

CAPACITATION OF SPERM

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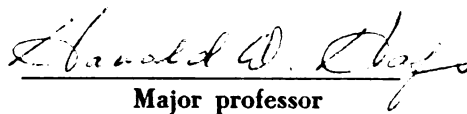
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ABSTRACT

CAPACITATION OF SPERM

by Kenneth T. Kirton

Incubation of sperm for 6 hrs in uterine fluid in vitro increased the fertility of sperm inseminated oviducally at the time of ovulation (40 of 95 ova fertilized) above the level of fertility of sperm incubated in buffered Lockes' solution in vitro (3 of 22 ova fertilized). Similar to the fertility results obtained with sperm incubated in utero, in vitro incubation for longer periods of time increased the fertility of sperm which were oviducally inseminated at the time of, or 1.5 hrs after ovulation. Sperm incubated in vitro in the macromolecular portion of uterine fluid were fertile (8 of 48 ova fertilized) whereas sperm incubated in the dialysable portion of uterine fluid were not (0 of 51 ova fertilized). Storage of uterine fluid at -20°C for 60 days reduced, and boiling destroyed the ability of uterine fluid to capacitate sperm in vitro.

Incubation of sperm in 0.1 or 1.0 mg % of α -amylase for 8 hrs increased fertility over levels obtained with control sperm incubated in buffer alone (3 of 11 and 5 of 13 ova fertilized, compared to 0 of 32 ova fertilized,

respectively). Increasing the in vitro incubation time to 10 or 12 hrs increased the fertility of sperm inseminated at the time of ovulation and at 1.5 hrs after ovulation. Sperm incubated in α -amylase, or in a combination of protease and β -amylase were also partially capacitated in vitro.

Rabbit blood serum contained more amylolytic activity (0.40 mg maltose equivalents liberated ml/min at 37°C) than rabbit uterine fluid (0.29 maltose equivalents), but less than rabbit seminal plasma (0.54 maltose equivalents). Amylolytic activity of uterine fluid decreased with increasing intervals of time after uterine ligation.

Elution of rabbit blood serum or concentrated rabbit uterine fluid from a column of Sephadex G-200 separated each fluid into three major ultraviolet radiation absorbing peaks. Agar gel diffusion tests revealed that the uterine fluid specific antigens were separated quantitatively by this chromatography. Agar gel electrophoresis revealed that the uterine fluid proteins were separated quantitatively, similarly to published reports for blood serum proteins of other species.

Addition of α - or β -amylase to bull semen extender did not affect motility adversely and the high level of α -amylase increased motility ($P < 0.01$). Fertility of bull sperm after storage at -196°C was significantly increased over control levels by addition of 0.1 or 1.0 mg % of

α - or β -amylase to the semen extender prior to freezing ($P \approx 0.07$). Fertility of semen treated with the high level of α -amylase was significantly higher than semen treated with the low level ($P \approx 0.07$).

In summary, the results of this thesis indicate that rabbit uteri retain the ability to capacitate sperm for at least 2 wks after ligation. Fluid recovered from ligated uteri at least partially capacitated sperm during in vitro incubation and β -amylase in phosphate-buffered Locke's solution mimiced uterine fluid in this regard. Increasing the in utero or in vitro incubation time from 6 to 12 hrs increased the degree of sperm capacitation. Addition of 1.0 mg % of α -amylase to bull sperm increase subsequent motility and fertility.

CAPACITATION OF SPERM

By

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BIOGRAPHICAL SKETCH

Kenneth T. Kirton was born at Kingman, Kansas on June 16, 1934. He received his elementary education at Independence School and was graduated from Iola High School in June, 1952.

In September 1952, he entered Kansas State College, and received a Bachelor of Science degree in Dairy Husbandry in June, 1957. After serving two years with the United States Army from August, 1957 to 1959, he operated a dairy farm at La Harpe, Kansas until accepting a graduate assistantship at Michigan State University in September, 1961. He received the MS degree in June, 1963 and Ph.D in March, 1967, in the Dairy Department with a major in Reproductive Physiology.

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INTRODUCTION

The available evidences for the mammalian species which have been studied indicate that freshly ejaculated sperm are not capable of fertilizing ova. Rather they normally develop the capacity to fertilize ova during a period of residence in the uterus or oviduct of the female. At first glance, such a reproductive process, whereby sperm are ejaculated in a nonfertile condition, seems inefficient. On the other hand, this mechanism provides the uterus with means whereby it regulates the ability of sperm to fertilize an ovulated egg and thereby prevents fertilization when optimal intra-uterine conditions for implantation and fetal development are not prevalent. Direct evidence for this fertility regulating mechanism has been demonstrated for the rabbit, (Chang, 1951, and Austin, 1951), the rat (Austin, 1952), and the mouse (Braden and Austin, 1954), and indirect evidence has been reported in several other species (Dukelow, et al., 1966a). This process by which sperm develop the ability to fertilize ova has been termed capacitation.

Chang (1957) demonstrated that sperm capacitation is reversible. Sperm that had been capacitated in utero, and subsequently re-suspended in as little as 5% seminal plasma for 30 minutes, had to be recapacitated in utero

before they were capable of fertilizing ova. The term decapacitation factor has been applied to the substance in seminal plasma which is capable of reversing the capacitation reaction.

The active substances involved in the capacitation and decapacitation reactions apparently are not species specific. For example, rabbit sperm may be capacitated in the uterus of an estrous dog or rat (Hamner, 1966). Furthermore, human, horse, rat and bovine seminal plasma contain a decapacitation factor which is active against capacitated rabbit sperm.

Because capacitation of sperm is prerequisite to fertilization, elucidation of the mechanism of capacitation could provide valuable insight to understanding the mechanism of fertilization. The objective of this study was to determine the nature of some factors which are normally responsible for or associated with capacitation of sperm and to devise a means of achieving capacitation of sperm in vitro where the process may be studied more easily.

REVIEW OF LITERATURE

Evidence of Necessity for Capacitation

Heap (1905) and several subsequent workers (Parker, 1931, Hammond, 1934, San Martin, 1951, Braden, 1953) reported that rabbit sperm normally arrive at the site of fertilization in the upper portion of the oviduct within 3 to 4 hours after coitus. However, other published researches indicated that sperm transport may be more rapid in the rabbit than had been believed formerly. For example, Chang (1952), Adams (1956), and Greenwald (1956), reported finding sperm in the upper portion of the oviduct as early as 1 hour after copulation. Braden (1953) found a maximal number of sperm present in this part of the oviduct within 6 hours after copulation, and the number of sperm there remained relatively constant for about 18 hours.

Chang (1951), and Austin (1951) found a maximum of 2,000 to 3,000 sperm in the vicinity of the ova at the time of fertilization, and Austin concluded that this may be the minimal number necessary to provide normal levels of fertility in the rabbit (1948). Undoubtedly, the difficulty in accurately enumerating the few sperm which arrive at the site of fertilization accounts for some of the disagreement on the speed of sperm transport in the female. In most other laboratory animals the transport of some

sperm to the oviducts takes place within minutes of copulation (Austin and Bishop, 1957). Therefore, sperm transport in the rabbit may also be more rapid than is now generally believed.

Barry (1839), and others (Heap, 1905, Walton and Hammond, 1928, Hammond, 1934, and Harper, 1961), demonstrated that ovulation in most rabbits normally occurs 9.5 to 11.5 hours after copulation or after the intravenous injection of an ovulating hormone. Clearly then, sperm in sufficient numbers for maximal fertility are normally present at the site of fertilization for at least 5 to 7 hours before ovulation occurs in rabbits. However, Hammond (1934) demonstrated a reduction in fertility of rabbits that were inseminated 5 to 6 hours after sterile copulation, or about 5 hours or less before the expected time of ovulation. Since the rabbit egg probably remains viable in the female tract for a period of only about 6 hours after ovulation (Hammond, 1934, Blandau, 1961), this indirect evidence indicates that rabbit sperm require a period of some 10-11 hours in the female reproductive tract to develop their maximum fertilizing capacity.

Subsequently Chang (1951) and Austin (1951), working independently, published the first direct evidence to show that sperm require a period of maturation in the female reproductive tract to develop the capacity to fertilize ova and Austin termed the process "capacitation."

These authors demonstrated, by performing oviducal inseminations at varying intervals before or after ovulation, that freshly ejaculated rabbit sperm require a period of at least 5-6 hours in the female reproductive tract to attain the capacity to fertilize an egg. The ease with which the time of ovulation may be determined in an induced ovulator such as a rabbit facilitates experiments on the mechanism of capacitation. Largely for this reason, a requirement for capacitation has been demonstrated unequivocally only in the rabbit.

Although evidence for the necessity of sperm capacitation is most direct in the rabbit, indirect evidence indicates that the sperm of several other species must also undergo a final maturation in the female reproductive tract before attaining the ability to fertilize ova. For example, Austin (1952) allowed rats to copulate between 7:00 and 7:30 on the morning of estrus and then examined the eggs 1 to 6 hours later for evidence of sperm penetration. The data from this experiment, and from Austin and Braden (1954) indicated that there is a delay of about 2 hours from the time of insemination to the initial penetration of ova by sperm. That sperm were present at the site of fertilization within 1 hour after insemination, indicates a final maturation of sperm is also prerequisite to fertilization in this species. Further evidence for the necessity of capacitation in rats was published by Noyes (1953). He obtained

a higher rate of fertilization with rat sperm inseminated into the uterus near the time of ovulation when the sperm had been previously incubated in the uterus of another estrous rat.

A similar experiment with mice (Braden and Austin, 1954) indicated that sperm of this species require at least 1.5 hours to attain fertilizing capacity. However, Brinster and Biggers (1965) obtained about 10% fertilization by incubating mouse ova and epididymal sperm in excised oviducts and Yanagimachi and Chang (1963) obtained a low rate of fertilization of Golden Hamster eggs in vitro by incubating the eggs in media with epididymal sperm (17%). However, the percentage of fertilized eggs was increased considerably (66%) with sperm that were incubated in utero for 4-5 hours prior to incubation in vitro with ova. Similar indirect evidence indicated a necessity for capacitation of ferret sperm (Chang and Yanagaimachi, 1963). Rowlands (1957) obtained a higher pregnancy rate when he inseminated guinea pigs by an intraperitoneal injection of sperm 1 to 12 hours after estrus (80%) than when inseminated 0 to 36 hours before estrus (33%). However, the interval from insemination to sperm penetration of the ova was not determined.

Trimberger and Davis (1943) determined that the average time of ovulation in dairy cattle was 10.5 hours after the end of standing heat. Artificial insemination

at varying times before and after ovulation revealed maximum fertility from inseminations made 13 to 18 hours before ovulation (86%), compared to significantly lower fertility (57%) for inseminations 6 hours or less before ovulation and 30 to 40% fertility when inseminations were performed 2 to 6 hours after ovulation. Extensive indirect evidence indicating the best time to breed cows for maximum conception is the middle to the end of standing heat (Barrett and Casida, 1946, VanDemark, 1952), further substantiates the results of Trimmerger. Since the minimal time for sperm transport has been determined to be as short as 2 to 3 minutes in the bovine (VanDemark and Moeller, 1951), bovine sperm apparently also require a period of exposure to the female genital tract to obtain maximum fertilizing ability. More recently Mahajan and Menge (1966) were unable to demonstrate directly that capacitation was necessary in the bovine. Sperm which had been incubated in utero were no more fertile than sperm which had been incubated in a semen extender in vitro, when used to inseminate cows at varying intervals up to 22 hours after ovulation. However, the few ova obtained and the manipulations of the sperm during this experiment made the apparent conclusions uncertain.

