted an antifertility effect that was observable through inhibition of fertilization and several stages of pregnancy. These investigators were, however, unable to determine the exact immune mechanism involved in the induction of embryo degeneration. Bell (1969) was unable to suppress fertility in rabbits by immunization with homologous spermatozoa in sodium alginate. He suggested that the induction of infertility observed could be accounted for by a failure of fertilization, implying a lethal effect on the spermatozoa themselves, rather than anaphylaxis as postulated by Katsh (1957, 1959), or death of the embryos as suggested by Menge (1967, 1969).

The degree of fertilization failure and embryo loss in female guinea pigs injected with guinea pig testis homogenate and Freund's adjuvant was measured by Kiddy and Rollins (1973). Controls were injected with saline and adjuvant. The fertilization rate was 91.6% in 26 control animals and 88.0% in 36 experimental animals. The average percentage of embryos surviving in 46 antigen treated females was 45.8 compared to 72.7 in 19 controls. These investigators suggested that the major cause of reproductive failure was early embryonic death. Like Menge et al. (1972), they failed to identify the mechanisms involved, but they presumed the loss of the embryo to be due to reaction of antibodies in the
uterus with the antigens borne by the embryo itself. Katsh (1959) postulated that the infertility might result from contractions of the uterus upon contact with semen. He has shown that specific contractions occurred in the uteri from immunized guinea pigs when challenged with guinea pig sperm in vitro. He suggested that such contractions might interfere with sperm transport or with implantation. The findings of Kiddy et al. (1973) would seem to cast doubt on the importance of the interference with sperm transport, since their investigation revealed a high incidence of ova fertilization. If specific uterine contractions are important, they might interfere with post-fertilization development.

Menge and his co-workers (1962) reported that there were no significant differences observed in the survival rates of embryos treated at 1 day or 9 days of age with normal sera, and immune sera produced against rabbit erythrocytes. The absence of an effect on embryo survival by antibodies produced against erythrocytes is significant in the light of the work of Chung et al. (1961), suggesting that ABO blood group incompatibility was a major cause of fetal death. The conditions of Menge's experiments were grossly different, however, from those existing in human pregnancy. The rabbit embryos were treated only once at a specific stage of development, whereas under normal conditions in human they may be challenged continually with naturally occurring and immune blood group antibodies.

In addition to laboratory animals, a fairly great amount of fertility studies has been done with the bovine species. Menge and Christian (1971) studied the effects on semen quality and spermatogenic activity of the testes in bulls that were auto- and iso-immunized with
semen and homogenized testis material combined with Freund's complete adjuvant. They reported that the effects ranged from little or none in three of the eleven bulls used, to complete depression of semen quality, and aspermatogenesis in three other bulls. They indicated that aspermatogenesis was induced only after prolonged periods of immunization and aspermic semen production. They postulated that production of aspermic semen after immunization appeared to be due to a heavy influx of leucocytes into the seminiferous tubules and rete testis, which prevented the passage of sperm into the epididymis. Previous work in bulls by Johnson et al. (1964) indicated no effects on semen quality and spermatogenesis after auto-immunization with semen, which is contradictory to Menge's findings.

With growing interest in the area of semen antigens and their effect on fertility, Menge (1967), decided to perform more extensive studies on their effects in heifers. Some heifers were iso-immunized with bull semen and homogenized testis combined with Freund's complete adjuvant. A temporary lowering of fertility was reported. Eleven heifers injected intradermally with semen and six heifers with testis required an average of 4.11 and 4.83 inseminations per conception respectively, compared with nine control heifers which averaged 1.11 services per conception. He also reported that four of the diagnosed pregnancies of the iso-immunized heifers underwent degeneration resulting in embryo loss. In addition to these evidences of impaired fertility, sperm agglutinins were observed in the serum, and uterine and vaginal secretions of all the iso-immunized heifers.
Antifertilizing effect of seminal vesicular fluid

The antifertilizing effect of the bull seminal vesicle fluid was reported by Matousek (1966, 1969). He reported the damage to spermatogenesis in mice, rats, guinea pigs, rabbits and rams after intramuscular or subcutaneous administration of bull seminal vesicular fluid. Injections of bovine seminal vesicle fluid was shown to produce a decrease in the index weight of the testes, a disturbance of spermatogenesis, and in more severe cases, a distortion and contraction of the seminiferous tubules. The inhibitory effect of this aspermatogenic substance was also reported in female mice and hens by Matousek and Petrovska (1969).

Matousek et al. (1972) performed a series of experiments to characterize the aspermatogenic substance, and to discover its sensitivity towards different temperatures and enzymes. They reported that the biosynthesis of the aspermatogenic substance in the bull's seminal vesicle was dependent upon the androgen level. This was supported by the fact that the aspermatogenic substance was not present in sexually immature bulls, but its synthesis could be evoked by exogenous administration of testosterone propionate. It was also shown by these investigators that castration, followed by testosterone treatment resulted in a resumption of the secretion of the seminal vesicle fluid and a restoration of the aspermatogenic effect. Various experiments showed this substance to be thermostable and resistant to digestion by a number of different enzymes including proteolytic enzymes. These authors believe that this aspermatogenic substance might be bound to a protein complex in order to induce aspermatogenesis. It was reported by these investigators that rabbits developed several antibodies against bull seminal vesicular
fluid treated with enzymes, as judged by double diffusion, immunoelectrophoresis and hemagglutination tests. Matousek (1969), demonstrated that antibodies produced by animals which were immunized subcutaneously did not cause sperm agglutination, immobilization or spermatoxic reactions. No difference in antibody response was observed in rabbits with damaged or undamaged testes after injections of the seminal vesicle fluid. Matousek and his co-workers (1972) suggested that the action of the aspermatogenic substance might not be immunological since it produced rapid degenerative changes in the testis which could be observed 3 days post-injection.

With a growing interest in the behavior of this designated aspermatogenic substance, Dostal et al. (1973), proceeded to make more detailed studies of its chemical properties. They isolated the aspermatogenic substance from the seminal vesicles of bulls by precipitation with acetic acid and ammonium sulfate and subjected it to column chromatography. It was shown to exist in two polymeric forms. The molecular weight of the monomer was reported to be 22,000 and that of the dimer 44,000. The aspermatogenic substance was shown to be pure as far as could be detected by disc electrophoresis, immunoelectrophoresis in agar gel and by ultracentrifugation. The N-terminal amino acid was lysine and the sedimentation coefficient was 2.7. Further work by Matousek and his colleagues (1973) revealed that fractions of bull seminal vesicle fluid containing ribonuclease activity significantly increased embryonic mortality in female guinea pigs and rabbits. It was found that the disturbances of embryogenesis in the rabbits occurred mainly in the post-implantation period.
Characterization of Testicular and Epididymal Fluids

Immunoelectrophoretic studies

The secretion and absorption of protein, changes in antigenicity of sperm undergoing maturation and other aspects of epididymal physiology have been elucidated by immunological studies done by Hunter and Hafs (1964), Matousek (1964), Hunter (1969), Barker and Amann (1970, 1971), and Johnson and Hunter (1972). In each of these investigations it was impossible to determine if antigens that were detected in the cauda epididymal plasma or antigens associated with sperm were of epididymal origin. According to Killian and Amann (1973) the antigenic composition of sperm and fluid entering the epididymis was not clearly established, so they designed experiments which would be investigative in this area. In their immunoelectrophoretic study of the antigenic characteristics of sperm and fluids recovered via cannulae implanted in the rete testis and proximal vas deferens of living bulls, these investigators revealed that certain antigens which were detected in the seminal vesicle fluid were also present on testicular as well as epididymal sperm. These authors also reported that certain antigens present in rete testis fluid, but not detectable in blood serum, were associated with testicular sperm, while others, common to seminal vesicle fluid, might be secretory products or enzymes originating in the seminiferous tubules. They also suggested that some antigens were selectively resorbed, utilized or altered within the epididymis, since some non-blood antigens in the rete testis fluid entering the epididymis were not detected in the cauda epididymal plasma. The detection of non-blood antigens in the rete
testicular fluid is in agreement with disc gel electrophoretic analyses of rete testis fluid and cauda epididymal plasma by Amann et al. (1973). Similarly, Koskimies et al. (1971) reported that the free flow primary secretion of rat seminiferous tubule contained a number of proteins not detected electrophoretically in blood serum or intratesticular lymph. Koskimies et al. (1971), concluded that non-blood proteins probably originated in the seminiferous tubules, while most blood serum proteins entered the testicular effluent in the rete testis.

Immunoelectrophoretic analyses of ram rete testis fluid by Johnson and Setchell (1968), and ram epididymal fluid by Alumot et al. (1971) have established that immune globulins are present in both fluids. Immunoelectrophoretic studies by Killian and Amann (1973) also showed the presence of immune globulins in similar bovine fluids. These investigators also reported that bovine cauda epididymal plasma contained several proteins found in bull blood serum including albumin, IgA, IgG₁, IgG₂, IgM and secretory IgA. With the exception of IgA, each of the other proteins was reported to be found in rete testicular fluid. Killian and Amann concluded that although these findings might lead to the inference that the rete testicular fluid is the primary source of blood serum antigens present in the cauda epididymal plasma, it cannot be considered the exclusive source. These investigators postulated that other antigens in the cauda epididymal plasma common to seminal vesicle fluid probably were secretory products of the epididymis. Studies made by these investigators on saline soluble antigens released by sonication of testicular and epididymal sperm revealed that sperm undergo antigenic modification during their epididymal passage. They suggested that some
antigens were probably lost from sperm into the surrounding fluid while others, possibly modified, remained associated with the cells. Loss of sperm antigens within the epididymis was believed by Dickey (1965) to result from membrane alteration, or by shedding of the enzyme containing cytoplasmic droplet (Dott and Dingle, 1968). Garner et al. (1972) suggested that this loss of antigens reflected the changes in acrosomal proteinase.

