ENDOCRINE CHANGES INFLUENCING SPERM CAPACITATION

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
ROBERT PAUL WETTEMANN

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ABSTRACT

ENDOCRINE CHANGES INFLUENCING
SPERM CAPACITATION

By

Robert Paul Wettemann

Three experiments were designed to study the influence of gonadotropins on sperm capacitation in the rabbit uterus and on simultaneous progestin secretion by the ovary. Mature estrous rabbits of mixed breeding were used in these studies.

In the first experiment rabbits (hereafter called incubator rabbits) were injected with 0, 50, 75, 100, or 300 IU of Human Chorionic Gonadotropin (HCG) or 100, 250, 500, 1000, or 2000 µg of National Institutes of Health-Luteinizing Hormone (LH) and 200 million sperm were surgically inseminated into the uterus. Sperm were recovered 3.5, 7, or 10 hours later and used to inseminate superovulated test rabbits, about 12.5 hours after an ovulating injection, to assay the degree of sperm capacitation.

When incubator rabbits were injected with 75 IU of HCG the fertilizing ability of sperm incubated in the uteri was increased (85 of 99 ova fertilized) relative to the rate of capacitation in estrous control rabbits (84 of 153 ova fertilized). But when incubator rabbits were injected with 300 IU of HCG at the time of insemination,

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capacitation was inhibited in the uterus (40 of 131 ova fertilized).

In contrast to the influence of HCG, sperm capacitation was enhanced by injection of low or high levels of LH, relative to the rate of capacitation in estrous control rabbits. This was true although quantities of LH equivalent in leuteotropic activity to over six times the high level of HCG were injected.

Mating and injection of gonadotropins enhance capacitation in the rabbit and both also cause increased synthesis of 20α-hydroxy-pregn-4-en-3-one (20α-ol) by the ovary. In the second experiment incubator rabbits were injected with 20α-ol to determine whether it may mimic the action of injected gonadotropin on capacitation. With limited observations, the rate of capacitation in the uterus was not significantly affected by the injection of 20α-ol.

In the third experiment rabbits were injected with either 75 or 300 IU of HCG or with 100 or 1000 μg of NIH-LH at various intervals before or after cannulation of ovarian veins. Ten-ml samples of ovarian venous blood were collected hourly for periods up to 7 hours. Progesterone and 20α-ol were quantified in these samples to determine if gonadotropins influence sperm capacitation by regulating the rate of secretion of these progestins.

The secretion patterns for 20α-ol were not influenced significantly by level or kind of injected gonadotropin. The maximum rate of secretion was about 1700 μg per gram of ovary per hour and occurred approximately 2 hours after gonadotropin stimulation. The secretion of 20α-ol returned to preinjection rates by about 8 hours after injection.

Levels of progesterone in the same samples of ovarian venous blood were only 3 to 4 per cent as large as levels of 20α-ol. But the secretion pattern of progesterone was similar to that for 20α-ol with the exception that high level of gonadotropin resulted in greater progesterone secretion.

There was no significant difference in progestin secretion after rabbits were injected with high level of LH or high level of HCG which could be interpreted to cause the difference noted in sperm capacitation. It appears that inhibition of capacitation which occurred when incubator rabbits were injected with high levels of HCG is not caused by excessive progestin secretion.

ENDOCRINE CHANGES INFLUENCING SPERM CAPACITATION

by

Robert Paul Wettemann

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BIOGRAPHICAL SKETCH

Robert Paul Wettemann was born on November 12, 1944, in New Haven, Connecticut. He attended public schools in Guilford, Connecticut and graduated in June, 1962. In September, 1962 he enrolled at the University of Connecticut, majoring in Dairy Science, and received a Bachelor of Science degree in June, 1966.

He accepted a graduate assistantship at Michigan State University in September, 1966, and he was granted an NIH Predoctoral Fellowship in March, 1968. He received the Master of Science degree in September, 1968.