The observation by Dauzier and Thibault (1959) that fertilization of sheep ova can be achieved in vitro with spermatozoa recovered from the genital tract of mated ewes, but not with freshly ejaculated semen, indicated that

ram spermatozoa may also undergo capacitation in the genital tract of the ewe. This conclusion was supported by the in vivo observations made by Mattner (1963). Sperm penetration of the zona pellucida never occurred in less than 1.5 hours after tubal insemination, indicating that this may be the minimal interval of time necessary for capacitation in sheep. Hunter and Dziuk (1966) recovered penetrated swine ova 3 hours after oviducal insemination, indicating that if capacitation is necessary in this species, the minimal interval of time required is 3 hours or less.

No direct evidence is available to indicate that capacitation is a prerequisite to fertilization in primates. However, van Wageningen (1945) determined that matings in a colony of Rhesus monkeys during the 24 hour interval from noon of day 11 to noon of day 12 of the menstrual cycle were more fertile than earlier or later matings. Hartman (1933) determined, by rectal palpation, that the greatest number of ovulations in this same species occurred on day 13, thus indicating that a period of sperm incubation in the oviduct or uterus is probably necessary for optimal fertility in this species also.

Factors Affecting Capacitation

Time Required for Capacitation

Chang (1955), incubated rabbit sperm in uteri of estrous rabbits for varying periods of time prior to

recovering and using these sperm to inseminate other rabbits 2 hours after ovulation. Maximal fertility was achieved (77%) when sperm were incubated in utero for 16 hours. Noyes (1960) indicated a lag of 5 to 6 hours between mating and the onset of fertilization in rabbits. Although capacitation of rabbit sperm may be accomplished in the uterus or oviduct, Adams and Chang (1962) concluded that the process required more time in the oviduct than in the uterus. In utero inseminations performed at a time approaching ovulation were more fertile than oviducal inseminations. These data could explain the marked discrepancy in the fertility of animals that were naturally mated and those in which semen was deposited into oviducts at corresponding times (Chang, 1951). Austin, (1951) also concluded that a minimum of 5 to 6 hours was necessary for capacitation of rabbit sperm to be completed in the uterus. Hammond (1934) and Dziuk (1965) indicated that sperm from certain strains of rabbits and certain bucks within a strain of rabbits, are either ejaculated in a more mature state or require less time to become capacitated in the uterus.

Data for the rat (Austin, 1952), mouse (Braden and Austin, 1954), and hamster (Chang and Sheaffer, 1957) indicated that capacitation of spermatozoa probably requires less time in rodents (1.5 to 2.5 hours) than in rabbits. However, indirect evidence in cattle (Trimberger

and Davis, 1943, and VanDemark, 1952) indicated that development of maximal fertility of bovine sperm may require as long as 14 to 16 hours in utero. In contrast, freshly ejaculated sperm of the golden hamster were apparently capable of fertilizing some ova in vitro without exposure to the female reproductive tract (17% of the ova were fertilized; Yanagimachi and Chang, 1963).

Large numbers of sperm cells have been found in the uterine horns of guinea pigs, rats, and dogs immediately after copulation (Florey and Walton, 1932, Hartman and Ball, 1930, Blandau, 1945, and Evans, 1933). Furthermore, the interval between mating and the arrival of the first few sperms at the site of fertilization is about 15 minutes or less for the mouse (Lewis and Wright, 1935), rat (Blandau and Money, 1944), golden hamster (Yamanaka and Soderwall, 1960), bitch (Whitney, 1933), cow (Van Demark and Moeller, 1951), and ewe (Starke, 1949). Therefore, the lag between the time of insemination and the time of fertilization in these species apparently is not due to the time required for sperm transport.

Endocrine Status of the Incubator Animal

Chang (1958) demonstrated that sperm were not capacitated when incubated in the uterus of pseudopregnant rabbits or in immature ovariectomized or estrous rabbits treated with progesterone. However, uteri of uninjected immature or uninjected ovariectomized rabbits did

capacitate sperm. He further demonstrated that capacitation in the oviduct was not qualitatively affected by the hormonal balance of the host animal. Noyes, et al. (1958) at least partially capacitated sperm in such isolated and seemingly unrelated organs as the colon, bladder, anterior eye chamber, or even the glandularis vesicularis of male rabbits. In the same experiment sperm were partially capacitated when they were confined within the excised uterine horns of a rabbit and when suspended with strips of endometrium in saline solution at room temperature. Smith (1951) incubated rabbit ova with oviducal mucosa scrapings and freshly ejaculated sperm for 1.5 to 2 hours. Subsequent examination of the ova after incubation in culture chambers revealed sperm heads or a developing male pronucleus within the vitellus. In similar experiments Moricard (1950) incubated sperm and ova in explanted oviducts under relatively anerobic conditions. He observed sperm penetration through the zona pellucida and into the oocyte cytoplasm. However, capacitation was not achieved when sperm were confined in a cellophane dialysis bag in the intact uterus of an estrous host or when incubated in vitro in the presence of washed red blood cells or dead spermatozoa (Noyes, et al. 1958). This evidence strongly implied that a macromolecular constituent of uterine or oviducal origin was involved in capacitation.

However, Hadek (1959) obtained data which indicated that washing sperm to remove seminal plasma may partially capacitate, or at least shorten the period of time required for in vivo capacitation of rabbit sperm. Insemination into the oviduct with whole semen 6 hours after sterile mating never resulted in fertilization. Insemination at this time with once-washed sperm resulted in fertilization and early cleavage, but the embryos degenerated in the blastocyst stage. Twice washed sperm caused a high rate of fertilization (18 of 23 ova) and early embryological development. However, Chang (1955) was unable to obtain any fertilized ova after insemination with centrifuged epididymal or ejaculated sperm 11.5 to 12.5 hours after injection of sheep pituitary extract. Several other attempts to capacitate sperm in vitro with red blood cells, blood sera, or white blood cells have been unsuccessful (Chang, 1955, Noyes, et al., 1958).

Capacitated sperm have been obtained by two different means. Either the sperm were surgically deposited directly into the uterus, or a doe rabbit was mated several times to increase the number of sperm found in the uterus above that found after a single mating. When the degree of capacitation of the sperm recovered from uteri of these two kinds of experiments was evaluated in terms of the percentage of fertilized eggs obtained following oviducal insemination of test animals, sperm recovered after surgical deposition result in lower

fertility than those recovered after natural mating (Adams and Chang, 1962). They found that sperm placed in the uterus by surgical deposition resulted in 16% fertilization of ova when used to inseminate does oviducal 10 hours after luteinizing hormone injection, considerably less than the 60% fertility obtained with sperm recovered after natural mating.

The most obvious difference between the two methods is the presence or absence of the stimulus of coitus, a phenomenon which causes the release of pituitary luteinizing hormone and subsequent ovulation. Another difference of possible significance is the presumed smaller amount of seminal plasma in the uterus following natural mating. Recently Soupart (1966) demonstrated a relationship between the amount of ovulating hormone (Human Chorionic Gonadotrophin) injected, and the ability of the rabbit uterus to capacitate sperm. Following the injection of 75 IU of HCG, epididymal sperm deposited surgically into uteri attained a high degree of capacitation (74.5% of ova fertilized). However, when lower or higher levels of HCG were injected, the degree of capacitation attained was much less.

It is unlikely that ovulating hormone directly caused these changes. Rather, these data probably indicate an effect on the ability of the uterus to capacitate sperm, by a secretion product of the ovary following

gonadotrophin stimulation. Hilliard et al. (1964) demonstrated in rabbits that ovarian secretion of the major reduction product of progesterone (20 α -hydroxy-pregn-4-en-3-one) increases rapidly postcoitally, remains elevated for 6 to 8 hours, and then drops to the non-detectable precoital levels by the time of ovulation. She also demonstrated a close relationship between the blood levels of ovulatory hormone and 20 α -hydroxy-pregn-4-en-3-one. This or a similar steroid may have a very rapid and direct effect on the secretion of the uterine endometrium. Such a phenomenon would not only provide for more rapid capacitation in uteri of copulated rabbits, but could also explain the data of Chang (1958) in which he was unable to capacitate sperm in the pseudopregnant uterus or in the uterus of rabbits under the influence of exogenous progesterone.

Seminal Plasma

Chang (1957) demonstrated that incubation of capacitated sperm in seminal plasma (diluted as much as 20 fold) for 30 minutes reversed the capacitation process. Sperm which were decapacitated in this manner could be recapacitated by another period of incubation in an estrous uterus. Bedford and Chang (1962) isolated the "decapacitation factor" (DF) from seminal plasma by centrifugation at 105,000 X g for 3 hours, or by precipitation with 100% ethanol. They further demonstrated that the active fraction

in seminal plasma was not destroyed by heat (65°C), or by dialysis. More recently Dukelow, et al. (1966c) determined that the activity of DF was destroyed by α -or β -amylase. Furthermore, the activity was stable to prolonged drying at 100°C, or storage at 37°C, (Dukelow et al., 1966a). After reaction of seminal plasma with pronase, the biological activity of DF largely remained in the supernatant fluid after centrifugation, probably indicating that the active portion had been cleaved from a large molecular weight protein. However, the activity of DF was not destroyed by incubation with pronase, lysozyme, hyaluronidase, glucose oxidase, or galactose oxidase. These data suggest that the active portion of DF is a relatively low molecular weight carbohydrate which is bound to a much larger protein carrier. In support of this concept, Hunter and Nornes (1966) reported that partial chromatographic separation and subsequent staining indicated that DF was a glycoprotein.

Weinman and Williams (1964) found DF activity in epididymal fluid, thus explaining the necessity for capacitation of epididymal sperm (Austin, 1951, and Chang, 1951). Weil (1962) demonstrated that ejaculated rabbit sperm were coated with antigens from the seminal vesicles. Hunter and Hafs (1964) demonstrated that some of these antigens are present on epididymal sperm and are not quantitatively removed by repeated washing. These

observations may be due to the similar embryological origin of the seminal vesicles and the epididymides. This may also explain why washed sperm need also be capacitated (Chang, 1955). In any case, decapacitation factor is at least partly derived from the epididymis and is probably firmly bound to the sperm and remains there through the ejaculatory process and during the initial ascent of the female genital tract by the sperm.

Bull, rabbit, stallion and human seminal plasma decapacitated rabbit sperm, but dog, and rooster seminal plasma did not (Chang, 1957, Dukelow et al., 1966c). Also, the estrous dog or rat uterus is capable of capacitating rabbit spermatozoa (Hamner, 1966). These data and those discussed above for other species suggest that capacitation has little if any species specificity.

Phenomena Associated with Capacitation

The process involved in capacitation may consist of several successive and closely related steps, rather than an all or none phenomenon, in that each stepwise change may be a necessary prerequisite for a corresponding reaction or process during fertilization. This theory was first proposed by Noyes (1960). In support of this concept, there are several reports of superficially diverse experiments which may, in fact, be related directly or indirectly with capacitation and which shed some light on the process of sperm capacitation. For example, Austin

and Braden (1953) detected an increased incidence of polyspermy in rats mated at 10:00 a.m. as compared with 9:00 a.m. on the day of estrus (9.2% and 1.5% respectively), and an increased polyspermy in rabbits mated 10 hours after administration of HCG (17.0% compared to a normal rate of 2.0%). Yanagimachi and Chang (1963) obtained ova with a higher incidence of polyspermy (33%) following in vitro incubation of ova with sperm which had been incubated with epididymal sperm (23%). In utero incubation provided sperm with increased ability to penetrate ova, but many of these penetrated sperm were unable to undergo formation of pronuclei and syngamy. However, other factors can also influence the incidence of polyspermy. For example, Austin (1955) determined that elevation of body temperature to 40.6°C for 6 hours following delayed mating increased the incidence of polyspermy in rabbits from 2.2% to 19.8%.