Immunofluorescent analyses

Barker and Amann (1971), studied the sites of protein absorption and secretion in the epididymis, and changes in sperm antigenicity during their maturation and senescence, using the immunofluorescent antibody technique. Tissue and sperm samples from normal bulls, unilaterally vasoligated bulls and bulls in which one epididymis was ligated distal to the caput epididymis were examined. Globulins from rabbit antisera against bull seminal plasma, plasma from the cauda epididymis, washed ejaculated spermatozoa, washed spermatozoa from the cauda epididymis and blood serum were conjugated with fluorescein isothiocyanate. Tissue sections and sperm smears were allowed to react with conjugated immune globulin by the direct method. Localization of fluorescence in positive reactions was verified by the indirect method using fluorescein labeled goat anti-rabbit immune globulin. These investigators reported that in mature spermatids, acrosomal immunofluorescence was homogeneous. Their studies also showed that after spermatozoa had traversed the first half of the caput epididymis, the apical body was especially prominent. They demonstrated an intense fluorescent reaction of the apical cytoplasm in
the proximal end of the cauda epididymis and the proximal vas deferens of normal bulls with conjugated anti-cauda epididymal plasma. Fluorescence was faint or absent, however, with conjugated anti-sperm sera. They suggested that in normal bulls sperm-specific antigens probably were not observed in those areas. Barker and Amann (1971), showed that for similar tissues from vasoligated bulls, apical fluorescence was consistently intense after reaction with conjugated anti-sperm sera as well as with anti-cauda epididymis plasma. The immunofluorescent reactions for these vasoligated bulls are consistent with the assumption that spermatozoa trapped by ligation are absorbed by the epithelium of the duct. Evidence reported by Grant (1958), Niemi (1965), Friend and Farquhar (1967) for rats, and by Nicander (1958) for stallions, bulls, and rams indicates that the epithelia of the corpus and cauda epididymis and vas deferens have absorptive capabilities. Immunodiffusion tests performed by Barker and Amann (1970) showed that some antigens in normal cauda epididymal plasma were absent from the fluid recovered from the spermatocoele in the caput epididymis. Barker and Amann (1971) suggested that under normal physiological conditions the corpus and the cauda epididymis probably secrete antigens in addition to those secreted by the testis or caput epididymis. These authors also suggested that evidence of immunofluorescence in the apical cytoplasm of the epithelium lining the corpus and proximal cauda epididymis was indicative of secretory activity. Similar fluorescence in the epithelium of the vas deferens might have resulted from the absorption of proteins.
Protein and immunoglobulin content

There is little information on the accessibility of the seminiferous tubule to serum proteins and especially to the glycoprotein hormones or to immunoglobulins. The Biuret reaction was used by Johnson and Setchell (1968) to determine the total protein concentration of rete testis fluid collected at different times of the year from rams showing wide seasonal variation in spermatogenesis. There was no significant difference between the protein concentration in rams with active spermatogenesis and those with poor spermatogenesis. These investigators identified many serum proteins in the testicular fluid including the C3 component of complement, Ig, G1, IgG2 and IgM. They demonstrated that these three immunoglobulins all possessed antibody activity, and in the guinea pig, complement-fixing IgG2 antibodies appeared to cause immunological aspermatogenesis. Johnson and his co-workers (1968) showed by immunoelectrophoresis that the protein precipitin line in rete testis fluid cross reacted with α2 macroglobulin in the first peak of a G-200 elution of ram serum and was presumably identical with it.

Johnson and Setchell (1968) determined the concentration of immunoglobulin in normal and concentrated rete testis fluid, ram serum and ram seminal plasma by radial immunodiffusion, using anti-ram IgG or anti-ram light chain antisera produced in rabbits. A standard IgG was prepared by DEAE-cellulose ion exchange chromatography and calibrated by the Biuret reaction and by diffraction densitometry. The concentration of immunoglobulin in the rete testis was estimated to be about 0.2 per cent of that in serum. The immunoglobulin content of ram seminal plasma was shown to be approximately 2 per cent of that in ram serum. Since
very little immunoglobulin was detected in the seminiferous tubules, Johnson et al. (1968) concluded that the entry of appreciable amounts of serum protein from the interstitial tissue of the testis into the seminiferous tubules is prevented by a barrier which may also prevent or impede the entry of other proteins such as LH and FSH. They suggested that the presence of this protein barrier might prevent spermatozoal antigens from leaking to the systemic circulation, thus reducing the incidence of autoimmunization. They claimed also that the complement-fixing IgG antibody, cytotoxic to homologous spermatozoa, which Edwards (1960) and Johnson (1968) reported to be present in low titers in normal sera of several species, would not enter the tubules in sufficient quantities to lyse the contents. With these concepts in mind Johnson et al. (1968) suggested that for antibodies, produced by testicular injection, to cause testicular damage, the protein barrier would have to break down.

Sexton et al. (1970) analyzed the rete testis fluid, the vas deferens plasma, accessory sex gland fluid and seminal plasma of the conscious bull. Fluids and spermatozoa were collected by cannulae in the rete testis and vas deferens, by ejaculation of the bulls prior to surgery, and by ejaculation of bilaterally vasoligated bulls. They reported the presence of sixteen free amino acids in each of the sperm free fluids. Glutamate was identified to be the predominant amino acid in the rete testis fluid, vas deferens fluid and seminal plasma. This is in agreement with reports by Setchell et al. (1967) which indicated that glutamate was the predominant amino acid in testicular fluid and epididymal plasma of rams. This report is also supported by Al-Hahim et al. (1970), Bhargava et al. (1959) and Hopwood et al. (1962) who all reported
glutamate as the main amino acid in the seminal plasma of bulls. Sexton and his colleagues (1970) also reported that alanine was the predominant amino acid in the accessory sex gland fluid. This is in agreement with data reported by Hopwood and Gassner (1962), for vasectomized bulls. For vasectomized rams, however, Setchell and his co-workers (1967) reported that glycine was the predominant amino acid in the cell free ejaculated fluid.

**Characterization of Seminal Plasma Proteins**

**Variations in constituents**

Seminal plasma contains a number of different proteins, some of which occur in the blood, and some of which do not. Menzoian and Ketchel (1966), claimed that these proteins are highly antigenic when injected into an animal of a different species, but not when injected into an animal of the same species. The members of a single species, therefore, appeared to be immunologically tolerant to the seminal plasma proteins of that species, just as they are to most blood plasma proteins. Menzoian and Ketchel (1966) demonstrated that the female reproductive tract shared with the male some protein or proteins which do not occur in the blood or certain tissues. It was suggested that the female might acquire immunological tolerance to seminal plasma proteins by producing the same proteins in those accessory glands of the female tract which are embryological homologues of the male accessory glands.

Immuno electrophoretic studies of human seminal plasma by Defazio et al. (1969), using antisera from rabbits, revealed minor but distinct
differences in the antigenic components of seminal plasma obtained from different individuals. They showed that the differences existed primarily in those proteins of seminal plasma which do not occur in blood and were independent of ABO secretor status. A total of nineteen antigens was demonstrated in the seminal plasma of eight human subjects studied, although the seminal plasma of any single individual contained no more than sixteen antigens. Defazio and her co-workers (1969) felt that the failure to detect some antigens in the seminal plasma of certain individuals might be due not to their absence, but to their low concentrations. They also demonstrated that the antigenic variations, based upon the examination of successive ejaculates over a 2 hour period, were inter-individual rather than intra-individual in nature. Differences in the antigenicity of components of the seminal plasma from different males have been reported by Searcy, Craig and Berquist (1964) and Weil and Roberts (1965). However, these investigators only discovered a total of fifteen or fewer antigens.

Since it has been suggested by many investigators that the chemical constituents of seminal plasma vary in proportion to sperm concentration, Nun et al. (1972) in evaluating this hypothesis investigated the variations in seminal plasma constituents from fertile, subfertile and vasectomized azoospermic men. Their results indicated that the mean levels of seminal acid phosphatase were significantly higher than normal in vasectomized azoospermic men as well as in two men with azoospermia despite varicocele surgery. The mean values of sialic acid in normal and post-vasectomy seminal plasma were similar, in contrast to lower values obtained in oligospermic patients. No significant differences in total
protein contents in the seminal plasma were observed among the three groups of men. The mean values obtained were in agreement with those reported by Moon and Bunge (1970). There were also no significant differences observed between the three groups of patients in alkaline phosphatase and fructose levels in the seminal plasma.

Most studies on seminal plasma proteins have been done with human seminal plasma. Much less work has been done with other species. Quantitative comparisons were made of the proteins of the seminal plasmas of bull, ram, rabbit and boar by Bennet (1965), in an attempt to determine the antigenic variations between three species. A quantitative agar gel zone electrophoretic method was used to establish the relative amounts and mobilities of the seminal plasma proteins. Bennet (1965), observed a striking species specificity in electrophoretic patterns. The number of components, relative mobilities, glycoprotein and lipoprotein contents and the relative amounts of the protein components were shown to vary markedly according to the species by this investigator. Bennet (1965) demonstrated by electrophoretic investigations that the seminal plasma proteins of the four species were of heterogeneous nature with eight protein components in the bull, six in the ram, twelve in the rabbits and four in the boar. Carbohydrates and sialic acid were shown to be present in all the major protein components. The presence of carbohydrates in seminal plasma has been observed by Boursnell et al. (1962) in the boar. Larson and Salisbury (1965), reported that carbohydrates were absent or present only in low concentration in bovine seminal plasma. Bennet (1965), reported that there was little variation among the electrophoretic patterns of seminal plasma collections.
obtained from five animals in any one species, and the variations in pattern among the collections from any one individual animal were also small. The species specific character of some seminal plasma has been emphasized by Weil (1961). He showed that serum antibodies against rabbit or human seminal plasma did not cross react with the seminal plasma of stallion, bull or ram.

Immunoelectrophoretic and immunodiffusion studies

The proteins of seminal plasma have been studied by various investigators. Herman (1959), has shown by means of immunoelectrophoresis that fresh human seminal plasma consisted of eight different proteins. However, he reported that none of these proteins could be identified with gamma globulin. Klopstock et al. (1963), using 102 semen samples both from normal and sick patients, reported that seven to ten different protein fractions could be observed by immunoelectrophoresis. The proteins from most of the seminal plasmas used were shown to fall within the ranges of albumin and alpha and beta globulin. These investigators also showed that the gamma-globulin in the seminal plasma was identical with serum gamma globulin but no correlation was observed between the physiological state of sperm and the presence or absence of gamma globulin in the seminal plasma.

Martinsson et al. (1972), studied the proteins of boar seminal plasma by immunoelectrophoresis and immunodiffusion tests. They identified four different proteins by immunoelectrophoresis, all of which seemed to be specific for seminal plasma. One of these proteins, with an electrophoretic mobility corresponding to gamma globulin, had no
identity with either IgG or fibrinogen. With the immunodiffusion tests, albumin, transferrin and IgG could be recognized and this was credited to the greater sensitivity of the immunodiffusion test.

**Electrophoresis**

The first electrophoretic analysis of human seminal proteins was done by Gray and Huggins (1942). They showed that the seminal plasma contained proteins of the albumin, and of the alpha, beta and gamma globulin type. In 1966, Mischler and Reineke reported that cellulose acetate or disc electrophoresis of human semen and seminal plasma revealed between seven and ten zones of migration. Disc electrophoresis of washed human spermatozoa resulted in a pattern of protein separation that could not be distinguished from those obtained by disc electrophoresis of semen or seminal plasma. This indicates that the proteins being removed from the spermatozoa are very similar to those present in seminal plasma.

Balbierz et al. (1973) analyzed the seminal proteins of bulls electrophoretically, using starch gel and an alkaline buffer. Twelve to seventeen fractions were obtained in five migration zones. Larson and Salisbury (1954) studied proteins of the bovine seminal plasma by means of moving boundary electrophoresis. Their electrophoretic patterns indicated the heterogeneous nature of the investigated proteins, showing the presence of three major and at least eight minor components. According to these investigators, the major components of seminal plasma exhibited mobilities similar to those of the $\alpha_1$, $\alpha_2$ and $\alpha_3$ globulins of blood.
In 1959 Vesselinovitch studied the proteins of bovine seminal plasma electrophoretically by means of filter paper and starch gel electrophoresis and made the following observations:

1. Filter paper electrophoresis revealed 7 protein components, of which only 2 migrated to the cathode.
2. Starch gel electrophoresis detected 16 protein components, four of which migrated to the cathode.
3. Both methods indicated that the major components had mobilities similar, but not identical, to alpha and beta serum proteins.
4. No difference was observed between the electrophoretic patterns of samples obtained from animals of normal fertility and those from infertile animals or those of low fertility.
5. No qualitative or quantitative relationship was found between seminal plasma proteins and blood serum proteins.