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INTRODUCTION

While the major infectious diseases of reproduction have been largely controlled, infertility persists as a major problem in livestock production. Although animal breeders are much better animal husbandmen than they were, poor management is still a major cause of infertility. But in the opinion of some authorities, abnormal endocrine conditions may be one of the major causes of reproductive failures. Cystic ovaries are one well known example of such causes of infertility and others may prove to be more significant economically.

A rapid sequence of important, but not completely understood endocrine events is initiated near the time of mating. These endocrine changes are, among other things, responsible for controlling or mediating (1) sperm transportation to the site of fertilization, (2) ovulation, (3) maturation of sperm and eggs, (4) fertilization, (5) movement of the zygote into the uterus, and (6) preparation of the uterus for implantation of the embryo. But knowledge of endocrine mediation in these events is meager and the extent to which failure of the normal events after insemination may be responsible for infertility is unknown. One of these events, preparation of sperm in

the female reproductive tract for fertilization and endocrine control of this event, is the topic of this thesis.

Sperm must be ready to fertilize ova at the time and site of fertilization because ova have a short fertile life. Within 4 to 8 hours after ovulation ova move past the middle of the oviduct (34, 42) and become much less fertile. Although sperm have been found at the site of fertilization within 2 minutes after insemination in bovine (67), fertility is very low when females of most species are inseminated following ovulation (12, 66). These findings suggest that sperm must reside in the female tract for about 4 to 6 hours, at least in rabbits, to develop the capacity to fertilize ova. This is the nature of the evidence that led to the notion that while residing in the female reproductive tract, sperm undergo a maturation process called "capacitation" (4). It is thought to consist of alteration of some macromolecular substance which normally coats ejaculated sperm and inhibits fertilization (53).

In some species lowered fertility may be caused by the failure of sperm capacitation. In other cases, poor management of insemination may not allow sperm capacitation. For example, when cattle are artifically inseminated late (near the time of ovulation), as many undoubtedly are, failure of sperm capacitation may be the cause of infertility. Preparing bull sperm for fertilization

by causing sperm capacitation before insemination of cattle should minimize this cause of infertility.

The major objective of this research was to study capacitation *in vivo* on the premise that such knowledge may lead to capacitation of sperm *in vitro* and thereby lead to higher fertility. In these experiments, the relationships of capacitation to mating, the consequent release of gonadotropin and alteration of ovarian hormones were studied. This approach was motivated by the observation that mating enhances sperm capacitation in the rabbit (2, 64), although the mechanism was unknown.

REVIEW OF LITERATURE

Necessity for Capacitation

Freshly ejaculated or epididymal spermatozoa of most mammals are infertile (1, 2, 3, 11, 12). These spermatozoa normally require a period of residence in the female reproductive tract to develop the ability to penetrate and fertilize ova. Changes occurring during this period which render sperm capable of fertilization are called sperm capacitation (4).

In 1926, Hammond (37) found that rabbits artificially inseminated more than 10 hours after sterile coitus were much less fertile than those bred during the previous 8 hours. Dairy cattle also have drastically reduced fertility when they are inseminated after the time of ovulation (66). Originally, many researchers speculated that this reduced fertility was caused by failure of sufficient sperm to arrive at the site of fertilization before the end of the fertile life of ova. But because sperm have been found in the ovarian portion of the bovine oviduct within 2 minutes after insemination (67), limitations on sperm transport do not appear to limit fertility in these circumstances. Rather, the reduced fertility of inseminations performed near the time of ovulation is now thought to be caused by failure of sperm capacitation,

which was first demonstrated independently by Chang (12) and by Austin (3) in 1951. Chang (12) established the necessity for capacitation in the rabbit by performing oviducal inseminations at varying intervals before or after ovulation. Austin (3) found that few ova (3 per cent) recovered from rabbits inseminated with epididymal sperm 1 to 3 hours after ovulation were fertilized, but if rabbits were similarly inseminated 6 to 8 hours prior to ovulation, 76 per cent of the ova were fertilized.

Rabbit spermatozoa must reside in the uterus of an estrous rabbit about 6 hours before they are able to penetrate ova (13). However, capacitation takes about 10 hours in rabbit fallopian tube (2). In the rat, capacitation is accomplished in about 4 hours either in the oviduct or periovarian sac (3). By employing methods similar to those used in these two laboratory animals, the necessity for capacitation also has been demonstrated in the ewe (56), ferret (19) and golden hamster (18).