Murphree et al., 1947, found that eggs from superovulated estrous, juvenile, and anestrus rabbits were fertilized at a normal rate (80 to 82%). However, superovulated ova from pseudopregnant rabbits were not fertilized. The authors later concluded that sperm transport accounted for much of the lutealphase infertility, but (in light of what we now know about capacitation) it may also have been at least partially attributable to the inability of the pseudopregnant uterus to capacitate

sperm (Murphree et al., 1951). Austin (1949) reported similar results and demonstrated that ova from pseudopregnant rabbits are capable of being fertilized when transferred to estrous rabbits. He also concluded that reduced sperm transport contributed to the lowered fertility in pseudopregnant rabbits. Hunter (1966) found a much lower level of fertility than normally expected when pseudopregnant gilts were ovulated by hormone injections and then artificially inseminated. This low fertility was probably also caused, at least in part, by the inability of the pseudopregnant uterus to capacitate sperm. Casida (1967) indicated that cattle inseminated shortly after parturition were not fertile. He suggested that the lack of fertility may be due to a lack of capacitation in the post-partum uterus.

Soupart and Clewe (1965), indicated that sialic acid residues may be instrumental in some way in the biochemical mechanism by which sperm penetrate ova. They found that enzymatic removal of these residues by incubating ova in neuraminidase for 60 minutes inhibited subsequent sperm penetration.

Bedford (1965) demonstrated a differential phagocytosis of rabbit sperm by leucocytes after incubation in the estrous uterus, but not after incubation in the pseudopregnant uterus or in the pleural cavity. He suggested that the change which occurred in the surface membrane of spermatozoa

in the estrous uterus rendering them acceptable to leucocytes may also constitute one phase of capacitation.

Bedford (1963) found changed or lose acrosomes on 5 to 58% of the sperm he examined after 9 to 11 hours of in utero incubation in estrous or pseudopregnant doe rabbits. He also determined that the acrosome was absent in sperm released from the perivitelline space of fertilized ova. However, he concluded the acrosome changes noted may not be a "morphological concomitant of capacitation, but more probably reflect impending sperm senility."

Theories on the Mechanism of Capacitation

Harter (1948) suggested that spermatozoa may dissolve the mucopolysaccharide of the zona pellucida by the local action of acid, since the zona of the rat ova dissolves below pH5. However, the zona of rabbit ova is insoluble above pH 3, and is not dissolved in vitro by large numbers of sperm. Although reducing agents dissolve the zona under less drastic conditions than acid, the environment within the oviduct is oxidative (Bishop, 1956). Whitten (1957), suggested that lactic acid produced by sperm metabolism may be important in conditioning the oviducal environment. Austin (1951), suggested that a mucolytic enzyme carried by the sperm may digest a path through the zona pellucida. He speculated that capacitation might involve removal of an enzyme inhibitor which was

located on the surface of the sperm. Austin and Bishop (1958) and Austin (1961, 1963) have speculated that ripening and shedding of the acrosome, thus exposing the perforatorium, may be involved in the process of capacitation. A similar reaction (Colwin, and Colwin, 1957) has been reported to be prerequisite to fertilization in several invertebrate species. Earlier, however, Austin and Bishop, (1957) suggested that capacitation involved the activation of an enzyme system which required some factor from the female tract.

More recently Piko and Tyler (1964) suggested that the acrosome reaction released an acrosomal enzyme which enabled the sperm to pass through the cumulus and zona pellucida. Their conclusions were substantiated by electron-micrographs of rat sperm in the epididymis and during ovum penetration. Williams, et al., (1965) suggested that the enzymatic alteration or removal of the seminal plasma decapacitation factor may constitute capacitation. Noyes (1960) speculated that capacitation progressed gradually to completion in each individual sperm and that the fully capacitated sperm are then able to penetrate the zona very rapidly. In an experiment conducted by double mating does to two bucks whose offspring were distinguishable from each other, Dzuik (1965) determined that sperm which had resided in the genital tract for a period of time had a competitive advantage

over sperm that had spent less time in the female. He further concluded that capacitation might be a quantitative as well as qualitative phenomenon. Soupart and Orgebin-Crist (1966) speculated that intimate contact of the sperm head and uterine endometrium may be necessary for capacitation.

Obviously then, the mechanism of capacitation is not completely understood at this time. In the rabbit the evidence available indicates complete capacitation can be achieved only in the uterus or oviduct of an estrous doe, and not during pseudopregnancy or while the animal is under the stimulation of exogenous progesterone.

Stevens et al., (1964a) demonstrated that fluid accumulated in ligated rabbit uteri. Their electrophoretic and immunochemical data indicated that some of the proteins present in the accumulated uterine fluid were specific secretions of the uterus, not mere transudates from blood. In view of the evidence which implicated a large molecular weight constituent from uterine secretions in capacitation, the method Stevens developed to accumulate quantities of uterine fluid provides an important means of studying capacitation. Therefore, the secretions of the estrous rabbit uterus, especially the large molecular weight components and their relation to capacitation, were the primary object of study for most of the experiments reported in this thesis.

EXPERIMENTAL PROCEDURES

General Sequential Design

Stevens et al., (1964a) demonstrated that ligated uteri of estrous rabbits accumulate fluid at rates of about 2.5 ml per cornu per week. Because sperm are readily capacitated in estrous uteri (Chang, 1951), the secretions which accumulated during uterine ligation may also capacitate sperm, unless ligation qualitatively altered the intra-uterine environment of the incubator animal. The initial experiment was designed to test this point and find if sperm could be capacitated in ligated uteri. Subsequently, uterine fluid was aspirated from ligated uteri to determine if this fluid could capacitate sperm in vitro.

Chang (1957) demonstrated that incubation of sperm in seminal plasma for 30 minutes apparently reversed the capacitation process. More recently Williams et al. (1965) found that the enzyme β -amylase destroyed the biological activity of this factor in seminal plasma which reverses capacitation. They further demonstrated that pronase apparently altered the structure, although it did not destroy the biological activity of the decapacitation factor. If capacitation consists of destruction or removal of decapacitation factor from seminal plasma, then in vitro capacitation may be effected with enzymes which destroy

decapacitation factor. Consequently, sperm capacitation was tested by in vitro incubation in amylase, protease, lipase, and all possible combinations of these enzymes.

Noyes et al. (1958) found that sperm were not capacitated when suspended in a dialysis bag in utero, thus implicating a macromolecule in the process of capacitation. Stevens et al. (1964b) demonstrated that at least two general classes of proteins, one with an electrophoretic mobility similar to pre-albumin and one similar to β -globulin, were specific to uterine fluid; their counterparts were not found in rabbit blood sera. Consequently, an attempt was made in this thesis to fractionate the proteins in uterine fluid and partially to isolate these two classes of uterine fluid specific proteins, to further characterize them, and to relate them to capacitation.

Indirect evidence (Trimberger and Davis, 1943, Van Demark, 1952) indicated that bovine sperm also must spend a period of time in the female reproductive tract to develop fertilizing capacity. At present, Michigan Animal Breeders Cooperative authorities estimate that, of all cattle inseminated, about 5% do not conceive because they were inseminated too late relative to estrus for optimal fertility. If some method can be devised to capacitate bovine sperm in vitro, some cows which would normally be artificially inseminated too late to conceive with normally ejaculated sperm may be impregnated when inseminated with capacitated

sperm. To test this hypothesis, trials were conducted to determine the motility and fertility of bull sperm with added amylase.

Capacitation of Sperm in Ligated Uteri

Uteri of estrous doe rabbits were ligated anterior to the cervix with a synthetic suture material.* This ligation was accomplished through a mid-ventral incision while the rabbits were under a general anesthetic (Stevens et al. 1964a). After various intervals of time up to 8 weeks after ligation, the volume of accumulated uterine fluid was adjusted to about 2 ml per cornua by surgically aspirating the excess with a syringe. Washed, ejaculated rabbit sperm were then injected into the fluid which remained in the cornua to obtain a concentration of about 25×10^6 sperm per ml of uterine fluid. After various periods of incubation in utero up to 12 hr (Table 1), the sperm and uterine fluid were aspirated from the uteri and held at body temperature until they were used for oviducal inseminations to test the degree of capacitation.

The sperm used in these experiments were collected with an artificial vagina (Gregoire et al. 1958) and centrifuged at room temperature at about 1,000 x g for 10 min. to remove most seminal plasma. They were then re-suspended to the original ejaculum volume by gentle agitation in physiological saline. Sperm concentration was

*Supramid, (Mittel), Jensen-Salsbery Laboratories, Inc., Kansas City 41, Missouri.

then determined hemacytometrically. In each experiment, ejaculations were pooled from at least three rabbits. Ejaculations which were obviously contaminated with urine and those which contained an abnormally large proportion of dead sperm were discarded. Care was taken to prevent cold shock to the sperm.

The rabbits used to test for the degree of sperm capacitation were injected intravenously with 2.0 to 2.5 mg. of Armour PLH (Equine Luteinizing Hormone, LH). Ovulation occurred about 10.5 hr later (Harper, 1961). At intervals of 6 to 12 hr after LH injection, the rabbits were inseminated by depositing about 0.05 ml of semen to a depth of about 2 cm from the upper end of the oviduct with a glass pipette. These procedures were performed through a 3 to 4 inch midventral incision while the rabbits were under general anesthesia. General anesthesia (ether) was preceded by a tranquilizing intravenous injection of about 1.5 ml of 3% Surital.*

The rabbits were sacrificed 24 to 36 hr after insemination, the oviducts were removed, and ova were flushed from the oviduct through the severed tubo-uterine junction with 0.85% saline at 37°C. Ova were collected on depression slides and examined by bright field microscopy for evidence of fertilization. In addition, some of the ova were examined by dark field and phase contrast

*Surital Sodium, Parke Davis and Company, Detroit, Michigan.

microscopy. Initially, some ova were fixed, stained and sectioned to determine the distribution of nuclear material in the dividing blastomeres. As a final precaution in the first two series of experiments, some rabbits were examined at 14 and 28 days after insemination to determine the extent of implantation and degree of embryonic death. These procedures were adopted to distinguish between fragmented ova or parthenogenically cleaved ova and normally fertilized ova. When sufficient experience accumulated to justify confidence in this judgment, ova were considered to be fertilized when they possessed blastomeres with equal size and nuclear material of normal appearance, and a number of blastomeres reasonable for the time lapsed since ovulation.

In vitro Capacitation of Sperm

Uterine fluid was aspirated from uteri which had been ligated not longer than 2 weeks. Freshly ejaculated, pooled (at least three ejaculates) and washed sperm were suspended in uterine fluid to a concentration of about 25×10^6 per ml. Incubation was performed for 6 to 12 hr in 10 ml Carrel flasks at 38-39°C. Horizontal circular agitation at 30-40 rpm was maintained throughout the incubation period. During the longer incubation periods, bacterial growth was inhibited by addition of antibiotics to the semen at the rate of 250 units of penicillin and 500 micrograms of streptomycin per milliliter. Similar

incubations in vitro of sperm in phosphate buffered Lockes' solution at pH 7.4 served as controls in these experiments. Amylolytic, proteolytic, and/or lipolytic enzymes were added to the phosphate-buffered Lockes' solution to test their effect upon sperm capacitation in vitro.