Lavon (1972) studied the seminal plasma proteins of boars, bulls, rams and rabbits by disc electrophoresis at pH 4.5 and pH 8.6 and by iso-electric focusing using ampholines of pH range 3 to 10. The results revealed ten proteins in boar seminal plasma at pH 4.5 and eleven at pH 8.6. The bull seminal plasma showed eleven and thirteen, the ram eight and seventeen, and the rabbit sixteen and twenty two, respectively. Due to better resolution, the number of proteins found in the same seminal plasma samples using iso-electric focusing was greater than that obtained with disc gel electrophoresis. Boar seminal plasma proteins were found
to be basic in character while those of the other animals possessed acidic, neutral, and basic iso-electric points.

**Chromatography**

Behrman and Amano (1967), investigated human seminal plasma by ion exchange chromatography. For cation exchange chromatography, carboxymethyl (CM) cellulose was used and this was prepared and buffered with pH 6.4 phosphate buffer. The size of the column was 2 x 40 cm, and 20 ml of pooled human seminal plasma was used in either case. The elution system of chromatography was applied with a sodium chloride gradient system ranging from 0 to 0.5 M. The protein distribution was checked by ultraviolet light absorption at 280 μm. The results showed that human seminal plasma proteins were separated into two major parts. The first portion consisted of two fractions designated 1 and 2, and this portion was eluted with a low concentration of sodium chloride. The second portion comprised three peaks designated 1, 2, and 3 and was eluted with 0.2 to 0.3 M sodium chloride.

Further studies of the human seminal plasma proteins were made by Amano and Behrman (1968) using Sephadex gel-filtration. A column of Sephadex G-100, 2.2 x 39.5 cm was used employing a 0.01 M sodium phosphate buffer with 0.15 M sodium chloride as the eluting solution. A sample of 4 ml of human seminal plasma was applied to the column. Fractions of 2.5 ml were collected and protein distribution was determined by u.v. light absorption at 280 μm. There were two major peaks with three minor ones in between them. It was shown also by these authors that pronase treatment of the seminal plasma did not destroy the major
antigens, instead it improved the chromatographic profile thus enhancing isolation of the antigens. These results are presented in Figure 1.

The estimated molecular weight of these fractions showed that fraction A was > 100 \times 10^3, B approximately 80 \times 10^3, C was 20 \times 10^3 and E was less than 10 \times 10^3. The protein and carbohydrate concentrations for each fraction were determined by the Biuret test and the Phenol-sulfuric acid method respectively and these results are shown in Table 1.

Table 1. Protein and Reducing Sugar Contents of Each Fraction.

<table>
<thead>
<tr>
<th>Fraction of Pronase</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>Sugar*</td>
<td>Protein</td>
<td>Sugar</td>
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<tr>
<td></td>
<td>mg/ml</td>
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<td>mg/ml</td>
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<tr>
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<td>1.25</td>
<td>0.053</td>
<td>1.12</td>
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<tr>
<td>0.2 mg</td>
<td>1.25</td>
<td>0.88</td>
<td>0.050</td>
<td>0.38</td>
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<tr>
<td>2.0 mg</td>
<td>0.62</td>
<td>0.072</td>
<td>0.072</td>
<td>0.25</td>
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</tbody>
</table>

* Not estimated

In 1973, Lenhardt et al. investigated bovine seminal plasma proteins by gel-filtration chromatography. They reported that bovine seminal plasma contained 6.37 per cent bound carbohydrate of which 3.40 per cent was neutral hexose, 1.35 per cent hexosamine and 1.59\% sialic acid. Sixty per cent of the total bound carbohydrate was shown to be localized in the main molecular weight regions. Fructose was found to be located in the reducing terminal position of the oligosaccharide.
Figure 1. Elution patterns from columns of Sephadex G-100 of human seminal plasma before and after treatment with pronase. Untreated (solid line) 0.2 mg per ml (dotted line) and 2 mg per ml (dashes).
Sperm Agglutinins

A report by Franklin and Dukes (1964), indicated that there was a high percentage of women with unexplained infertility possessing sperm agglutinating antibody. Bratanov and Dikov (1960) observed sperm agglutinins in the serum of cows that were resistant to pregnancy in titers as high as 1:512. Natural sperm iso-agglutinins were found to be present in the serum of bulls and rams but there was no evidence of them affecting the fertilizing capacity of the spermatozoa produced by these animals.

Matousek (1964), reported the presence of agglutinins in the normal sera of bulls, rams and sows to both homologous and heterologous spermatozoa. He showed that immune sera obtained by immunizing rabbits with bull and ram spermatozoa not only agglutinated homologous spermatozoa, but also produced cross reactions with germ cells of other species. Padma (1972) demonstrated the presence of sperm agglutinins in the normal serum of rabbits to homologous spermatozoa. He indicated, however, that these sperm agglutinins did not appear before 50 to 60 days of age.

Wilson (1954) reported the presence of sperm agglutinin in the seminal plasma and blood serum of two sterile men. Their sera had high antibody titers, and head and tail agglutination was seen in the early stages, but tail agglutination was predominant in the larger clumps.

It has been known for a long time that extracts of certain seeds, known as lectins or phytoagglutinins, agglutinate erythrocytes of human as well as of animal origin. With this concept in mind, Kashiwabara et al. (1965) designed experiments to investigate the action of soybean
extracts on fresh bull spermatozoa. Their results indicated that the lectins produced tail to tail agglutination in most of the cases tested. Badel and Brilliantine (1969) were able to induce the agglutination of the spermatozoa of clams and of human A, B and O blood groups by phyto-agglutinins. The head to head type of agglutination was observed in the spermatozoal suspension.

**Erythrocyte Antigens and Semen Antigens**

In 1964 Edwards, Ferguson and Combs reported that they were able to get spermatozoa from secretors to participate in ABO specific mixed agglutinations whereas those from non-secretors would not. They concluded that ABO antigens on seminal spermatozoa were derived from the seminal plasma and that ABO antigens on spermatozoa were dependent on the secretor status of the donor. This conclusion was contrary to those of Shahani and Southam (1962) who supported Gullbring's (1957) findings that spermatozoa possess only the ABO antigens controlled by the gene they carry. Boettcher (1968) could not detect blood group antigens on human spermatozoa from non-secretors, but he was able to demonstrate the presence of these antigens on spermatozoa from secretors, thus agreeing with Edwards and his colleagues. He also showed that spermatozoa would absorb ABO antigens from aqueous solution, and this led him to conclude that these antigens were only present in the seminal plasma and adsorbed to the sperm on their way through the reproductive tract.

Docton et al. (1952) reported that iso-immune serum, containing antibodies for bovine erythrocytes, reacted specifically with bovine
spermatozoa. They also reported that antibodies produced in sheep against bovine spermatozoa caused agglutination of bovine spermatozoa and also produced specific lysis of erythrocytes of certain cattle. Matousek (1964) was unable to demonstrate the presence of the erythrocyte antigens of cattle either in the seminal or the epididymal spermatozoa of bulls of the Red Spotted breed. This is in disagreement with the earlier findings of Docton et al. Padma (1972) was unable to find any common antigens between red cells and spermatozoa in rabbits.

Hemolytic Factor of Seminal Plasma

A powerful hemagglutinin in boar seminal plasma has been described by Boursnell and Combs (1966). This factor has been shown by Nelson and Boursnell (1966) to be associated with the presence of a number of basic proteins. Investigations on the combination of the hemagglutinins with red blood cells and red cell ghosts and some of their reactions with homologous and heterologous spermatozoa were carried out by Boursnell (1967). He reported that the hemagglutinins in boar seminal plasma were absorbed by ejaculated washed motile ram, bull and rabbit spermatozoa. Washed epididymal spermatozoa also absorb the hemagglutinin, but to a lesser extent. He also reported that considerable motility of the heterologous spermatozoa was still present after 15 minutes contact with boar seminal plasma. Mixed cell agglutination of bull spermatozoa and red cells gave a reticulate agglutination that could be detected microscopically. It was shown that the hemagglutinins are absorbed at least as well by washed pig red blood cell ghosts as by the intact washed
erythrocytes.

Millar (1956) also discovered a hemolysin in semen from unhealthy bulls. His findings suggest that semen samples from healthy, fertile bulls in use are non-hemolytic immediately after collection. Following collection, however, these non-hemolytic samples of semen were shown to acquire the hemolytic properties as the viability on storage decreases. Millar claimed that the discovery of this hemolytic factor might have some future value either in the evaluation of the genital health of bulls or in the assessment of individual samples of semen intended for storage at artificial insemination centers.

Kysilka (1972) isolated the hemolytic factor from bull seminal vesicle fluid and partially analyzed its biochemical characteristics. It was shown that the molecule has only one N-terminal amino acid, aspartic acid. It was demonstrated that the hemolytic activity is greatly decreased in the reduced, reduced and carboxamidomethylated, and oxidized protein. Since N-terminal analysis showed that the hemolytic factor was probably composed of the identical subunits, Kysilka suggested that a decrease of hemolytic activity results from a splitting of disulfide bonds. He also indicated that no proteolytic activity was demonstrated by the hemolysin but there was evidence of phospho-lipolytic activity.

Histocompatibility Antigens and Spermatozoa

Histocompatibility antigens of the type H-2 have been found on most cells of the mouse, but there are conflicting reports concerning
their expression on spermatozoa. Using the indirect immunofluorescence technique, Barth and Russell (1964) found no H-2 antigen on spermatozoa. Using the techniques of antibody absorption and indirect immunofluorescence, Vojtiskova et al. (1969) concluded that H-2 antigens were probably detectable on spermatozoa of mouse by all three procedures. Goldberg et al. (1970) using the cytotoxicity test were able to demonstrate the presence of the H-2 antigens on mouse spermatozoa, also disagreeing with the findings of Barth and Russell. The use of the cytotoxicity test by Goldberg et al. (1971) demonstrated the existence of the H-Y antigenic system on the mouse spermatozoa. This antigen was reported to be carried by male cells and was believed to be responsible for the rejection of male tissues by females of the same inbred strain.

Fellous and Dausset (1970) reported the presence of HL-A antigen on human sperm, and on the basis of the proposed homology between the HL-A and the H-2 loci it seems probable that H-2 antigens might be present on spermatozoa. These investigators suggested that the HL-A antigens were expression of the haploid genome on spermatozoon membrane. If the haploid expression of HL-A antigen is confirmed, Fellous and Dausset firmly believe that this would enable one to determine directly the haplotypes and genotypes of the HL-A system in males. These authors also suggested that confirmation of the haploid expression of the HL-A system would make it possible for gametic selection based on the use of cytotoxic antibodies which recognize HL-A or other antigens. If this becomes a reality then one might even speculate on the prevention of hereditary diseases governed by linked loci.
Local Antibody Production to Seminal Antigens

Experiments concerned with local antibody production have resulted in a growing series of tests in tissues where antibody synthesis has been demonstrated. The reproductive tract has been examined by several workers including Kerr (1955) and Strauss (1965) whose results have suggested that antibodies might be produced locally by the cervix or vagina. Bell (1969) did some detailed studies on the immunological control of fertility in mice by comparing different immunization schemes to determine if the route of injection had any significant effects. When homologous spermatozoa were injected intraperitoneally into the mouse, this proved to be a reliable method for inducing infertility in this species. This is in agreement with earlier reports by McLaren (1964 and 1966), and Edwards (1964). Immunization with the same homologous spermatozoa by the intravaginal method failed to alter the fecundity of mice or enhance the effect of systemic injections. Contrary to this report, Behrman and Otani (1963) claimed to have induced an immune response in the guinea pig by the intravaginal method. Behrman and Nakayama (1965), in addition to reporting an immune response from this method, also claimed that there was an adverse effect upon fertility. Edwards (1960) failed to evoke an immune response in rabbits after intravaginal injection of rabbit spermatozoa, although antibody against both egg yolk and bull spermatozoa could be elicited by this procedure.