Other kinds of evidence suggest that sperm capacitation may also be prerequisite to fertilization in other species. For example, Chang demonstrated that incubation of capacitated sperm in seminal plasma reversed the capacitation process and that these decapacitated sperm could be recapacitated with another period of incubation in the uterus (14). Later Bedford and Chang (9) sedimented the "decapacitation factor" from seminal plasma by

centrifugation at 105,000 x g. The presence of decapacitation factor in seminal plasma of domesticated animals, when assayed in a rabbit test system (70), has been used as an indicator that capacitation is required.

Decapacitation activity is assessed quantitatively in a rabbit test system by inseminating rabbits at the time of ovulation, with capacitated rabbit sperm that has been treated with decapacitation factor isolated from seminal plasma of another species (70). If ova recovered 24 hours after insemination are not cleaved, it is assumed that the species tested has decapacitation activity, so capacitation is required.

Another technique used as an indirect indicator of capacitation is based on the observation that sperm absorb the fluorescent compound, tetracycline (31). Ericsson suggested that tetracycline may mimic decapacitation factor in two ways. Both tetracycline and decapacitation factor have affinity for the sperm cell membrane and both are removed from sperm under conditions where capacitation normally occurs in laboratory animals (31). Thus the coating of sperm with tetracycline and its removal may indicate the requirement for capacitation. It has also been noted that capacitated sperm respire at a much greater rate than freshly ejaculated sperm (40, 58). Thus when the respiration rate of sperm from large animals incubated in the uteri of laboratory animals is increased,

this is also an indication that capacitation has occurred. Based upon data derived from these indirect indicators, it is suggested that capacitation is necessary in cattle (27, 32), horses (24, 27), monkeys (23, 27), swine (27, 50), and man (23, 32).

Some phases of capacitation may be initiated by agents which are not species specific. For example, rabbit sperm can be partially capacitated in the rat (10) or dog (39) uterus. When rabbits were inseminated 12 to 13 hours after an ovulating injection with rabbit sperm incubated in the estrous rat uterus for 5-14 hours only 4 of 88 ova recovered were fertilized. However, when rabbits were inseminated 0 to 5 hours after ovulating injection with similar sperm 29 of 37 ova were fertilized (10). Thus complete sperm capacitation involves at least one step which may be species-specific.

Decapacitation Factor

That capacitated rabbit sperm can be made infertile (decapacitated) by incubating them *in vitro* with rabbit seminal plasma (14) suggested that a seminal plasma factor necessitated sperm capacitation. Although this seminal plasma substance cannot be removed from sperm by washing (25, 53), rabbit sperm can be recapacitated by another period of incubation in the uterus of an estrous rabbit. The time required for recapacitation of decapacitated

sperm is similar to that for capacitation of freshly ejaculated sperm (28).

Decapacitation factor can be removed from seminal plasma (9) by ultracentrifugation, indicating that it is a macromolecule. As might be expected on the basis of common embryological origins of epididymides and secondary sex organs (which produce seminal plasma), rabbit epididymal fluid also contains decapacitation factor (68). Decapacitation factor has been found in bull, boar, stallion, human, and monkey seminal plasma, but not in rooster or dog seminal plasma (23, 27).

Even though the decapacitation factor is stable to long-term storage and dehydration, it can be destroyed by β-amylase (27). Other enzymes such as pronase, lysozyme, glucose oxidase, and hyaluronidase do not destroy decapacitation factor (25, 27). Treatment with pronase, however, apparently cleaves decapacitation factor and it can no longer be sedimented by centrifugation (105,000 x g)--it remains in the supernatant fluid (69). This suggests that decapacitation factor may consist of a protein carbohydrate complex bound to the sperm head and the active part is the carbohydrate portion.