Similarly to the experiments in vivo, a pool of three or more ejaculates was always used in each experiment in vitro. After centrifugation to remove seminal plasma, the sperm cells were resuspended in the media which was to be used during subsequent incubation. Oviducal insemination and recovery of ova to test the degree of capacitation were performed as described above for the in vivo experiments.

Addition of Amylase to Bull Semen

Initially, the motility of bull sperm was determined with 0.0, 0.1, or 1.0 mg α - or β -amylase per 100 ml of semen extender. In the first experiment, these levels of β -amylase were added to the non-glycerol portion of the routine semen extender and semen samples from 20 ejaculations from bulls at Michigan Animal Breeder Cooperative (MABC) were extended with these extenders. The semen was then glycerolated and a portion of each sample was frozen by routine procedures at MABC. Motilities of each sample of unfrozen semen were estimated in duplicate at day 0 and day 7 after ejaculation during storage at 5°C. The frozen semen was stored for 4 days at -195°C and then thawed in 5°C water. Motility of sperm was determined in duplicate

from each sample by each of two observers at 0 and 72 hr after thawing during storage at 5°C. The second experiment was similar except that α -amylase was used and the semen was not frozen.

Subsequently, a preliminary fertility trial was conducted with bull semen to determine if addition of 1 mg of α - or β -amylase per 100 ml of extender would severely reduce fertility. One ejaculum from each of four of the more popular Holstein bulls in use at MABC was selected, and α -amylase was added (1.0 mg per 100 ml) to a portion and β -amylase was added (1.0 mg per 100 ml) to the remainder of each ejaculation. The experimental semen was routinely cooled, glycerolated, frozen, and shipped to four breeding technicians. These technicians were chosen to facilitate supervision of semen distribution and compilation of fertility records. Each of the four technicians received experimental semen from each bull, two received semen treated with α -amylase and two with β -amylase. A total of about 100 first services were obtained with semen from each treatment. The fertility results consisted of 60- to 90- day non-return percentages, which criterion is routinely used to assess fertility at MABC.

Once it was determined that fertility was not greatly impaired by the amylase treatments imposed in the preliminary experiment, a larger fertility trial was designed. Either 0.0, 0.1, or 1.0 mg of α - or β -amylase per

100 ml semen extender was added to 99 ejaculations from 16 bulls at MABC. The order of application of the amylase treatments was randomized. One treatment was imposed on all of the semen samples collected on any given day of collection and each treatment was imposed on two collection days. Treatment effects on fertility were measured on the basis of 30- to 60- day and 60- to 90- day non-return percentages and analyzed by analyses of variance.

Analyses of Uterine Fluid

Amylase Assays

Uterine fluid was assayed for amylolytic activity by the method of Bernfeld (1955) as described in Appendix Procedure A. To determine the amount of amylolytic activity in uterine fluid relative to blood sera, uteri of five estrous doe rabbits were ligated. Two weeks later, the accumulated uterine fluid was aspirated into a syringe through a mid-ventral incision, and blood samples were obtained by cardiac puncture from each of these five rabbits. The blood samples were allowed to clot at 5°C, and the resulting sera were removed. In an effort to obtain an indication of the type of amylase in uterine fluid, and blood sera, the amylolytic activity of these fluids was assayed in buffers of optimal pH and ionic strength for either α -amylase (pH 6.9, 0.02 M phosphate with 0.0067 M sodium chloride) or β -amylase (pH 4.8, 0.0016 M acetate) as outlined by Bernfeld (1955). The

fractions collected following separation of uterine fluid proteins on a column of Sephadex G-200 as described below were assayed for total amylolytic activity by the method of Bernfeld (1955) as modified by Ujihira et al. 1965.

Column Chromatographic Separation of Uterine Fluid

In an attempt to separate and partially characterize the proteins specific to uterine fluid, uterine fluid was concentrated in an Ultra-filter* or lyophilized and resolubilized in buffer to contain 50 mg protein per ml. These solutions were chromatographed on a Sephadex G-200 column (see Appendix Procedure B for the method of column preparation and elution). Fractions of about 10 ml were collected from the column and were monitored for protein content by measuring absorbance of light at 280 m μ in a Beckman DB spectrophotometer. The fraction containing protein were then lyophilized to reconcentrate the protein. The degree of separation of the protein components in the uterine fluid was determined by the agar-gel double diffusion method of Ouchterlony (1958), by agar-gel electrophoresis (Weime, 1965) and by immunoelectrophoresis (Crowle, 1961).

Salt Fractionation by Ammonium Sulfate

Uterine fluid proteins which had been concentrated, lyophilized, and resolubilized as for chromatography were

*LKB vacuum dialysis apparatus, A. H. Thomas, Co.

precipitated by ammonium sulfate fractionation at 33, 50, 67, and 100% saturation at 5°C. For this purpose, the uterine fluid solutions were placed in dialysis tubing and dialyzed against the appropriate salt concentration. Each precipitate was resolubilized and reprecipitated once. The precipitates were then solubilized and stored at -20°C until analyzed. The degree of separation of the uterine fluid proteins was determined by the same procedures used after chromatographic separations.

SECTION I

RESULTS

Capacitation in vivo

Initially, sperm were incubated in uterine fluid which had accumulated in utero following ligation of the cervical end of the uterus. The objective of this experiment was to determine if ligation had altered the ability of the uterus to capacitate sperm.

The results presented in Table 1 were obtained with washed rabbit sperm which were inseminated without incubation or after incubation in ligated uteri for the periods indicated. Non-incubated sperm inseminated at the time of injection of luteinizing hormone (positive control treatment) were highly fertile as expected; 22 of the 23 ova recovered were cleaved. Washed, freshly ejaculated sperm inseminated into oviducts at the time of, or up to 2 hrs after ovulation (negative control treatment) were not fertile; none of 78 ova were cleaved. However, when does were inseminated at the time of ovulation with sperm that had been incubated in ligated uteri for 6 hr, 30% of the ova recovered were fertilized. Comparison of the fertility obtained with sperm from the various treatments in Table 1 indicated the longer periods of in utero incubation resulted in more complete capacitation. To be

Table 1.--Capacitation of sperm in utero.

Incubation Time	Time of Insemination**	Rabbits	Ova Recovered	
			Total	Fertilized
			(No.)	(%)
0*	0.0	6	23	22 96
0*	10.0	12	49	0 0
0*	12.0	6	29	0 0
6	10.5	17	80	24 30
6	12.5	9	41	3 7
10	12.0	8	28	11 39
12	10.5	10	7 preg., 21 embryos	

*Washed sperm, non-incubated.

**Oviducal inseminations; hours after injection of luteinizing hormone (LH).

certain that the recovered ova were normally fertilized, ten oviducally inseminated rabbits were examined surgically at 14 and 27 days after insemination. Seven were normally pregnant and possessed a total of 21 embryos (Table 1).

Stevens et al. (1964b) demonstrated that the protein components of uterine fluid vary qualitatively and quantitatively with the interval of time after ligation. Therefore, sperm were incubated in uteri which had been ligated for various periods of time to determine if duration of ligation affected the ability of the accumulated fluid to capacitate sperm. Although the limited results listed in Table 2 are not entirely definitive, the data reveal a marked decrease in the ability of ligated

Table 2.--Capacitation of sperm in uteri ligated for various periods of time.

Ligation Period	Rabbits Inseminated	Ova Recovered		
		Total	Fertilized	
	(No.)	(No.)	(%)	
8 weeks*	8	30	0	
3 weeks*	6	35	5	
2 weeks*	11	45	19	
10 days**	8	28	11	

*Incubation time 6 hr and insemination time 10.5 hrs after LH.

**Incubation time 10 hr, insemination time 12 hrs after LH.

uteri to capacitate sperm when the uteri were ligated for more than 2 weeks.

Some of the published evidence indicated that the postcoital release of endogenous luteinizing hormone LH (Chang, 1958), or intravenous injection of an ovulating quantity of human chorionic gonadotropin (HCG) quantitatively increased the ability of the uterus to capacitate sperm. However, the author was not aware of any published attempt to simulate the effect of coitus with injections of LH, which is probably the ovulating hormone. To test whether LH influenced capacitation, some incubator doe rabbits were injected intravenously with luteinizing hormone prior to injecting sperm into ligated uteri. These experiments, summarized in Table 3, although quite variable, indicated

that injection of LH increased the ability of ligated uteri to capacitate sperm similarly to coitus (Chang, 1958) or injected HCG (Soupart, 1966). But even with this improvement, fertility did not approach the 96% obtained with the positive control (Table 1.)

Table 3.--Capacitation of sperm in utero, with or without prior injection of LH into the incubator doe.

Experiment No.	Injected		Non-injected	
	Ova Recovered	Ova Fertilized	Ova Recovered	Ova Fertilized
	(No.)	(%)	(No.)	(%)
1	12	42	23	0
2	20	55	25	32
3	13	31	15	47
Total	45	44	63	24

Capacitation in Uterine Fluid

Initially sperm were incubated in uterine fluid which had been aspirated from ligated uteri to determine whether it could capacitate sperm in vitro as it apparently did in vivo. Sperm were incubated in the aspirated uterine fluid at 38-39°C for 6 to 12 hr. Sperm incubated in phosphate-buffered (0.016 M; pH 7.40) Locke's solution served as a negative control and resulted in very low fertility; 5 of 116 ova were fertilized (Table 4). However, when sperm were incubated for 6 hr in uterine

Table 4.--Capacitation of sperm in Locke's solution (phosphate-buffered) and in uterine fluid in vitro.

<u>Sperm Incubation</u>			<u>Ova Recovered</u>			
Environment	Hr.	Time of Insem.*	Rabbits	Total	Fertilized	
				(No.)	(%)	
Locke's	6	10.5	7	22	3	14
Locke's	6	12.0	5	33	2	6
Locke's	8	10.5	7	32	0	0
Locke's	8	12.0	5	29	0	0
Uterine Fluid	6	10.5	39	95	40	42
Uterine Fluid	6	12.0	6	47	1	2
Uterine Fluid	8	10.5	6	29	15	52
Uterine Fluid	8	12.0	6	32	2	6
Uterine Fluid	12	12.0	7	22	11	50
Uterine Fluid (Dialy.)**	6	10.5	11	51	0	0
Uterine Fluid (macro.)**	6	10.5	11	48	8	17
Uterine Fluid (decap.)+	7	10.5	4	17	0	0
Uterine Fluid	6	10.5	6	3 preg., 3 embr.		

*Oviducal insemination; hours after injection of luteinizing hormone (LH).

**Dialysate (dialy.) and macromolecular (macro.) portions of uterine fluid.

+Sperm capacitated in uterine fluid and re-suspended in seminal plasma for 30 minutes.

fluid and oviducally inseminated at the time of ovulation, 40 of 95 ova recovered were fertilized. These 95 ova were from 4 separate experiments which are detailed in the first four lines of Table 5 and discussed below. They are combined in Table 4 for ease of comparison of the incubation intervals and insemination times. In contrast to the level of fertility for these 95 ova, however, when sperm incubated in uterine fluid for 6 hr were inseminated about 1.5 hr after ovulation, only 1 of 47 ova was fertilized. In vitro incubations for 8 hr rather than 6 hr resulted in slightly higher fertilities.