Kiddy et al. (1959) reported that intrauterine injections of cattle erythrocytes into heifers produces antibodies in the serum but none in the vaginal secretions. This suggests that the antigens pass
into circulation and that there is no antibody production in the reproductive tract. Omran and his co-workers (1971), unlike Kiddy and his colleagues, were able to induce local antibody secretion in the bovine uterine cervix using bovine spermatozoa. These authors immunized the cervices of three heifers with a pure protein, Keyhole Lympet Hemocyanin, after which precipitating antibody to this protein appeared in the cervical mucus but not in the blood serum. The cervices of these heifers plus three additional ones were then immunized with washed bovine spermatozoa. Again, a precipitating antibody against sperm appeared later and disappeared in four to six weeks. The animals were exposed to pregnancy, when antibodies were detected, by mating with a bull of proven fertility. The effects of the immunization on these heifers were not fully assessed at the time of their publication. From these studies and others one cannot rule out the possibility that there is local antibody production to seminal antigens, but more research needs to be done in this area in order to resolve the various conflicts.
MATERIALS AND METHODS

Source and Preparation of Bovine Semen

The semen that was used for the immunization was obtained from Select Sires Inc., Plain City, Ohio. Pooled semen from bulls belonging to different breeds was collected with an artificial vagina and stored at -20°C until required for use. The frozen semen was thawed prior to the preparation of the inoculum, and the required amount was removed while the rest was rapidly refrozen. In order to minimize the number of times that the samples were frozen and thawed, storage was done in 25 ml quantities.

Iso-immunization

Iso-immunizations were performed to demonstrate the iso-antigenicity of semen antigens and to obtain immune sera to aid in the characterization of bovine iso-antigens. The immunization scheme consisted of seven injections. A total of twenty virgin heifers was involved in the immunization. The heifers were divided into an experimental and a control group, consisting of 10 heifers each, with average ages of 14.05 and 14.45 months, respectively. These heifers were selected from the five breeds of dairy cattle that comprise the Ohio State University dairy herd. The experimental group consisted of 3 heifers belonging to the Ayrshire
breed, 1 to the Guernsey breed, 1 to the Jersey breed, 1 Brown Swiss and 4 belonging to the Holstein breed. The control group was distributed in a similar manner.

A total of seven injections was administered to each animal over a nine week period. The inoculum given to the experimental group consisted of 2 ml undiluted semen pooled from bulls belonging to various dairy breeds, and 2 ml of Freund's incomplete adjuvant. The first four injections in this group were given subcutaneously at several sites on the side of the neck, but owing to excessive development of abscesses the rest of the immunization scheme was continued with intramuscular injections in the region of the hip. Weekly injections were made at seven day intervals for the first six immunizations, and then there was a break of two weeks after which a booster injection was administered.

For the control group, weekly injections with 2 ml of Freund's incomplete adjuvant and 2 ml of 0.005M phosphate buffered saline were administered. Like the experimental group, there was a break in the routine for 14 days after the sixth week, and then a final dose was given on the ninth week. Only subcutaneous injections were employed in the immunization of this group since no abscesses were induced.

Production of Iso-immune Sera and Collection of Mucus Samples

Serum samples were collected from each animal, by bleeding from the jugular vein, prior to the first injection and also prior to subsequent injections. This method was utilized to ensure that there were control samples for the immunological tests and to determine the
progressive development of antibody production. Seven days after the last injection large quantities of blood were collected, and the sera separated and stored at -20°C until needed.

In order to determine if any of the antibodies that might be formed in response to the immunization could be detected in the reproductive tract, mucus samples from the anterior vagina and the mouth of the cervix were collected from each animal of both groups prior to each immunization. These samples were removed by inserting a speculum inside the vagina, and with the aid of a flashlight to improve visibility, cotton swabs placed on a wire rod were used to remove mucus from the anterior vagina and the mouth of the cervix. Since these heifers were all virgins they all possessed very small cervices, and therefore no effort was made to enter the cervical canal to obtain uterine secretions. These swabs, upon removal, were placed in 0.005 M phosphate buffered saline, pH 7.4, and the mucus removed, homogenized with the saline, and stored at -20°C until required for the immunological tests.

Preparation of Materials for Immunological and Biochemical Investigations

The materials which served as sources for antigenic studies included samples from the reproductive tracts of bulls, seminal plasma from pooled semen, whey proteins from bovine colostrum and bovine dry mammary gland secretions and blood plasma from bulls belonging to three different breeds. It was the hope of this investigator to use these antigenic sources as a means of determining the origin and nature of bovine iso-antigens and their degree of immunological cross-reactivity.
Reproductive tracts from bulls of unknown history were obtained from Coil Packing Company, Columbus, Ohio, fifteen minutes after slaughter, and transported to the laboratory ten minutes later. Antigenic components were harvested from the cauda epididymis, the caput epididymis, the seminiferous tubules and the seminal vesicles. Cauda epididymal fluid was obtained by incising the tail of the epididymis and flushing out the fluid with 0.005 M phosphate buffered saline. The tail of the epididymis was next macerated with a Waring Blender, suspended in phosphate buffered saline, centrifuged and the supernatant was saved. The cauda epididymal fluid was centrifuged for 30 minutes at 23,500 g in a Serval RC-2 super centrifuge, and the plasma separated from the sperm and stored at -20°C. The cauda epididymal sperm were resuspended in phosphate buffered saline and subjected to ultrasonication in order to obtain saline soluble sperm antigens for the immunological tests. This was achieved by using a Biosonic III ultrasonicator, (Bronwill Scientific, Rochester, New York), equipped with a standard probe set at maximum on the intensity scale. Sonication was done in a 50 ml lusteroid tube placed in a cold water bath. An average of 120 seconds per sonication was required to accomplish the removal of the soluble antigens.

Fluid was also removed from the head of the epididymis and the plasma was removed by centrifugation at 23,500 g for 30 minutes. This was stored at -20°C and used later in various immunological tests.

Testicular fluid was collected by making a longitudinal incision through the testes, flushing the seminiferous tubules with 0.005 M phosphate buffered saline, and aspirating it with a Pasteur pipette.
The testicular fluid was sonicated in the Biosonic III ultrasonicator for 90 seconds to increase homogeneity of the suspension. Testicular spermatozoa were separated from the fluid suspension by centrifugation at 23,500 g for 30 minutes, and the supernatant was stored at -20°C until required.

Seminal vesicular fluid was obtained from the seminal vesicles by gently flushing with phosphate buffered saline and aspirating the contents with a 10 cc syringe equipped with a 14 gauge needle. Any debris which might have been collected in the process was removed by centrifugation at 23,500 g for 30 minutes, and the supernatant stored at -20°C until needed for further use.

Seminal plasma for immunological and electrophoretic testing was removed from the pooled semen that was used in the immunization scheme. This was achieved by centrifugation at 12,100 g in a Serval centrifuge for 30 minutes. The samples were maintained at -20°C until they were required.

Mammary secretion was obtained from a 14 day dry cow belonging to the Jersey breed. This was subjected to centrifugation at 1,475 g for 20 minutes to remove the fats. The lower layer, which contained whey proteins and casein, was stored at -20°C for further use in the immunological tests.

Bovine colostrum was collected from a cow belonging to the Holstein breed immediately after parturition, and the whey proteins were extracted. The colostrum was centrifuged at 1,475 g for 20 minutes at 0°C in order to remove the fat and cellular material. The defatted sample was warmed for 20 minutes at 35°C. Casein was precipitated by
acidification at pH 4.5 with concentrated acetic acid. Acid was added one drop at a time with constant stirring. The precipitate was removed by centrifugation at 29,000 g for 20 minutes, and the supernatant which consisted of whey proteins was frozen and stored at -20°C.

Blood plasma from five bulls belonging to the Hereford, Shorthorn and Angus breeds was prepared from blood samples submitted to the Immuno- genetics Laboratory, of The Ohio State University, for typing. These samples were utilized in immunological tests to determine if there was any evidence of cross reactivity between bovine semen iso-antigens and blood plasma.

**Immunological Tests**

**Agar gel-diffusion**

The agar gel-diffusion test was performed as described by Lazear (1958). Ionagar No. 2, purchased from Difco Laboratories, was the agar used. It was dissolved in 0.005 M phosphate buffered saline, pH 7.4, and made to a concentration of 1.2%. Merthiolate in a concentration of 0.01% was added to the agar to inhibit bacterial growth. Wells were bored in the agar approximately 0.5 cm apart. The test serum in all cases was placed in the central well with the antigenic components distributed in the peripheral wells. Incubation was allowed to occur in a humid chamber at room temperature and readings were recorded after three days. Pictures were taken of the precipitin lines that were found.
Immunoelectrophoresis

Immunoelectrophoretic studies were done on whole bovine seminal plasma and the antigenic preparations obtained from various areas of the reproductive tract of the bull. The technique was a slightly modified form of the one described by Campbell et al. (1964). Gel was made to a concentration of 1% with Ionagar No. 2 and barbitone acetate buffer obtained from Colab Laboratories Inc. The buffer was adjusted to pH 8.6 and an ionic strength of 0.05. Merthiolate (0.01%) was incorporated into the gel to prevent bacterial growth. Glass slides (75 X 25 mm), mounted on a plexiglass frame, were coated with gel and allowed to cool for one hour at room temperature. Approximately 0.01 ml of antigenic suspension was placed in each of the wells that were cut on either side of a trough, and resolved in an electric field for 100 minutes at 100 v (35 m.a.). Immediately after electrophoresis, the test sera were placed in respective troughs. All of the antigenic preparations with the exception of seminal plasma were concentrated with Lyphogel, (Gelman Instrument Co.) to increase the strength of the immunological reaction. Ten ml of each sample were added to 1.5 grams of Lyphogel for 2.5 hours to produce a final volume of 4 ml.

Diffusion was allowed to occur in a humidity chamber at room temperature for 3 days, after which the results were recorded. The coated slides were then left in physiological saline for 3 days to wash away any unreacted proteins that might be present. Following this, they were stained with 1% amido black 10B for 1 minute and then destained with 7% acetic acid for 2 days. Pictures of the gels were taken after destaining.
Passive hemagglutination test

The passive hemagglutination test, as described by Stavitsky (1954), was utilized as a highly sensitive technique in detecting the presence of antibodies in the test sera and mucus samples. Tannic acid-cells were prepared by incubating sheep red blood cells with tannic acid diluted 1:20,000 in buffered saline, pH 7.2. These cells were sensitized by treatment with equal volumes of the antigenic suspension to be used in the test. This tannic acid-protein complex was washed and suspended in normal rabbit serum diluted 1:100 with saline.