Noyes et al. (1958) were unable to capacitate sperm inside a dialysis bag in utero, indicating that either macromolecules in uterine fluid or direct contact with the uterine epithelium is necessary for capacitation. Consequently, uterine fluid was concentrated by ultrafiltration, and the macromolecular portion and the dialysate were both tested to determine if either or both could capacitate sperm similarly to fresh uterine fluid. Eight of 48 ova were fertilized by sperm which had been incubated in the macromolecular portion (Table 4) while none of 51 were fertilized by sperm which had been incubated in the dialysate.

Chang (1957) reported that sperm which had been capacitated in utero could be decapacitated by addition of 5% seminal plasma to the sperm before oviducal insemination. If sperm capacitation in uterine fluid in vitro is similar

Table 5.--Capacitation of sperm in uterine fluid in vitro.

<u>Sperm Incubation</u>		<u>Ova Recovered</u>		
Environment	Hr.	Time of Insemination	Rabbits	Total Fertilized Fertilized
Frozen-thawed*	6	10.5	4	10
pH adjusted**	6	10.5	11	39
S.P. not removed+	6	10.5	5	21
Fresh U.F.	6	10.5	19	25
Anerobic++	12	11.5	3	20
U.F. heated+++	12	11.5	5	16
Fresh U.F.	12	11.5	7	22
				(No.)
				(%)
				30
				23
				39
				80
				0
				6
				50

*U.F. stored at -20°C for 60 days.

**pH of fresh UF adjusted to 7.6 with CO₂.

+Whole semen incubated in UF.

++Sperm incubated in uterine fluid under mineral oil.

+++Fresh uterine fluid heat at 100°C for 10 minutes.

to that change demonstrated in vivo, a similar decapacitation reaction could be expected to take place when these sperm are exposed to seminal plasma. The results presented in Table 4 indicate that sperm capacitated in uterine fluid in vitro do indeed lose their fertilizing ability when incubated in seminal plasma before oviducal insemination; none of 17 ova were fertilized.

Brackett and Williams (1966) found that relatively anerobic conditions (i.e., high CO₂ tension and/or low O₂ tension) are necessary to obtain fertilization of rabbit eggs in vitro. Stevens et al. (1964) indicated that the in vivo pH of rabbit uterine fluid of 7.6 is probably maintained chiefly by its high bicarbonate content. These observations and the observation that capacitation of sperm in uterine fluid in vitro was less complete than that in utero suggested that relatively anerobic in vitro incubation may more closely approximate in vivo conditions. Therefore, fresh uterine fluid was placed under mineral oil for subsequent incubation of sperm. However, the sperm incubated in uterine fluid under these conditions were infertile (Table 5), none of 20 ova recovered were fertilized.

To determine if the factor in uterine fluid normally responsible for capacitation was heat labile, uterine fluids which had accumulated in three rabbits for 1 wk were pooled and divided into two parts. One part was placed in a

boiling water bath for 10 min. before cooling to body temperature. Both boiled and unheated uterine fluid was then used to incubate sperm. The sperm incubated in the heated uterine fluid were relatively infertile (Table 5); only 1 of 16 recovered ova was fertilized. In contrast, the sperm incubated in the unheated uterine fluid were relatively fertile, 11 of 22 ova recovered were fertilized.

Capacitation with Enzymes in vitro

Williams et al. (1965) reported that the enzyme β -amylase destroyed the decapacitation factor in seminal plasma. Therefore, an experiment was designed to determine if this enzyme could mimic the in vitro capacitation described above for uterine fluid. An initial experiment determined that phosphate buffered (0.016M, pH 7.4) Lockes' solution maintained rabbit sperm motility at near ejaculatory levels during 12 hr of in vitro incubation at 36-37°C.

The data presented in Table 6 illustrated the fertility of sperm after incubation for various periods of time in phosphate buffered Lockes' solution with three levels of a crude β -amylase preparation.* Assay of the amylolytic

*Crystalline α -amylase: aqueous suspension, from Hog Pancreas lot 106B-1600-1; Crystalline β -amylase: Ammonium sulfate suspension, from sweet potatoe lot 16B-1901, Sigma Chemical Co; Crude β -amylase lot 6060, crude α -amylase lot 5594 Nutritional Biochemicals Corp.

Table 6.--Capacitation of sperm with crude β -amylase in vitro.

<u>Sperm Incubation</u>		<u>Ova Recovered</u>		
Level of Amylase	Hr.	Time of Insemination*	Rabbits	Total Fertilized Fertilized
10 mg %	8	10.5	3	(No.) (%)
1 mg %	8	10.5	4	8 13
0.1 mg %	8	10.5	4	11 27
0.1 mg %	8	12.0	4	13 39
0.1 mg %	10	10.5	4	18 22
0.1 mg %	10	12.0	4	18 44
0.1 mg %	12	10.5	6	17 29
0.1 mg %	12	12.0	4	21 48
				18 28

*Oviducal inseminations; hours after injection of LH.

activity in uterine fluid and in the enzyme preparations used for in vitro sperm incubation indicated that 1.0 mg of β -amylase/100 ml contained a quantity of amylolytic activity (20 mg maltose equivalents released /ml/min at 37°C) similar to that in 100 ml of uterine fluid. However, of the three levels of enzyme used for in vitro sperm incubation the highest fertility was obtained with 0.1 mg of enzyme per 100 ml buffer. Consequently, this level of enzyme was added to buffers for other incubation and insemination intervals (Table 6). Increasing the period of incubation in vitro increased the fertility of the incubated sperm and this trend was most apparent when oviducal inseminations were performed at the time of ovulation. These data indicate that the sperm were partially capacitated with β -amylase in vitro and that capacitation was completed in the oviducts of the oviducally inseminated does.

The data presented in Table 7 were obtained with sperm incubated in α -amylase* or β -amylase* of two different purities. The total amylolytic activity of the incubation medium for each experiment was adjusted to the value obtained by assay of 0.1 mg/100 ml crude β -amylase. Although the fertility was lower than that obtained in earlier experiments, the fertility of sperm incubated with

*Ibid.

Table 7.---Capacitation of sperm in α - and β -amylase preparations.

<u>Sperm Incubation</u>		<u>Ova Recovered</u>		
Enzyme	Hr.	Time of Insemination*	Rabbits	Total Fertilized Fertilized
Crude β -amylase	11.0	11.5	6	23 2 9
Purified β -amylase	11.0	11.5	6	25 0 0
Crude α -amylase	11.0	11.5	5	14 2 14
0.1 mg %	11.0	11.5	5	22 0 0
1.0 mg %	11.0	11.5	6	28 3 11
Control**				

*Oviducal inseminations; hr after injection of LH.

**Phosphate buffered Locke's solution.

amylase was higher than that of control sperm incubated in buffer alone. However, the more highly purified β -amylase did not produce as high fertility as the crude preparation (Table 7) which had been used previously. Also, the capacitating activity of crude α - or β -amylase differed little.

The data illustrated in Table 8 were obtained with sperm incubated in different combinations of protease,* lipase,* and/or amylase. Although there was apparent trend toward higher fertility with sperm incubated with amylase and protease, definitive conclusions are not possible from these data. Complete capacitation of sperm should result in 80 to 90% fertilized ova, a result which was achieved with none of the treatments.

*Crude lipase from wheat germ (lot 5969), and Crude protease from pancreas (lot 4780), Nutritional Biochemicals Corp.

Table 8.--Capacitation of sperm in different combinations of protease, lipase or β -amylase.

<u>Sperm Incubation</u>		<u>Ova Recovered</u>			
Enzyme*	Hr. Insemination***	Rabbits	Total	Fertilized	Fertilized (%)
β -amylase	12	6	25	7	28
protease	12	6	23	4	17
lipase	12	6	25	5	20
amylase +					
protease	12	6	30	11	37
amylase +					
lipase	12	6	33	2	6
protease +					
lipase	12	6	31	1	3
protease +					
lipase +					
amylase	12	6	35	11	31
Control**	12	6	25	1	4

*The concentration of each enzyme in the incubation media was 1.0 mg per 100 ml.

**Phosphate buffered Lockes' solution.

***Oviducal inseminations, hours after injection of LH.

DISCUSSION

Capacitation in vivo

The results presented in Table 1 indicated incubation in utero for 6 hr resulted in only partial capacitation. When these sperm were inseminated at 10.5 hr after LH injection, 30% of the ova were fertilized whereas only 7% were fertilized when these sperm were inseminated at 12.0 hr after injection of LH. These data suggest that in the first case capacitation was completed in the oviduct during the brief fertilizable life of the ova whereas in the second case the partially capacitated sperm did not have sufficient time in the oviducts to achieve the same degree of fertility before the ova became infertile. Longer periods of incubation in utero (10 hrs) resulted in a higher degree of capacitation, as indicated by a larger proportion of fertilized ova (39%).

These data are comparable with Chang's (1955) data on sperm recovered from unligated uteri of doe rabbits 12 hrs after mating; 54% of the ova he recovered following insemination 12 hrs after an intravenous injection of pituitary extract were fertilized. The rabbits which were examined for pregnancy at 14 and 27 days (Table 1) indicated that the ova which were considered by microscopic examination to be fertilized were capable of developing into viable

embryos. The data of Table 2 indicated that the sperm capacitating ability of uterine fluid decreased with increased duration of uterine ligation. These apparent physiological changes in uterine fluid may reflect a change in the macromolecular constituents of uterine fluid during prolonged periods of ligation, as suggested by Stevens et al., (1964b). Recently Soupart (1966) indicated that administration of HCG intravenously to the incubator rabbit had a dramatic stimulating effect on the ability of the uterus to capacitate sperm. The evidence presented in Table 3 also indicated that prior administration of an ovulating injection of LH increased the ability of the uterus to capacitate sperm.

Because we are unaware of any known direct effect of LH on the uterus, these data suggest that some response of the ovary to the injected LH may influence the uterus directly and rather rapidly. Such a change could be that described by Hilliard et al., (1964). She demonstrated that 20 α -hydroxy-pregn-4-en-3-one was secreted by the rabbit ovary beginning almost immediately after coitus and ending by the time of ovulation. If such an ovarian secretion is directly related to sperm capacitation, it is reasonable to assume that the immediate effects of such a substance would be less dramatic in ligated uteri because of dilution of the resultant uterine secretion in the previously accumulated fluid. Longer durations of

uterine ligation may result in greater dilution because of greater volumes of accumulated fluid.

As another possible explanation of the rather low fertility after in utero capacitation, the effects of uterine ligation and of the resultant accumulation of fluid on the endocrine balance of the individual are not known. The presence of a relatively small object in the uterus of an animal can have a very dramatic effect on the endocrine balance of that animal, at least in some species. For example, Hansel (1960) demonstrated that a small (30 ml) balloon in the uterus caused aberrant estrous cycles in dairy heifers. And, of course, the developing zygote in the uterine lumen causes maintenance of the corpus luteum of pregnancy. Therefore, ligation of uteri and the subsequent accumulation of fluid could alter the hormonal balance of the animal. However, the data of Table 2 indicated that the rabbit uterus retains the ability to capacitate sperm for at least 2 wks after ligation of the uterus.

Capacitation in Uterine Fluid

The level of fertility achieved by incubation of sperm in uterine fluid in vitro (Table 4) agree, in general, with that Chang (1955) reported from incubation in utero. Sperm which had been incubated in buffered Lockes' solution (negative controls) and subsequently used to inseminate rabbits at about the time of or shortly after ovulation were

relatively infertile. However, when sperm were incubated in uterine fluid and subsequently used to inseminate rabbits at about the time of ovulation, the fertility was markedly increased over corresponding control groups.

The data in Table 4 reveal, in general, that the longer sperm were incubated in uterine fluid, the greater was the degree of capacitation and that fertility was also enhanced with inseminations performed well before the end of the fertile life of ova. Thus, similar to the results obtained with sperm incubated in utero for 6 hrs (Table 1), these data indicate that the sperm were only partially capacitated at the time of insemination. When insemination was performed at the time of ovulation, the capacitation process was apparently completed in the oviducts during the brief fertilizable life of the ova, but when insemination was performed about 1.5 hrs after ovulation, there was insufficient time to complete capacitation before the ova became infertile. However, this trend was at least partially offset by longer intervals of incubation in vitro (12 hr) which resulted in a much higher level of fertility, even with sperm inseminated 1.5 hrs after ovulation (Table 4). The data suggest that periods of incubation in vitro longer than those tested here may result in higher fertilities perhaps approaching those for the positive controls (Table 1). The results of this experiment agree with published evidence (Hammond, 1934; Chang, 1955) that

development of maximal fertilizing capacity of rabbit sperm in utero may require as much as 14 hrs.

Noyes et al. (1958) demonstrated that sperm were not capacitated in vivo when suspended in the uterus within dialysis tubing, thus implicating a macromolecule of uterine origin in sperm capacitation. The data obtained in the present experiment (Table 4) provide more direct evidence than heretofore available and demonstrate that it is specifically the macromolecular portion of uterine fluid which is associated with capacitation. Similarly to in utero incubation, however, the macromolecular portion of uterine fluid apparently resulted in only partial capacitation. The data in Table 4 also indicated that the partial capacitation in vitro obtained in the present experiment was similar to capacitation in vivo, because subsequent incubation of these sperm in seminal plasma reversed the capacitation process in both cases.

The data in Table 5 reveal that the capacitating ability of uterine fluid is relatively unstable in vitro. Prefreezing the uterine fluid or adjustment of the pH of uterine fluid to the in utero value with CO_2 apparently reduced the capacitating ability of the uterine fluid. The results obtained with sperm incubated in uterine fluid under mineral oil suggested that relatively high O_2 tension is essential for sperm capacitation. The lack of capacitation of sperm by uterine fluid which had been

heated indicated that the capacitation factor in uterine fluid is heat labile.

Capacitation with Enzymes in vitro

Williams et al. (1965) demonstrated that the enzyme β -amylase destroyed the decapacitation factor found in seminal plasma. If capacitation consists of removal or destruction of the seminal plasma decapacitation factor found on ejaculated sperm, capacitation in vitro by incubation of sperm with the enzyme β -amylase should be possible. The data in Table 6 provide convincing evidence for this hypothesis.

When sperm were incubated in vitro for various intervals in various concentrations of amylase, their fertility was similar to that after comparable intervals of in utero incubation. Similar to the data for sperm incubated in uterine fluid in vitro, sperm incubated with β -amylase in vitro were only partially capacitated. Therefore, at each of the incubation times listed in Table 6, insemination at the time of ovulation resulted in a larger percentage of fertilized ova than did insemination about 1.5 hrs after ovulation. When compared to control inseminations with sperm incubated in buffer alone (Table 4), β -amylase significantly increased fertility, even at the shortest incubation time.

Initially, a relatively crude amylase preparation was used (Table 6) for in vitro incubation. However, the

partial capacitation obtained with this enzyme could have been due to the amylase activity per-se, or to some contaminant (enzyme) present in the crude enzyme preparation. Therefore, a commercial highly purified amylase preparation was compared with the crude preparation. Unfortunately, the data (Table 7) do not provide an adequate test because of the overall low fertility in that experiment. Nevertheless, the data suggest that purification may have removed some capacitation ability from the crude β -amylase.

Although the enzyme α -amylase is apparently widely distributed in animals, (Fischer and Stein, 1960) β -amylase has been reported only in plants (French, 1960). It seems likely, therefore, that α -amylase should capacitate sperm in vitro if amylolytic activity of uterine or oviduct secretions is normally responsible for capacitation. Unfortunately, because of low fertility, this experiment like that described above, was inadequate. Nevertheless, the data obtained with α -amylase and β -amylase (Table 7) are very similar; only partial capacitation was achieved with either of these amylases in vitro. These data, and those presented in Tables 6 and 7 indicate that another factor or factors which may be present in the female reproductive tract are necessary, in addition to amylase, to achieve complete capacitation in vitro.

Williams et al. (1966) indicated that pronase, a general proteolytic enzyme, altered the molecular size of

the decapacitation factor. If capacitation involves a non-specific enzymatic alteration of the surface of sperm cells, then protease, lipase, amylase, or a combination of these enzymes might be expected to effect capacitation in vitro. The data presented in Table 8 indicate that none of the enzyme combinations used in this experiment achieved complete capacitation in vitro. These data suggest that other factors, perhaps even other enzymes, which are present in the female genital tract may be necessary for complete capacitation of sperm.

SECTION II

RESULTS

Assays for Amylolytic Activity

Williams et al. (1965) indicated that β -amylase destroyed decapacitation factor, and Dukelow et al. (1966b) reported that rabbit uterine fluid contained about four times as much amylolytic activity as homologous blood sera. Therefore, it seemed likely that the high amylolytic activity found in secretions of the female tract may be important to the degree of sperm capacitation. However, the data presented in Table 9 indicated that the total amylolytic activity of rabbit uterine fluid 2 weeks after ligation, assayed by the method of Bernfeld (1955), was less than that of homologous blood sera. In fact, a pooled sample of rabbit seminal plasma contained more amylolytic activity than the average of the five uterine fluid samples assayed (0.54 and 0.29 mg maltose equivalent, respectively). The amylase in the incubation media for the in vitro capacitation experiments described above was used at levels (1.0 mg per 100 ml) similar to those of uterine fluid (0.20 maltose equivalents).

Uterine fluid and blood serum were assayed at the optimal pH and molarity of buffer for each type of amylase in an effort to determine the type of amylolytic activity

Table 9.--Assay of rabbit uterine fluids and rabbit blood sera for amylolytic activity.*

Rabbit	Uterine Fluids	Blood Sera
1	0.30	0.38
2	0.28	0.38
3	0.30	0.18
4	0.28	0.74
5	0.28	0.32
Average	0.29	0.40

*Mg. maltose equivalent liberated from starch per ml per min at 37°C.

present in each fluid. The results of this experiment, illustrated in Table 10 indicated that both rabbit uterine fluid and rabbit blood sera display more amylolytic activity in the buffer of optimal pH and molarity for α -amylase activity. The values for uterine fluid and blood serum in Table 9 and 10 were obtained from the same five rabbits. However, the uterine fluids used to obtain the values presented in Table 10 were from a subsequent collection 2 wks after the fluids were collected for the data in Table 9. Therefore, it appears that the amylolytic activity of uterine fluid decreased with increasing period of time after uterine ligation.

Rabbit uterine fluid was concentrated by ultra-filtration and separated into three fractions on a column of Sephadex G-200. The protein in each of the three fractions was assayed for amylolytic activity. Although

Table 10.--Assay of rabbit uterine fluids and rabbit blood sera at pH 6.9 and 4.8 for amylolytic activity* of α - and β -amylase varieties, respectively.

Rabbit	Uterine Fluids		Blood Sera	
	pH 6.9	pH 4.8	pH 6.9	pH 4.8
1	0.00	0.03	0.23	0.18
2	0.17	0.04	0.14	0.00
3	0.02	0.04	0.34	0.40
4	0.12	0.04	0.35	0.03
5	0.05	0.00	0.30	0.15
Average	0.07	0.03	0.27	0.15

*Mg. maltose equivalent released from starch per ml per min at 37°C. α -amylase optimal activity at pH 6.9 and β -amylase at 4.8.

the concentrated uterine fluid contained amylolytic activity (0.10 mg maltose equivalent per ml per min), none of the fractions from the column contained any detectable amylolytic activity when assayed by the method of Bernfeld (1955) as modified to detect smaller quantities by Ujihira et al. (1965).

Physiochemical Analyses of Uterine Fluid

Published data for the rat (Ringler, 1961) and rabbit (Stevens et al., 1964b) indicated that most of the antigenic and electrophoretic components found in the uterine fluids of these species are derived from blood serum. However, both reports indicated that some of these components in uterine fluid are specific secretions of the uterus without counterparts in blood serum. Noyes et al. (1958) implied that a macromolecule of uterine origin was necessary for

sperm capacitation. Since Chang (1955) was unable to capacitate rabbit sperm in homologous blood sera, one could speculate that one of the uterine fluid specific components was normally responsible for capacitation in utero. Therefore, the available evidence indicates that a large molecule, probably a protein found specifically in the uterus or oviduct, is necessary for capacitation.

Since many of the uterine fluid proteins are found in proportions similar to those found in blood serum and because procedures were available for separation of blood serum proteins on Sephadex (Flodin, 1963), this resin was used for column chromatography of uterine fluid proteins. The results of elutions of 1.5 ml of concentrated rabbit uterine fluid (50 mg protein per ml) and rabbit blood serum (60 mg protein per ml), from a 2 by 155 cm column of Sephadex G-200 are illustrated in Figure 1. Similar elution patterns were obtained with uterine fluid and blood serum. In agreement with previously reported data (Flodin, 1963) three major ultraviolet radiation absorbing peaks were obtained from blood serum.

The eluted fractions with each of the three major peaks obtained from uterine fluid were pooled and lyophilized to reconcentrate the protein. The degree of separation of the antigens and/or electrophoretic components in these fractions was determined by agar-gel double diffusion (Figure 2), electrophoresis in agar (Figure 3) and by immuno-electrophoresis (Figure 4). Preparation of the antisera