All serum and mucus samples were first heat inactivated in a water bath at 56°C for 30 minutes. Absorption of each was made with an equal volume of packed sheep red blood cells. Serial dilutions of the antiserum and mucus samples were made using normal, absorbed rabbit serum diluted 1:100 with saline. The dilutions were made in 13 X 100 mm test tubes so that each tube contained 0.5 ml of test serum or mucus. To each tube was added 0.05 ml of tannic acid-protein cells. The tubes were shaken and allowed to incubate at room temperature for 12 hours, after which readings of the extent of agglutination were recorded.

Three sets of controls were employed in the test to ensure accuracy of results. These controls included: (1) tannic acid-saline cells plus antiserum or mucus in diluent (2) suspension of tannic acid-protein cells in known positive sera (3) tannic acid-protein cells suspended in buffered saline.

Sperm agglutination tests

Sperm agglutination was studied by the method utilized by Matousek
(1964). Sera from control and immunized animals were inactivated at 56°C for 30 minutes to destroy complement activity. Dilutions were done in a geometrical series in 0.05 ml physiological saline in test plates used for inhibition tests by the Immunogenetics Laboratory of The Ohio State University. Semen was collected by an artificial vagina from bulls of excellent fertility status and then transferred in a thermos with a constant temperature of 33°C. Microscopic examination of the sample was always made prior to the setting up of the test, to ensure that adequate motility was present.

To each well in the serological plates was added 0.025 ml of semen averaging 2.35 X 10^8 cells. These plates were slightly shaken and allowed to incubate at 33°C for 15 minutes in a water bath. After incubation the mixture of serum and spermatozoa was shaken, and two drops were placed on each slide, by means of a Pasteur pipette, and examined microscopically for agglutination.

**Immunofluorescent antibody study**

The indirect immunofluorescence antibody technique was used in an attempt to determine the point of origin of the bovine iso-antigens which were detected in the testicular fluid. The method utilized was a modification of the one described by Coons et al. (1950). Cross sections were made from the testes of bulls, 30 minutes after slaughter, and these were frozen and stored in liquid nitrogen at -196°C until required. For the immunofluorescent study, sections ranging from 4 to 6 microns thick were prepared on a microtome cryostat (International Equipment Co.). Cutting of the sections was performed according to the method of Burns
(1962). These sections were placed on fluorescent antibody slides, coated with 0.25% gelatine, and placed in a refrigerated chamber to prevent rapid thawing. Storage in this manner continued for not more than 12 hours before use.

The sections were treated with a few drops of unlabeled serum and incubated in a wet chamber at 37°C for 30 minutes. Following incubation, the slides were washed in 0.005 M phosphate buffered saline for 5 minutes, rinsed in double distilled water for 5 minutes to get rid of excess serum, and then air dried. Approximately 0.1 ml of rhodamine was next added to the slide preparation and washed off after 10 minutes with double distilled water. Several drops of 1:8 diluted conjugated rabbit anti-bovine immunoglobulin, (Miles Laboratories), were applied to the preparation, and incubation was carried out for 30 minutes at 37°C in a humid chamber. At the end of the incubation period, the preparation was again washed with phosphate buffered saline, rinsed with double distilled water and air dried. Microscopic examination followed in u.v. light, and all the details were noted. A saline control was used in which saline was used instead of the test serum. Serum from non-immunized animals was also used in this manner as another form of control.

**Sephadex Gel-filtration**

Gel-filtration with Sephadex G-200 was used to separate the macromolecules of pooled seminal plasma. A column 2.6 X 100 cm was utilized in the process. Blue Dextran 2000 was applied to the column to check its homogeneity and to determine the void volume. Column separa-
tions were done at 4°C with Tris-sodium chloride buffer, pH 8.0, as the eluant. A sample of 2.0 ml of seminal plasma was applied to the column, and the effluents were collected on a GME fraction collector in increments of 5 ml. Relative protein concentration in the effluent was determined by measuring the ultra-violet light absorption at 280 m\(\mu\) with a Beckman model DB spectrophotometer. The various fractions that were eluted from the column were pooled and dialyzed against double distilled water, lyophilized, and stored at -20°C until required. They were reconstituted in double distilled water when needed for use.

**Molecular Weight Estimation**

An estimation of the molecular weights of the peaks eluted from the G-200 Sephadex column was performed as described by Andrews (1964). A solution of 0.2% Blue Dextran 2000 was used to determine the void volume of the column, after which purified standard compounds were added. The compounds comprised the following: (1) aldolase (molecular weight 158,000), (2) ovalbumin (molecular weight 45,000), (3) chymotrypsinogen A (molecular weight 25,000) and (4) ribonuclease A (molecular weight 13,700). A standard curve was constructed by plotting the elution volumes of these proteins against the logarithm of their molecular weights. This curve served to estimate the molecular weights of the unknown seminal plasma proteins.

**Ion Exchange Chromatography**

Pooled bovine seminal plasma was subjected to ion exchange
chromatography in an attempt to separate antigenic components. Anion exchange chromatography was performed with DEAE Sephadex A-50, (Pharmacia Fine Chemicals, Piscataway, N. J.), on a column 2.5 X 45 cm. The Sephadex was equilibrated in 0.01 M sodium phosphate buffer (pH 8.0) for 48 hours. After packing, the column was equilibrated by allowing 1000 ml of the initial buffer to flow through. Protein was eluted from the column by a continuous gradient of sodium phosphate buffer, pH 8.0, ranging in molarity from 0.01 to 0.4 M. The initial 0.01 M buffer was placed in a 2000 ml sealed flask located on top of a magnetic stirrer. A second 2,000 ml flask contained 0.4 M buffer which flowed continuously into the first flask. Constant mixing by the stirrer ensured the maintenance of a proper concentration gradient.

The chromatographic separation was done at room temperature and the hydrostatic pressure was regulated occasionally to maintain a constant flow rate of 30 ml per hour. Samples ranging in size from 5 to 15 ml were equilibrated against 0.01 M phosphate buffer for 36 hours before they were added to the column. After addition of the samples, approximately 15 ml of the initial buffer (0.01 M) was used to wash it into the gel. The effluent was collected in 5 ml quantities and absorbance was measured at 280 μm in a Beckman DB spectrophotometer. Pooled fractions were prepared, dialyzed against double distilled water, lyophilized and used for immunological and electrophoretic testing.

**Protein and Carbohydrate Determination**

Protein determination of the various seminal plasma G-200 column
fractions was performed by the method described by Lowry et al. (1951). A standard curve was constructed by utilizing the absorption spectra obtained from human crystalline albumin (Calbiochem, San Diego, California), and the percent protein in the fractions was calculated by extrapolation from this curve. The per cent carbohydrate in each of the seminal plasma fractions was determined in a manner similar to that described by Dubois et al. (1956). In both the protein and carbohydrate determinations, tests on each sample were done in triplicate.

**Heat Stability Studies of Semen Iso-antigens**

The stability of semen iso-antigens to various temperatures was tested. It was hoped that this method would serve to aid further physical characterization of these iso-antigens. Pooled bovine seminal plasma was subjected to three different temperatures for varying lengths of time. Incubation of the seminal plasma was conducted under the following conditions:

1. 56°C for 30 minutes
2. 65°C for 15 minutes
3. 65°C for 30 minutes
4. 75°C for 15 minutes
5. 75°C for 30 minutes

After incubation the samples were tested against antiserum to bovine semen to assess the effects of the temperature treatments, and the results were recorded.
**Electrophoretic Studies**

Horizontal electrophoresis with polyacrylamide gel was utilized in order to: (1) examine the electrophoretic relationships between proteins of bovine seminal plasma, cauda epididymal plasma, cauda epididymal sperm, caput epididymal plasma, testicular fluid and seminal vesicle fluid, (2) make qualitative comparisons of these components with bovine serum proteins.

A 5 per cent polyacrylamide gel was used for electrophoresis in a discontinuous buffer system. The gel consisted of Cyanagum 41, (Fisher Scientific Co.), dissolved in a buffer solution containing 0.38 M glycine and 0.05 M Tris (hydroxymethylaminomethane), diluted 1:10 in double distilled water, and the pH adjusted to 9.0. The reservoir buffer was prepared by dissolving 18.6 grams of boric acid and 4.0 grams sodium hydroxide in a final volume of 1 liter. Electrophoresis was performed at room temperature. The power supply was set at 175 volts for 2 hours and then raised to 200 volts until the Bromphenol Blue marker had migrated a distance of 13 cm. The gels were stained in 1% amido black 10B for 5 minutes and destained in a mixture of methanol, water and acetic acid for 3 days, after which pictures were taken and the results recorded.

**Enzyme Studies**

Lyophilized fractions of bovine seminal plasma components obtained from gel-filtration were treated with pronase and alpha amylase (Calbiochem). Gel-filtration fractions one and two of the seminal plasma
were utilized in this study, since these fractions proved by agar-gel-diffusion to be the source of the iso-antigens. It was hoped that by subjecting these fractions to proteolytic enzyme or amylase digestion, some progress would be made in determining the nature of the iso-antigens.

Each enzyme was dissolved in 0.01 M phosphate buffer (pH 7.1) to give concentrations ranging from 0.02% to 1.6% (w/v). One part of enzyme solution was incubated with 5 parts of a 4% (w/v) suspension of the respective seminal plasma fraction at 37°C for 90 minutes. After incubation, each digested sample was utilized for immunological tests by the agar-gel-diffusion technique. The gel-diffusion tests were performed at room temperature (25°C) and at 4°C. Incubation at the refrigerated temperature should help to ensure that the enzyme, present in the digested fractions, would be fairly inactive and thus would not be capable of destroying the antibodies in the antiserum.
RESULTS AND DISCUSSION

Iso-immunization

The iso-antigenic potency of bovine semen was demonstrated when it was administered to dairy heifers both subcutaneously and intramuscularly. The various immunological tests used substantiated this finding. These results are in agreement with Menge (1967) who was able to evoke antibody response by immunizing heifers with bovine semen. The progressive development of antibody production was studied by the sperm agglutination test and passive hemagglutination test. The passive hemagglutination tests are presented in Table 2. All sera from the control animals failed to show any hemagglutination, indicating that the antibodies produced by the experimental animals were evoked by semen antigens. The seminal vesicular fluid failed to produce any hemagglutination titer when tested against antiserum produced by immunization with bovine semen. This could be an indication of lack of iso-antigenicity by this component. The very sensitive hemagglutination test also failed to detect any antibodies in the mucus samples. This is rather surprising since work by many reporters including Omran et al. (1971) suggested antibody production in the female bovine reproductive tract. Previous work in our laboratory revealed the presence of antibodies in the uterine fluid after subcutaneous and intra-uterine injections with bovine spermatozoa. Failure to detect antibodies in the cervical mucus
Table 2. Hemagglutination Titers of Sera From Animals Immunized With Bovine Semen and Adjuvant.