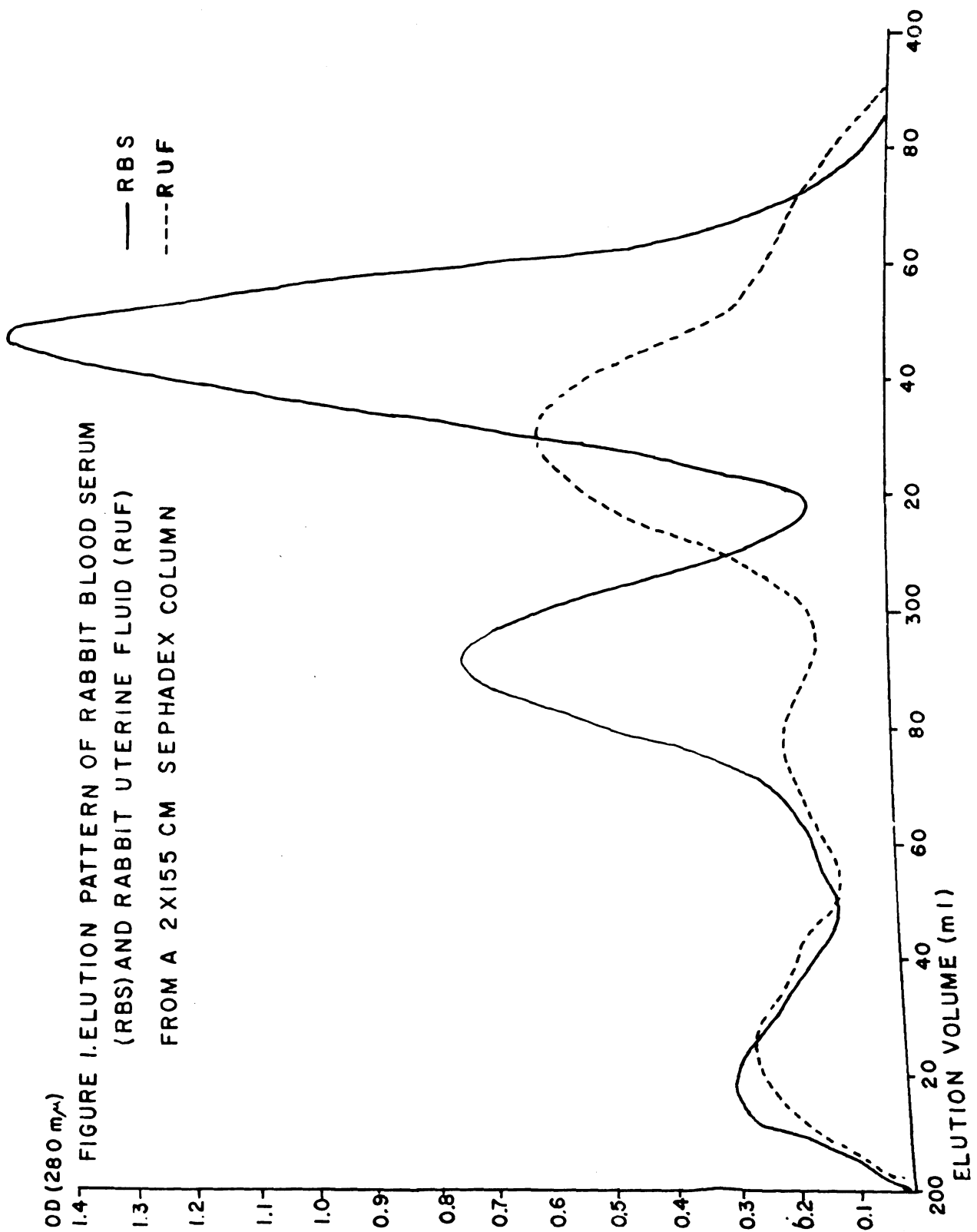


Figure 2

Agar gel double diffusion reaction of absorbed guinea pig antisera to rabbit uterine fluid (AUF) with rabbit uterine fluid (RUF) and the three fractions of uterine fluid eluted from a Sephadex column (F-1, F-2, and F-3).

Center well was filled with 0.25 ml of normal rabbit blood sera (RBS) and 0.25 ml of (AUF) 12 hrs later. Peripheral wells contained 0.1 ml (RBS), (F-3), (F-2), and (F-1) counterclockwise from 12:00 o'clock, as labeled. All fluids contained about 6% protein.

Figure 3

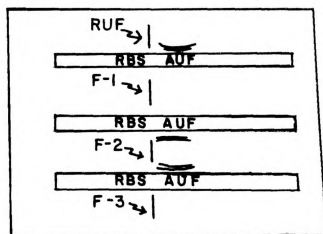
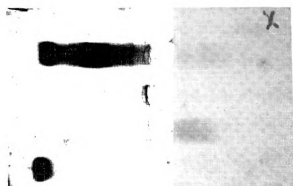
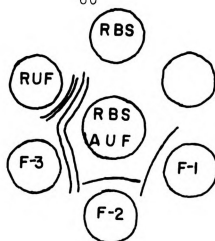
Agar gel electrophoresis of rabbit uterine fluid (RUF), and the three Sephadex fractions of (RUF); F-1, F-2, and F-3 top to bottom.

About 0.5 mg of protein was placed in each slit. Electrophoresis was conducted 40 min at 150 V, anode was at left. Agar fixed and stained with Amidoschwartz protein stain.

Figure 4

Immunoelectrophoresis reaction of (AUF) and Sephadex fractions of RUF against (AUF).

Agar gel electrophoresis was conducted as for Figure 3. Then the three intermediate troughs were cut in the agar and filled with AUF absorbed with RBS. Each slit contained about 0.5 mg of protein, and about 0.25 ml of RBS and AUF were placed in each trough.



and the procedures used in the above analyses were similar to those outlined by Stevens, et al. (1964b).

The pattern of precipitin lines illustrated in Figure 2 was obtained by filling the center well with normal rabbit blood serum, allowing it to diffuse out into the agar for 12 hr at 5°C and then re-filling the center well with antisera. This procedure caused all antibody to blood serum proteins to precipitate in the immediate vicinity of the center well. The peripheral wells were then filled with the antigens indicated. The antigens and antibody were then allowed to diffuse out into the agar at 5°C, and the precipitin lines which formed were recorded photographically. Agar gel diffusion indicated that precipitating antigens found in uterine fluid (and not in blood serum) were separated by the Sephadex column. The first fraction contained 1 antigen, the second fraction 1 antigen and the third fraction 2 antigens which were specific to uterine fluid.

Agar electrophoresis separates proteins on the bases of molecular size and charge density, the smaller more highly charged molecules moving most rapidly. Separations of rabbit uterine fluid electrophoretic components and each of the three fractions obtained by elution of uterine fluid from Sephadex are illustrated in Figure 2. The separations obtained are in close agreement with data presented by Flodin (1963) for blood sera. Very little protein staining material was visible in fraction #1.

Fraction #2 contained mostly globulins, while fraction #3 contained most of the albumin-like proteins present in uterine fluid.

Stevens et al. (1964b) determined that at least two classes of proteins are specific to rabbit uterine fluid. Data obtained in the present experiment (Figure 4) indicate the components which are specific to uterine fluid and possess electrophoretic mobility similar to beta-globulin were contained in the second peak eluted from a Sephadex column. However, the components with mobilities similar to pre-albumins were not detectable in any of the three elution fractions by the techniques used in the present experiment.

Ammonium Sulfate Fractionation of Uterine Fluid Proteins

The uterine fluid proteins which were precipitated by ammonium sulfate fractionation were resolubilized and the degree of separation of the uterine fluid specific antigens was determined by agar-gel diffusion.

Each of the antigens was present in detectable quantities in at least two of the ammonium sulfate fractions. Since uterine fluid proteins were apparently separated more efficiently by Sephadex, and because this method of separation should offer a less severe challenge to the physiological integrity of the protein molecule, further characterization of the ammonium sulfate fractions was not attempted.

DISCUSSION

Assays for Amylolytic Activity

Several unsuccessful attempts to capacitate sperm in vitro in homologous blood sera and other biological fluids have been reported (Chang, 1955; Williams et al. 1964). However, as assayed by our procedure rabbit blood sera contained more amylolytic activity than rabbit uterine fluid and rabbit seminal plasma contained a level of activity comparable to blood serum. Although decapacitation factor is present in seminal plasma (Bedford and Chang, 1962) and is destroyed by β -amylase (Williams et al. 1965), it is apparently unaltered by the amylolytic activity in seminal plasma. Although the amylolytic activity present in both seminal plasma and uterine fluid was measurable by the assay used in the present experiment, it is possible that some qualitative difference exists between the amylase in the two fluids, to which the assay used in this experiment was insensitive. Nevertheless, the evidence justifies the conclusion that amylase, if involved at all, is not solely responsible for sperm capacitation under normal conditions.

Since β -amylase has been found only in plants (French, 1960), the ability of this enzyme to destroy decapacitation factor apparently only mimicked the

action of factors which are normally responsible for sperm capacitation in uterine fluid in vitro or in the female reproductive tract. Furthermore, since both rabbit blood sera and rabbit seminal plasma contain appreciable amylolytic activity, but neither is capable of capacitating sperm, sperm capacitation in the uterus and oviduct may involve factors other than amylase.

The data illustrated in Table 10 indicated that the level of amylolytic activities found in blood serum and seminal plasma are similar. The higher activity at pH 6.9 indicated that α -amylolytic activity is predominant and this is the type which is widely distributed throughout the animal kingdom (Fischer and Stein, 1960).

The lack of amylolytic activity in any of the fractions of uterine fluid eluted from a Sephadex column indicated the enzyme was inactivated during the elution process or was not eluted from the column.

Physiochemical Analyses of Uterine Fluid

The gel filtration separations of rabbit blood serum in the present experiment were quantitatively and qualitatively similar to published separations of blood serum proteins of other species (Flodin, 1963). The separation obtained with concentrated rabbit uterine fluid was qualitatively similar to but quantitatively different from blood serum. In the elution pattern obtained with uterine fluid the first eluted peak was larger than the

second peak, the opposite of the results obtained with rabbit blood sera. The larger proportional area and trailing edge of the third peak from uterine fluid are probably indicative of a larger percentage of small molecular weight protein components in uterine fluid than in blood serum. These data agree with the comparative quantitative distribution of electrophoretic components found in uterine fluid and blood serum by Stevens et al. (1964b). Since macromolecules are apparently involved in the process of capacitation, and uterine fluid proteins were separated by Sephadex, one would predict that the capacitating activity of uterine fluid should be found largely in one of the three fractions eluted from the Sephadex column.

The data obtained by agar gel double diffusion indicate that the antigens which were present in rabbit uterine fluid were separated from each other by Sephadex. The precipitation pattern (Figure 2) indicated that each uterine fluid-specific antigen was present in only one elution fraction, at least in a quantity sufficient to react with absorbed antisera in the system used.

Agar gel electrophoresis of the three fractions eluted from uterine fluid indicated that the electrophoretic components of rabbit uterine fluid were separated by Sephadex in a manner quantitatively very similar to published data with blood serum of other species (Flodin, 1963). The electrophoretic pattern of

the reconcentrated proteins in each of the three fractions indicated that the uterine fluid-specific pre-albumins which Stevens reported (1964b) would probably be in the third elution fraction and the uterine fluid-specific-beta-globulins would probably be in the second elution fraction. Immunoelectrophoresis substantiated the location of the uterine fluid specific beta-globulins. However, the uterine fluid specific pre-albumins were not detectable in either of the fractions as tested by immunoelectrophoresis or by agar-gel electrophoresis. It is likely that the uterine fluids used in this research possessed no prealbumin component. Stevens, et al. (1964b) indicated that this component appeared in uterine fluid only after the fluid had accumulated in ligated uteri for more than 4 weeks. All uterine fluids used in this experiment were accumulated for no more than 2 weeks.

SECTION III

RESULTS

Addition of Amylase to Bull Semen

The available indirect evidence (Trimberger and Davis, 1943; VanDemark, 1952) indicated that capacitation of bovine sperm is prerequisite to fertilization. Therefore, in vitro capacitation of bull sperm prior to insemination should improve fertility. Because of the evidence described above that β -amylase mimicked the action of uterine fluid in capacitation of rabbit sperm and because capacitation is probably qualitatively similar in several species (Dukelow, et al. 1966 c), an initial motility trial was designed to determine the effects of α - or β -amylase on bull sperm motility. The results (Table 11) indicated no detrimental effects of the treatments imposed. Rather, the high level of α -amylase significantly increased motility after 1 day of storage ($P < 0.01$). Addition of 0.1 or 1.0 mg % of β -amylase did not significantly alter motility of sperm during storage at -196°C ($P > 0.25$).

Consequently, preliminary to a larger experiment, a small fertility trial was conducted to be certain that addition of 1.0 mg % of α - or β -amylase would not

Table 11.--Motility of bull sperm with added amylase.

Treatment	Level of Amylase added		
	0.0	0.1 mg/100 ml	1.0 mg/100 ml
α -amylase	57*	57	64
β -amylase	52	60	59

*Average motility on the first day of storage at 5°C. Each figure is the average of 80 separate motility estimations.

reduce fertility drastically. The fertility of semen from each treatment, based on 60-to 90-day non-returns, is listed in Table 12. Neither treatment lowered fertility. If anything, the amylase treatments increased fertility above the level normally expected (about 70%) with routine artificial inseminations by MABC.

Table 12.--Fertility of bull semen with added amylase.

Treatment	First Services	Returns	60- to 90-day non-returns
	(No.)	(No.)	(%)
α -amylase, 1 mg/100 ml	114	32	72*
β -amylase, 1 mg/100 ml	98	24	76*

*Comparable control values were about 70 percent.

Therefore, a larger fertility trial was conducted. Either 0.0, 0.1 or 1.0 mg of α - or β -amylase per 100 ml of semen extender were added to all semen samples collected from 16 bulls on 10 consecutive semen collection days (5 weeks). Fertility of semen, based on 30- to 60-day and 60- to 90-day non-return percentages, are illustrated in Table 13. Analysis of variance of the 60- to 90-day data revealed that fertility with control semen was significantly less than the average fertility of the other 4 treatments ($P \approx 0.07$), when bulls were considered as a fixed source of variation in the analysis. Furthermore, fertility of the semen treated with the high level of α -amylase was significantly greater ($P \approx 0.07$) than the fertility of semen treated with the low level of α -amylase, but no significant difference was detected in the fertility of the semen treated with two levels of β -amylase ($P > 0.10$).

Table 13.--Fertility of bull semen with added amylase.

Treatment	Number of First Services	Percent Non-Return to Service	
		30-to-60-day	60-to 90-day
α -amylase (0.1 mg %)	1,260	74.4	70.0
α -amylase (1.0 mg %)	1,547	76.7	73.7
β -amylase (0.1 mg %)	1,679	77.7	73.0
β -amylase (1.0 mg %)	1,295	74.9	71.9
Saline Control	1,318	72.7	69.4

DISCUSSION

Addition of Amylase to Bull Semen

Relatively few experiments have reported the effect of exogenous enzymes on subsequent sperm motility or fertility. Hafs (1961) reported that added catalase increased the fertility of bull sperm stored at -196°C in skim milk extender. The beneficial effect of this enzyme was explained quite logically on the basis of detoxification of peroxide formed from metabolism of aromatic amino acids by sperm. However, the reason for enhanced motility of bovine sperm after addition of α -amylase in the present experiment is not readily interpreted. It could have been caused by action of the enzyme on components in the medium to liberate a metabolizable substrate, or by direct enzymatic action on the sperm.

It is also hazardous to speculate on the mechanism of action of amylases in increasing bull sperm fertility. Of course, one might be inclined to attribute the increase to capacitation of the sperm due to the added amylase. This may be actually the case. But, more direct evidence than provided by this experiment is required to show that the increased fertility was due to capacitation. At this point, we can only conclude that amylase increases fertility. Conclusions regarding the mechanism of the

increase are unwarranted. If the effect was due to in vitro capacitation then the evidence indicates that α -amylase more effectively capacitated bull sperm than did β -amylase.

Comparison of the motility of sperm in extenders which contained amylase (Table 11) with subsequent fertility of semen after addition of similar levels of enzyme (Table 13) reveal an identical relationship among treatments. In each experiment the response to the high level of α -amylase was greater than the response to the low level. However, the response with β -amylase was reversed. The lower level of β -amylase resulted in higher motility and in higher fertility, although neither motility or fertility was significantly higher with the lower level.

SUMMARY AND CONCLUSIONS

Initially, rabbit sperm were incubated in utero about 2 weeks after ligation of the cervical end of the uterus of estrous rabbits to determine if ligation altered the ability of the uterus to capacitate sperm. Oviducal inseminations with these sperm at the time of ovulation indicated that in utero incubation for 6 hours resulted in only partial capacitation: 30% of the ova were fertilized, whereas only 6% were fertilized when these sperm were inseminated at about 2 hours after ovulation. Longer periods of in utero incubation (10 hours) resulted in a higher degree of capacitation, 39% of the ova were fertilized. Seven of ten rabbits which had been oviducally inseminated with similar sperm, were normally pregnant and possessed a total of 21 embryos at 14 and 27 days after insemination. Fertility obtained with sperm incubated in uteri which had been ligated for different intervals indicated that the sperm capacitating ability of uterine fluid decreased markedly with increased intervals of time after uterine ligation. This decrease probably indicated qualitative change in the macromolecular constituents of uterine fluid during prolonged periods of ligation. Administration of LH prior to incubating sperm in ligated uteri increase the ability of the uterus to capacitate sperm. These data and other

published evidence indicate that ovulating hormone probably triggers an ovarian response which directly affects the ability of the uterus to capacitate sperm.

Sperm were incubated in uterine fluid which had been aspirated from ligated uteri to determine if this fluid could capacitate sperm in vitro. Similar to the data on sperm incubated in utero, sperm incubated in uterine fluid in vitro for 6 hours were apparently only partially capacitated, 40 of 95 ova were fertilized by oviducal inseminations at the time of ovulation. Fertility was increased by longer in vitro incubation, indicating that sperm may normally require at least 12 hr of incubation in utero to attain maximal fertilizing capacity. These data agree with published evidence for in vivo capacitation. Sperm incubated in the macromolecular portion of uterine fluid were capacitated while sperm incubated in the dialyzable portions of uterine fluid were not, thus demonstrating that capacitation is specifically associated with the macromolecular portion of uterine fluid. The decreased capacitation ability of uterine fluid after storage at -20°C or after heating in boiling water indicate that the capacitation factor is relatively unstable and heat labile.

When sperm were incubated in vitro in phosphate-buffered Lockes' solution containing 0.1 or 1.0 mg % of β -amylase, subsequent fertility was similar to that attained after comparable intervals of incubation in

utero. Fertility was increased by increased periods of incubation, and after each incubation time, insemination at the time of ovulation resulted in a larger percentage of fertilized ova than did insemination about 1.5 hr after ovulation. Although the sperm incubated in vitro in uterine fluid or in buffer containing β -amylase were apparently only partially capacitated, fertility was markedly higher than the fertility of sperm incubated in buffer alone (negative control). Incubation of sperm in β -amylase, or in a combination of protease and β -amylase in vitro also partially capacitated sperm.

Uterine fluid collected 2 weeks after ligation contained less amylolytic activity (0.29 mg maltose equivalent liberated per ml per min at 37°C) than blood sera from the same does (0.40 mg maltose equivalent). The amylolytic activity of uterine fluid decreased with increasing intervals of time after uterine ligation. A pooled sample of rabbit seminal plasma contained relatively high levels of amylolytic activity (0.54 mg maltose equivalent). This seminal plasma amylolytic activity apparently does not destroy decapacitation factor, as β -amylase reportedly did in vitro.

Rabbit uterine fluid and rabbit blood serum were separated into three ultraviolet radiation absorbing peaks by elution from a column of Sephadex G-200. The three peaks contained 1, 1 and 2 uterine fluid specific antigens, respectively, as determined by agar-gel

double diffusion against anti-uterine fluid serum. Agar-gel electrophoresis revealed that uterine fluid proteins were separated by Sephadex similarly to previous reports for blood serum proteins of other species. Immunoelectrophoresis revealed that two uterine fluid specific immunoelectrophoretic components were contained in the second eluted fraction. These components possessed electrophoretic mobilities similar to β -globulins. None of the three eluted peaks contained detectable amylolytic activity, but the ability of each of these fractions to capacitate sperm in vitro was not tested.

Addition of 0.1 or 1.0 mg/100 ml of α - or β -amylase to yolk-citrate bull semen extender was not detrimental to sperm motility, and the high level of α -amylase increased motility after one day of storage at 5°C ($P < 0.1$). Consequently, a fertility trial was conducted. The fertility of semen to which 0.1 or 1.0 mg of α - or β -amylase per 100 ml extender had been added was significantly greater than the fertility of control inseminations ($P \approx 0.07$). Furthermore, fertility of the semen treated with the high level of α -amylase was greater than fertility of semen treated with the low level of this enzyme ($P \approx 0.07$). Further research is necessary to determine if the increased fertility was attributable to in vitro capacitation of the sperm or to some other beneficial effect on the sperm.

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APPENDIX

APPENDIX

Procedure A. Assay for Amylolytic Activity (Bernfeld, 1951).

Reagents

Substrate.--For α -amylase, 1% soluble starch in 0.02M sodium phosphate, pH 6.9 containing 0.006M Na Cl. For β -amylase, 1% soluble starch in 0.016M sodium acetate, pH 4.8.

Color Reagent.--Add 1 g 3,5-dinitrosalicylic acid to 20 ml of 2 N sodium hydroxide and 50 ml of water. Stir until dissolved. Add 30 g potassium-sodium tartrate (Rochelle Salt) and dilute the solution to 100 ml. Prepare fresh daily.

Procedure

Add 0.5 ml enzyme solution to 0.5 ml of substrate. Incubate at 20°C for 3 min. Add 1 ml color reagent. Heat in a boiling bath for 5 min and then cool to room temperature. Add 10 ml of water and read OD 540 in a spectrophotometer against a blank containing buffer without enzyme. A calibration curve is made with maltose (0.1 to 1.5 mg/ml) treated as substrate, and a regression equation is calculated from this curve and used to convert optical density to mg of maltose-equivalents. The values determined in this manner are corrected to the net amount of maltose

liberated by an enzyme solution. For this purpose, the optical density is determined for one tube in which enzyme and substrate are incubated for 3 min, and in a similar manner in another tube except that color reagent is added to the substrate immediately after addition of enzyme solution. The difference in maltose equivalents of these two solutions was considered to be due to enzymatic liberation of maltose from the starch substrate.

Procedure B. Sephadex Column Preparation and Elution

Reagents

Buffer.--0.1 M tris (trishydroxymethylaninoethylmenthane) containing 0.1 M sodium chloride. This buffer was used to swell the resin and to elute the sample from the column.

Procedure.--The dry gel (Sephadex G-200), was added, with constant rapid stirring, to a volume of buffer 2 to 3 times the expected final volume of the gel after swelling. After swelling for 24 hr at room temperature, the gel was again stirred, allowed to settle for about 1 hr, and the supernatant fluid decanted off and replaced with fresh buffer. This procedure was repeated at 24 hr intervals for at least 3 days. Prior to filling with gel, the column was filled about half full with buffer. A small ball of glass wool was placed in the bottom of the column and about 1.25 cm of glass bead (200 mesh) were placed on the glass wool. The remainder of the column was then

filled with a slurry of gel. A funnel was attached to the top of the column with rubber tubing and filled with the slurry of gel. A mechanical stirrer was placed in the lower part of the funnel to continuously agitate the gel during packing of the column. When a layer of gel 3-4 cm deep had formed at the bottom of the column over the glass beads, the outlet of the column was opened. When the column had filled with packed gel, the funnel and stirrer were removed from the top of the column. The top of the column was then attached, through a short siphon hose, to a buffer vessel mounted beside the top of the column at a level so the bottom of the buffer vessel was about level with the top of the gel in the column. After allowing buffer to percolate through the column for at least 24 hrs, samples were placed on the gel. For this purpose, the siphon hose was disconnected from the top of the column and the supernatant buffer gently aspirated off of the gel. The sample was then layered on the gel and allowed to enter the bed with the column outlet open. At the moment it disappeared through the surface of the gel, a small amount of buffer was again layered on the gel, and allowed to disappear into the gel. The column outlet was closed, the column was filled with buffer over the gel, and the siphon tube reconnected to the buffer vessel. Five ml fractions were collected on a timed fraction collector and flow rate was about 5 ml per hour.

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