<table>
<thead>
<tr>
<th>Cows</th>
<th>C.E.P.</th>
<th>C.E.S.</th>
<th>Ca.E.P.</th>
<th>T.F.</th>
<th>S.V.F.</th>
<th>S.P.</th>
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<td>1:16</td>
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<td>1:256</td>
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<tr>
<td>37A</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>48H</td>
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<tr>
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<td>1:16</td>
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<td>0</td>
<td>1:64</td>
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</tbody>
</table>

*Aver-age titer* 1:64 1:56 1:25 1:19 0 1:85

0 - No hemagglutination
C.E.P. - Cauda Epididymal Plasma
C.E.S. - Cauda Epididymal Sperm
Ca.E.P. - Caput Epididymal Plasma
T.F. - Testicular Fluid
S.V.F. - Seminal Vesicular Fluid
S.P. - Seminal Plasma

*Average hemagglutination titer calculated from the log to the base 2 of the various dilutions.*
of these animals in this investigation might be somewhat related to the overall debilitated condition of the reproductive tract as a consequence of the immunization. Cervical mucus secretion was extremely low in all of the experimental animals, and this might be due to the lack of cyclicity exhibited among these animals. Consequently, the antibodies present might be in such low concentration that they were not able to be detected by the immunological tests used.

Sperm agglutinins specific for homologous spermatozoa were found in both the immune sera and the control sera, but to a lesser extent in the latter. The head to head type of agglutination was the only kind demonstrated by the control sera which reacted positively. This might be indicative of non-specific agglutination since this pattern was also observed for some of the saline controls. Two types of agglutination were observed with all of the immune sera tested. These were the head to head and the tail to tail type of agglutination. Results from the sperm agglutination test are shown in Table 3.

Pronounced abscesses developed at the injection sites in all immunized animals, and a deterioration of general well-being occurred which was probably the cause of reduced reproductive function. The immunized animals failed to cycle normally, and rectal palpation revealed abnormally small reproductive tracts and ovaries, with noticeable lack of tonicity. There were two suspected cases of metritis in two of the immunized animals, #52H and #53H, at the end of the immunization, and they were treated by the veterinarian. Freund's adjuvant has been known to produce abscesses when administered with an antigentic substance but the deterioration of the animals' performance quite likely has been
Table 3. Sperm Agglutination Titers of Immune and Normal Sera.

<table>
<thead>
<tr>
<th>Animal</th>
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<th>Animal</th>
<th>Titer</th>
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<tbody>
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<td>1:32</td>
<td>23J</td>
<td>1:4</td>
</tr>
</tbody>
</table>

0 = No titer.
due primarily to the effects of the semen. The semen used for the immunization was untreated so that the integrity of the antigens would not be adversely affected. Bacteriological tests of a specimen of the semen, performed by the Clinical Laboratory of the College of Veterinary Medicine, revealed the presence of diphtheroids and organisms belonging to the genus Bacillus. On the basis of these findings it seems likely that these organisms might have triggered a systemic infection to disturb the normal physiological functions of these animals.

The control group of heifers which received sham injections of saline and adjuvant developed no abscesses, cycled normally, and were bred and conceived routinely. As late as six months post immunizations, the three Ayrshire heifers and a Holstein heifer #52 belonging to the experimental group failed to resume their reproductive cycles. The other experimental animals which resumed their cyclicity were bred, and conception compared favorably with the control group. Although antibody titers were detected in all experimental animals sixty days after the last immunization, in most cases the titers were markedly lower. If the strength of an antibody titer is positively correlated with the induction of infertility, the above finding might suggest that one will not be able to control population immunologically on a long term basis without repeated immunizations.

Agar gel-diffusion

The agar gel-diffusion test revealed the presence of four precipitin lines formed by the reaction of bovine seminal plasma with antisemen sera. A similar number of precipitin lines was formed against cauda
epididymal plasma, caput epididymal plasma, cauda epididymal sperm and testicular fluid, and all of these were identical to those produced by the seminal plasma. These results are presented in Figure 2. The pattern of immunological reaction was the same for all immune sera tested, thus providing no evidence of polymorphic differences among the ten experimental animals. These results support the conclusion that the testis is the primary, and possibly the sole, source of the bovine semen iso-antigens. These iso-antigens, according to Menge (1969), probably arise in the testes after the spermatogonial stage of development since immature testis failed to evoke any iso-antibody response. No precipitin lines were produced by the seminal vesicular fluid even after it was repeatedly concentrated with Lyphogel (Gelman Instrument Company), suggesting that it is non-isoantigenic despite its great contribution to the volume and total number of protein components of the seminal plasma.

Although the testes seem to be the point of origin for the semen iso-antigens, it is still not clear as to the exact point of their secretion. This is subject to various speculations. Since the testicular fluid which was obtained from the seminiferous tubules also produced four precipitin lines it seems probable that spermatozoa might get their full complement of iso-antigens in these tubules while they are being produced. This is supported by the fact that the phosphate buffered saline soluble sperm antigens also produced four common precipitin lines. The observation of identical iso-antigens in other secretions might suggest that secretions from the seminiferous tubules are extruded along the ductular system of the reproductive tract. Alternatively these antigens may have been released by physiologically normal spermatozoa on
their passage through the reproductive tract or they may represent end products of sperm dissolution.

**Immunoelectrophoresis**

The immunoelectrophoretic study was done primarily to confirm the results of the agar gel-diffusion. With all ten immune sera tested the immunological pattern was the same. The results for the test serum obtained from animal #35A are illustrated in Figure 3. Two precipitin arcs were observed against cauda epididymal plasma. One of these arcs seemed identical to the one that was formed by the caput epididymal plasma and the cauda epididymal sperm. Four precipitin arcs were produced against the pooled seminal plasma, and two of these appeared common to those produced by the cauda epididymal plasma. The additional two precipitin lines produced by the seminal plasma could not be traced to their point of origin. It is interesting to note that no precipitin arcs were observed for the testicular fluid, although its antigenicity was clearly defined by the gel-diffusion test. It seems apparent that the antigenic concentration of the testicular fluid might have been very low, although repeated attempts were made to increase its concentration with Lyphogel. The difference in visible reactions between the immunoelectrophoretic technique and the gel-diffusion test might be due to a difference in sensitivity of the two systems.

The two most concentrated proteins of the seminal plasma appeared not to move from the origin, which indicates that they are probably high molecular weight substances. The other two precipitin arcs appeared to migrate towards the anode. The precipitin lines observed against the
Figure 3. Immunoelectrophoretic patterns of bovine seminal plasma and other constituents of the bovine reproductive tract. C.E.P.: cauda epididymal plasma, C.E.S.: cauda epididymal sperm, Ca.E.P.: caput epididymal plasma, S.P.: seminal plasma.
cauda epididymal sperm, cauda epididymal plasma and caput epididymal plasma all remained at the origin.

**Immunofluorescent detection of semen iso-antigens**

The localization of semen iso-antigens, in the seminiferous tubules, by the immunofluorescent antibody technique is illustrated in Figures 4 and 5. Fluorescent staining was pronounced in the extratubular spaces of the seminiferous tubules as well as the areas occupied by the Sertoli cells. This would indicate that the Sertoli cells might be the site of synthesis for these iso-antigens.

The Sertoli cells are regarded as supporting cells, which probably provide nourishment for the spermatids with which they are intimately associated. Not much is known of their secretory activity, but since they are implicated in estrogen secretion it is likely that they might also be associated with the synthesis of these iso-antigens. A few scattered fluorescent spots could be observed in the lumen of some of the seminiferous tubules. These areas were not distinct enough to produce any conclusive evidence. The sparsity of fluorescence in the lumen might suggest that these antigens are all soluble in nature and were removed during the preparation of the slides.

**Sephadex gel-filtration**

Bovine seminal plasma components were fractionated into five fractions. These fractions were designated 1 to 5. Fraction 1 was eluted first and fraction 5 last. Typical elution patterns are presented in Figure 6. Fractions 1, 2, 3 and 5 are major seminal plasma protein components but a minor constituent 4 was resolved. The four
Figure 4. Immunofluorescent antibody technique demonstrating the possible origin of semen iso-antigens. X100.
Figure 5. Immunofluorescent antibody technique demonstrating the possible origin of semen iso-antigens. X100.
Figure 6. Separation of pooled bovine seminal plasma by gel-filtration on Sephadex G-200 column.
precipitin lines that were formed by the seminal plasma in the gel-diffusion study are associated with fractions 1 and 2.

Molecular weight estimation

The elution volumes of the four protein standards used were plotted against the logarithm of their respective molecular weights and a linear relationship was obtained. This indicated that the column was separating the proteins effectively. The plot of the elution volumes against molecular weights is presented in Figure 7. On the basis of this linear relationship the molecular weights of fractions 2 and 3 were estimated to be 92,000 and 18,000 respectively. It was not possible to obtain good estimates of the molecular weights of fractions 1, 4 and 5, since they fell outside the range of the standards used. Nevertheless, some molecular weight characterizations are justified. Fraction 1 appeared to possess a relatively large molecular weight, since it fell outside the exclusion limit of the G-200 Sephadex and was collected in the void volume. It appears, therefore, that fraction 1 has a molecular weight which not only exceeds that of the largest marker employed (158 x 10^3), but is probably in fact considerably larger, equalling or exceeding G-200 protein void volume expectations. Similarly it may be safely stated that fractions 4 and 5 have molecular weights of less than 13,700, the weight of the smallest marker employed.

Ion exchange chromatography

The results of the DEAE Sephadex A-50 chromatography are presented in Figure 8. Five major peaks and 2 minor ones were resolved for bovine
Figure 7. Molecular weight estimation of Fractions 2 and 3 of the pooled bovine seminal plasma obtained by extrapolation from the standard protein curve.
Figure 8. Chromatographic separation of bovine seminal plasma with DEAE Sephadex A-50.
The first major peak, designated fraction A, was eluted with a low molar concentration of phosphate buffer, while fractions B to G were eluted with increasingly higher concentrations of eluting buffer. The agar gel-diffusion tests revealed the presence of iso-antigens in all fractions with the exception of fractions E and F.

**Protein and carbohydrate determination**

The protein and sugar content of each of the lyophilized seminal plasma gel-filtration fractions are shown in Table 4. The highest concentration of protein (63.45%) was found in fraction 2. The protein concentration was about the same in fractions 1 and 3, and each of these fractions contained approximately 50 per cent of the concentration present in fraction 2. It is rather surprising not to find a greater concentration of proteins in the first three major fractions of the seminal plasma. This might be explained on the basis of the major limitations of the Folin reaction. It has been shown by Lowry (1951) that the amount of color formed by the Folin reaction varies with different proteins. On the basis of this finding it is probable that the proteins of the seminal plasma reacted differently than the proteins used in the standard thus resulting in an underestimation of the protein concentration per fraction.

The highest concentrations of the sugars were found in the first three major fractions of the seminal plasma. Fraction 1 possessed 3.115% which is at least twice the concentration of carbohydrates found in either fraction 2 or 3. The sugar concentration, like the protein concentration, was almost negligible in fractions 4 and 5. It is likely
### Table 4. Percent Protein and Carbohydrate Contents of Each Seminal Plasma Fraction.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% Protein</th>
<th>% Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.25</td>
<td>3.115</td>
</tr>
<tr>
<td>2</td>
<td>63.45</td>
<td>1.25</td>
</tr>
<tr>
<td>3</td>
<td>30.37</td>
<td>1.35</td>
</tr>
<tr>
<td>4</td>
<td>1.56</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>0.20</td>
</tr>
</tbody>
</table>
that these fractions might consist mainly of lipids although there is no evidence at this time to support this. The high concentrations of proteins in fractions 1 and 2 might be responsible for their iso-antigenicity and large molecular sizes.

**Heat treatment**

The stability of bovine semen iso-antigens to various temperatures was also investigated, and the results are presented in Figure 9. All of the semen iso-antigens appeared to be destroyed by heating at 65°C for 30 minutes. One of these iso-antigens is very heat labile and was completely destroyed with a temperature of 56°C for 30 minutes. The other three antigens exhibited a greater degree of stability. Two of these remaining iso-antigens were destroyed at 65°C for 15 minutes, but when the treatment time at this temperature was increased to 30 minutes all antigens were destroyed.

There is still some doubt regarding the nature of these iso-antigens. In general, mucoproteins are heat stable, (Amano and Behrman 1968), so the observed heat lability of these iso-antigens makes it appear more likely that they are either glycoproteins or lipoproteins or mixtures of both than that they are mucoproteins.

**Electrophoretic comparisons**

With the use of polyacrylamide gel electrophoresis at least the protein components were discernible in the bovine seminal plasma, and probably more were obscured by the more concentrated components. All of these components migrated to the anode, suggesting that they are
Figure 9. Effects of temperature treatments on semen iso-antigens. 1: Untreated seminal plasma, Seminal plasma treated: (2) 56°C for 30 minutes (3) 65°C for 15 minutes (4) 65°C for 30 minutes (5) 75°C for 15 minutes (6) 75°C for 30 minutes.
negatively charged. The mobilities of the various electrophoretic components in relation to bovine serum are presented in Figure 10. It is apparent from the patterns of electrophoretic mobility that most of the detected seminal plasma proteins qualitatively are not identical to blood serum proteins. Based on many investigations one cannot rule out the possibility that serum proteins might be present in the seminal plasma in very small quantities not detectable by the electrophoretic system utilized in this study. Many investigators including Vesselinovitch (1959), however, showed that there were no qualitative or quantitative relationships between bovine seminal plasma proteins and blood serum proteins. In order to characterize the seminal plasma proteins in relation to serum proteins and components from the various areas of the reproductive tract, all components were run side by side on the same electrophoretic gel under the same conditions.

Component 1 was designated the fastest constituent observed for any material in the electrophoretic field and the slower bands were labeled sequentially in ascending order. Consequently the slowest band of the seminal proteins was designated 10 and that for the serum proteins designated 8. The major components of the seminal plasma, i.e. 4, 5, 6 and 7, exhibited mobilities faster than the beta globulin of bovine serum but slower than serum albumin. Components 5 and 6 of seminal plasma had mobilities similar to the alpha globulins of the blood serum. Materials with the same mobility of blood serum albumin were observed for the seminal plasma and the various constituents tested from the male reproductive tract. None of this mobility, however, was observed in the gel-filtration fractions of the seminal plasma which might suggest that
Figure 10. Electrophoretic patterns of bovine seminal plasma proteins, bovine serum and the various preparations from the male bovine reproductive tract. a: cauda epididymal plasma, b: cauda epididymal sperm, c: caput epididymal plasma, d: testicular fluid, e: seminal vesicle fluid, f: whole bovine seminal plasma, g to k: F1-5 of seminal plasma fractions, l: bovine serum.
the concentration was very low. The seminal plasma, the seminal vesicle fluid and the testicular fluid all exhibited electrophoretic migration identical to the gamma globulin of the blood serum. Unlike previous reporters, this investigator was unable to observe any migration to the cathodal end of the gel. This might be due to the pH and buffer system that were used in this study.

Electrophoretic evidence shows that the majority of the bovine seminal plasma proteins probably arose from the seminal vesicles, although the seminal vesicular fluid failed to demonstrate any form of iso-antigenicity. An electrophoretic mobility in the pre-albumin region was observed for the caput epididymal plasma, testicular fluid, the seminal vesicle secretion, the seminal plasma and fractions 1 and 2 of the seminal plasma. No such migration was observed for the bovine serum proteins. Three of the major proteins of seminal plasma were found in fraction 2, while two were seen in fraction 3 despite the fact that the latter failed to produce any visible precipitin lines in the immunodiffusion test. It is rather surprising that more electrophoretic bands were not observed in the cauda epididymal plasma and the cauda epididymal sperm. This odd behavior might be explained on the basis of concentration differences.

A considerable amount of protein was detected at the point of origin of samples including the whole seminal plasma, fractions 1 and 2, the seminal vesicle fluid and the testicular fluid. This protein was not identified, but its failure to migrate is indicative of a very high molecular weight. Fraction 1 of the seminal plasma failed to show any electrophoretic migrating proteins other than the one appearing in the
region of the pre-albumin. It seems obvious that most of the proteins in this fraction may be of such high molecular weight that electrophoretic mobility was impaired. Two of the electrophoretic bands observed in the seminal plasma could not be traced to their point of origin. One of these bands had the same electrophoretic mobility as the faster alpha globulin of the bovine serum while the other one showed a mobility slightly less than serum albumin. The point of origin of these bands at this time is highly speculative, but they may have originated from the other accessory sex glands.

Electrophoretic comparisons were also made of the DEAE Sephadex column fractions of seminal plasma. These results are presented in Figure 11. Fractions A, B and C each produced 1 electrophoretic component. Fraction A exhibited migration to the cathodal end of the gel, although no such migration was noted for the whole seminal plasma. This indicates that fraction A contains one or more protein components which are positively charged under the employed electrophoretic conditions. They are evidently concentrated in fraction A to a point where they are detectable, whereas the lower concentration in whole seminal plasma was not detected. Fractions B and C exhibited mobilities similar to the gamma globulin of bovine serum. Two bands were observed for fraction D. The electrophoretic mobility of one was identical to that of serum gamma globulin while the faster band was slightly slower than those of the serum beta globulins. Fraction E produced two bands, one of which migrated to the cathode. The other band showed mobility identical to fraction F. These two fractions, however, failed to demonstrate any positive reaction by the immunodiffusion technique. Two components were
Figure 11. Electrophoretic comparisons of the ion exchange A-50 fractions of seminal plasma with bovine serum.  
A-G: fractions of seminal plasma  
S.P.: whole seminal plasma  
B.S.: bovine serum.
produced by fraction G. One had an electrophoretic mobility similar to bovine serum albumin, whereas the other constituent corresponded to one of the major proteins of seminal plasma.

**Enzyme digestion**

The immunodiffusion tests that were performed at room temperature (25°C) revealed no reactivity for the pronase digested fractions 1 and 2 of the bovine seminal plasma. This would indicate that the enzyme completely destroyed or inactivated the iso-antigens. When these tests were run at 4°C, however, the results were different. At this temperature the two outermost precipitating lines produced by fractions 1 and 2 were absent from the enzyme digests. The two innermost lines, however, persisted although they were very faint. The results of the pronase digestion are illustrated in Figures 12-14. There is thus evidence that the pronase is affecting these iso-antigens to some extent. The complete removal of these precipitin lines, when incubation was performed at room temperature, might suggest that the enzyme was attacking the antibodies. Since the 4°C temperature reduces, but does not entirely eliminate the possibility that pronase is destroying antibody activity, the results should be treated with some caution. The concentration of the enzyme seems to be very critical in these digestion studies, since 0.02% (w/v) of pronase failed to have any visible effects on fractions 1 and 2.

Alpha amylase in similar concentrations as pronase failed to alter the immunological reactivity of fractions 1 and 2. These results are shown in Figure 15. This might indicate that these iso-antigens are predominantly proteins in nature.
Figure 12. Gel-diffusion precipitation at room temperature showing enzymic digestion of bovine seminal plasma fractions with 1.6% pronase. $F_1U$: fraction 1 undigested, $F_2U$: fraction 2 undigested, $F_1D$: fraction 1 digested, $F_2D$: fraction 2 digested.
Figure 13. Gel-diffusion at 4°C showing enzymic digestion of bovine seminal plasma fractions with 1.6% pronase. $F_1U$ and $F_2U$: fractions 1 and 2 undigested, $F_1D$ and $F_2D$: fractions 1 and 2 digested.
Figure 14. Gel-diffusion precipitation showing proteolytic enzyme digestion of bovine seminal plasma fractions with 0.02% pronase. \( F_1U \): fraction 1 undigested, \( F_2U \): fraction 2 undigested, \( F_1D \): fraction 1 digested, \( F_2D \): fraction 2 digested.
Figure 15. Gel diffusion precipitation showing enzymic digestion of bovine seminal plasma fractions with 1.6% alpha amylase. $F_1 U$: fraction 1 undigested, $F_2 U$: fraction 2 undigested, $F_1 D$: fraction 1 digested, $F_2 D$: fraction 2 digested.
Pronase, which is a broad spectrum protease, is known to be a mixture of four proteinases (Amano et al. 1968). Two of these proteinases are active at neutral pH so these may have been involved in the digestion of the seminal plasma proteins. Pronase is noted for its broad specificity attacking all types of peptide linkages and shows both endo- and exopeptidase activity. The results obtained from the pronase digestion might indicate that the enzyme is acting on the antigenic molecule, partially breaking it up into haptenic molecules or removing some of the antigenic reactive sites.

Electrophoretic tests showed that pronase completely destroyed four of the major proteins of seminal plasma. These results are shown in Figure 16. Two electrophoretic migrating components were resolved for fractions 1 and 2 after pronase digestion but these two lines persisted throughout the entire gel thus indicating an electrophoretic trailing of the pronase enzyme. The alpha amylase failed to produce any noticeable effects in either fraction. No electrophoretic mobility was observed for alpha amylase but pronase produced four bands, two of which showed up in all the treated fractions and bovine serum. It is probable that some of the seminal plasma components which were destroyed by pronase are associated with the semen iso-antigens.

Immunological comparison

All five Sephadex gel-filtration column fractions of bovine seminal plasma were compared immunologically. Fractions 1 and 2 were the only fractionated components of the seminal plasma that produced precipitin lines, and these were formed against all ten immune sera
Figure 16. Electrophoretic examination of enzyme digested and undigested components. 1: seminal plasma untreated, 2: pronase digested seminal plasma, 3-4: pronase treated fractions 1 and 2 respectively, 5-6: α amylase treated fractions 1 and 2 respectively, 7: bovine serum, 8: pronase, 9: α amylase.
tested. In all cases, each of these two fractions produced four common precipitin lines which were identical to those produced by the seminal plasma. The immunological comparison is illustrated in Figure 17. Despite the fact that fraction 3 shares three of the electrophoretic bands produced by fraction 2, it failed to produce any visible reaction.

Fraction 4 and 5 in all cases failed to produce any noticeable reaction against the test sera, indicating their lack of iso-antigenicity. Owing to the presumably great molecular size of fraction one it was not possible to estimate its molecular weight. Fraction 2, which is considerably smaller, however, produces the same number and kind of specificities.

Immunological investigation of the seven seminal plasma components from the DEAE Sephadex column revealed the presence of iso-antigens in five of these fractions. Figure 18 shows the immunological analysis of these fractions. Fraction C which was one of the largest protein peaks of seminal plasma formed three precipitin lines with the test sera. Fraction B also formed three precipitin lines, and these shared common identity with those of fraction C. Fractions A and D shared two precipitin lines with fractions B and C. The innermost line of A was identical with the outermost line of D. Only one precipitin line was formed against fraction G, and this was identical with the innermost line produced by the whole seminal plasma, fractions B, C and D. No visible reaction was observed for the two minor peaks, fractions E and F.

Immunological analysis with the agar gel-diffusion test showed no degree of cross reactivity between bovine seminal plasma and blood plasma from bulls belonging to three different breeds. These results are shown
Figure 17. Gel-diffusion precipitation showing cross reactivity between whole bovine seminal plasma and fractions 1 and 2. W.S.P: whole seminal plasma. F₁-₅: gel-filtration fractions of bovine seminal plasma.
Figure 18. Immunological comparisons of seminal plasma components from DEAE Sephadex A-50 column.
in Figure 19. Failure to observe any precipitin lines indicates that the detected semen iso-antigens are probably not shared with blood plasma.

Comparative immunological studies made between bovine seminal plasma and cauda epididymal plasma with dry mammary gland secretion and colostrum indicated that there was no cross-reactivity between the reproductive fluids and the mammary secretions. The results are presented in Figure 20. It has been demonstrated in man and rabbits that the seminal plasma contains a strongly antigenic substance which coats the sperm. Roberts et al. (1969) identified it as an iron binding protein sharing immunological characteristics with lactoferrin. Bovine mammary gland secretion is an excellent source of lactoferrin, so it was reasoned that an immunological comparison between these components would indicate whether a sperm coating antigen with the above characteristics exists also in the bovine species.

Evidence from these results suggests the following possibilities: a similar sperm coating antigen from the seminal plasma of the bovine species might not be iso-antigenic. This could be supported by the fact that the seminal vesicle fluid, which is the chief source of the sperm coating antigen, was shown in this investigation to be non-isoantigenic. (2) bull semen may not contain a sperm coating antigen comparable with that of man and rabbits, which is immunologically related to lactoferrin.
Figure 19. Immunological comparison of seminal plasma and male bovine blood plasma.

A: blood plasma from Angus bull
H: blood plasma from Hereford bull
S: blood plasma from Shorthorn bulls.
Figure 20. Gel-diffusion precipitation indicating lack of cross reactivity between bovine semen isoantigens and bovine mammary gland secretions.

C.E.P.: cauda epididymal plasma
D.M.S.: dry mammary gland secretion
S.P.: seminal plasma
C.: colostrum
SUMMARY AND CONCLUSIONS

The iso-antigenicity of bovine semen was demonstrated by the immunization of ten heifers from several different dairy breeds. All of the immunized heifers developed high serum antibody titers. The agar gel-diffusion technique revealed the same pattern of immunological reaction for all immune sera tested, thus providing no evidence of polymorphic differences among the immunized heifers. Sera from the control group of animals all reacted negatively to bovine semen antigens by the immunological tests used. There were no detectable antibodies in the cervical mucus of the experimental animals.

The sperm agglutination test revealed the presence of both head to head and tail to tail types of agglutination by the immune sera. The immunoelectrophoretic test demonstrated the presence of four precipitin arcs in seminal plasma, two in cauda epididymal plasma, one in cauda epididymal sperm and one in caput epididymal plasma. None was observed in testicular fluid despite its positive reaction by the gel-diffusion test, which might suggest that the immunodiffusion test was more sensitive.

Pronounced abscesses developed at the injection sites in all immunized animals. There was deterioration in the animals' general well being, and their reproductive cycles became abnormal. Rectal palpation
revealed abnormally small reproductive tracts and ovaries, with a lack of tonicity. The control heifers receiving injections of saline and adjuvant developed no abscesses, cycled normally, and were bred routinely.

The agar gel-diffusion test revealed the presence of four common precipitin lines in the seminal plasma, cauda epididymal plasma, cauda epididymal sperm, caput epididymal plasma, and testicular fluid. No precipitin lines were observed in seminal vesicular fluid, indicating its lack of iso-antigenicity. This was supported by the very sensitive passive hemagglutination test which failed to reveal any positive reaction for this component. The results of the gel-diffusion test indicated that the semen iso-antigens originated in the testes. Immunofluorescent antibody studies revealed the presence of these antigens in the extratubular spaces of the seminiferous tubules as well as the areas containing the Sertoli cells.

In the process of further characterizing the semen iso-antigens, the seminal plasma was first fractionated on the basis of molecular size by the Sephadex G-200 column. Four major fractions and a minor constituent were resolved. The molecular weights of the second and third column components were estimated to be 92,000 and 18,000 daltons respectively. It was not possible to obtain a good estimate of the weight of fraction 1 because it fell outside the range of the standards employed. However, it is obvious that the molecular weight exceeds $158 \times 10^3$ (the weight of largest standard used). Fractions 4 and 5 fell outside the lower limit of the standards used, and therefore have molecular weights of less than 13,700.
DEAE Sephadex anion exchange chromatography was used in an attempt to separate some of the antigenic components of seminal plasma. Five major and two minor protein peaks were detected in the effluent from this column. The first major fraction was weakly adsorbed to the column and was eluted with a low molar concentration of buffer, while the remaining fractions were eluted later with increasingly higher concentrations of eluant.

Protein studies on the lyophilized gel-filtration fractions of seminal plasma showed that most of the protein was located in the first three fractions. Carbohydrate studies indicated that most of the sugars likewise resided in the first three fractions.

All of the detected semen iso-antigens were destroyed by heating at 65°C for 30 minutes. Two of these iso-antigens were completely destroyed with a temperature of 56°C for 30 minutes. Of the remaining two iso-antigens, one was destroyed by heating at 65°C for 15 minutes while the other was destroyed at the same temperature but with the treatment time increasing to 30 minutes.

Comparative electrophoretic analyses of the bovine blood serum and seminal plasma proteins indicated that there was little qualitative relationship between the two systems. The major components of seminal plasma exhibited mobilities faster than beta globulin of bovine serum but slower than serum albumin. Seminal vesicle fluid was shown to contribute at least seven proteins to the seminal plasma despite its lack of iso-antigenicity. The seminal plasma produced at least ten components which all migrated towards the anode. Fraction 1 showed little or no electrophoretic mobility, probably because of its large
molecular size. Two of the electrophoretic bands observed in the seminal plasma could not be traced to any of the investigated components of the reproductive tract, indicating that they might have originated in other accessory sex glands.

Electrophoretic comparisons of DEAE Sephadex column fractions of seminal plasma showed that fractions A and E exhibited some electrophoretic mobility towards the cathode. All other fractions showed migration only to the anodal end of the gel.

Enzyme digestion studies suggested that pronase (1.6%) might be altering the reactive state of some or all of the semen iso-antigens whereas alpha amylase produced no noticeable effects. Pronase might be exerting its inhibitory effect on the immunological reaction by attacking the reactive sites of the antigens, or it might be cleaving the antigenic molecules into haptenic components. The additional possibility of pronase destruction of antibody integrity could not be ruled out, although low temperature immunological tests minimized this possibility. Electrophoretic examination of the enzyme digested seminal plasma showed that the enzyme destroyed four of the major protein bands. No electrophoretic migration was observed for the pronase digested gel-filtration fractions 1 and 2. Although the enzyme studies provided no conclusive information concerning the biochemical nature of the iso-antigens, the indications were that peptide chains were possibly involved in the molecular structure while no evidence of polysaccharide contribution was detected.

Immunological examination of the five gel-filtration fractions of the seminal plasma demonstrated that fractions 1 and 2 each had four
antigenic components. The other 3 fractions failed to produce any detectable immunological reactions. Immunological analyses of the DEAE Sephadex fractions showed that fractions B and C contained at least three antigenic components, fraction G showed one, while fractions A and D each contained two. No iso-antigenicity was demonstrated by fractions E and F. A summary of the characteristics of the bovine semen iso-antigens is presented in Table 5.

The detection of each iso-antigen in two well separated portions of the gel filtration column effluent, together with their presence in multiple well-defined anion exchange column peaks, suggests the possibility of some relationship and inter-conversion among the different forms. It was observed that although all four iso-antigens were found in the first two G-200 peaks, there appeared to exist a subtle shift in relative concentrations from peak one to peak two, paralleling a more pronounced shift in proportions in succeeding anion exchange column effluents. These changes were coincident with changes in heat stability and pronase vulnerability. The somewhat fragmentary picture emerging from these characterizations, then, is of antigen 1 as possible being associated with a relatively smaller molecule, more net negatively charged at basic pH levels, relatively heat stable and pronase resistant, while the other antigen-associated molecules, considered in order of increasing identification number, may be progressively larger, less net negatively charged under basic conditions, more heat labile, and more vulnerable to pronase attack.

Immunological studies demonstrated that there was no cross
Table 5. Summary of the Characteristics of the Bovine Semen Iso-antigens.

<table>
<thead>
<tr>
<th>Iso-antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Effluent peaks in which antigens were detected with:</th>
<th>Heat lability (Destroyed by:)</th>
<th>Pronase treatment effect</th>
<th>Amylase treatment effect</th>
<th>Probable immuno-electrophoretic behavior&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sephadex G-200</td>
<td>DEAE Sephadex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1,2</td>
<td>B,C,D,G</td>
<td>65°C, 30 min.</td>
<td>Intact</td>
<td>Intact</td>
</tr>
<tr>
<td>2</td>
<td>1,2</td>
<td>A,B,C,D</td>
<td>65°C, 15 min.</td>
<td>Intact</td>
<td>Intact</td>
</tr>
<tr>
<td>3</td>
<td>1,2</td>
<td>None*</td>
<td>65°C, 15 min.</td>
<td>Digested</td>
<td>Intact</td>
</tr>
<tr>
<td>4</td>
<td>1,2</td>
<td>A,B,C</td>
<td>56°C, 30 min.</td>
<td>Digested</td>
<td>Intact</td>
</tr>
</tbody>
</table>

<sup>a</sup> Isoantigens were numbered in order of the proximity of the corresponding precipitin lines to the antiserum well in the immunodiffusion test.

<sup>b</sup> The correspondence between immuno-electrophoretic precipitin arcs and antigens identified by immunodiffusion characteristics is based upon antigenic concentration similarities.

* Location of iso-antigen 3 in DEAE Sephadex column effluent could not be determined.
reactivity between seminal plasma or cauda epididymal plasma and bovine mammary gland secretions. Neither was any cross reactivity observed between semen iso-antigens and blood plasma from bulls of three different breeds.

This investigation clearly demonstrated that iso-antigens do exist in semen. Their role in the reproductive process is still questionable, however. Owing to the deterioration in the health of the experimental animals and their subsequent acyclicity, no satisfactory analysis could be made of the direct effects of the immunization on their reproductive function. Such relationships can perhaps better be determined after the detected iso-antigens are further purified and characterized.

The demonstrated potency of semen iso-antigens, together with the development of preliminary techniques for partial purification, would seem to indicate the desirability and feasibility of continued investigation in this field.