Recently Hunter reported immunochemical and immunoelectrophoretic analysis of decapacitation factor, isolated by centrifugation, which migrated in agar with the mobility of a slow serum beta globulin and stained as a glycoprotein (49). Using column chromatography, Hunter

also isolated an immunoelectrophoretic component from seminal plasma with a molecular weight of at least 200,000. This glycoprotein contained decapacitation activity, migrated in agar as a slow beta globulin, and was a sperm coating antigen.

Although seminal plasma will cause decapacitation of capacitated sperm this has little significance to the understanding of capacitation because capacitated sperm do not contact seminal plasma under normal physiological conditions. The purpose of the glycoprotein found in seminal plasma and coating sperm is not to decapacitate capacitated sperm but may be to maintain fertility of sperm until fertilization. It appears that glycoprotein attaches to the sperm head while in the epididymis or seminal plasma, thus protecting the sperm until conditions are favorable for fertilization. After the glycoprotein has been removed from the sperm (capacitation) the fertile life of the sperm is decreased (28). Instead of the term decapacitation factor, possibly the glycoprotein should be called "fertility factor."

Endocrine Influences on Capacitation

Sperm capacitation is dependent upon the hormonal state of the female rabbit. When estrogen is the predominant gonadal steroid, the uterus is most capable of sperm capacitation. But sperm capacitation in the uterus is inhibited by endogenous or exogenous progesterone, for example during psuedopregancy (15, 17, 38). On the other

hand, progesterone has little effect on capacitation in the oviducts (15, 38). Sperm capacitation occurs in uteri of immature rabbits with or without gonadotropin or estrogen treatment, but not as rapidly as in mature estrous rabbits. The ability for capacitation to occur is also reduced after ovariectomy (15). An ovariectomized rabbit has similar capacitating ability as an immature rabbit, but both will capacitate sperm more completely than when the uterus is under the influence of progesterone. If an ovariectomized rabbit is injected with estrogen (15, 63), the uterus does not totally regain its normal capacitating ability. This suggests that estrogen alone cannot produce all the conditions necessary for normal capacitation in the uterus.

Since changes in gonadal hormones are dependent upon changes in levels of gonadotropins, these could be related indirectly to capacitation. In rabbits, there is a rapid decrease in pituitary levels of adrenalcorticotropic hormone (ACTH), prolactin, and luteinizing (LH) but not follicle-stimulating hormone (FSH) after copulation (20). Levels of these hormones remain low for 0.75 to 7 hours, but return to precopulatory levels within 11 hours after mating. ACTH is usually associated with control of adrenal corticoid levels. Prolactin is responsible for proliferation of the mammary gland and, at least in some species, prolongation of the functional life of the corpus

luteum. Thus ACTH and prolactin are unlikely to affect capacitation. But a different picture has emerged for LH. The sudden release of LH after copulation in rabbits probably acts with basal levels of FSH to cause ovulation and organization of corpus lutea. Although these are the functions usually attributed to these three pituitary hormones, one cannot ignore the possibility that they may have other functions after copulation.

When spermatozoa are recovered from the rabbit uterus several hours after mating they have higher fertility (degree of capacitation) than sperm that were surgically deposited in the uterus for a similar period of time (2, 64). Thus it appears that some endocrine changes, which occur as a result of mating, influence the rate or degree of sperm capacitation. Injections of moderate levels of HCG also increase the rate of sperm capacitation in the rabbit uterus (62). When rabbits are injected with 75 IU of HCG at the time sperm are deposited in the uterus, the fertilizing ability of these sperm is similar to that of sperm recovered from the uterus after mating. But when rabbits are injected with 400 IU of HCG, fertility is reduced relative to that after injection of 75 IU of HCG (62).

Another endocrine change in the rabbit which occurs after mating and simultaneously with sperm capacitation is the rapid increase in synthesis of 20α-hydrozy-pregn-4-en-3-one (20α ol) by the ovary. Levels of this

progestin increase from 75 μg per gram of ovary per hour before mating to 1000 μg within 1 or 2 hours after mating (46). Then there is a decrease in the synthesis of 20α-ol to precopulatory levels by the time of ovulation. Levels of circulating gonadotropins appear to be correlated with the levels of progestins synthesized in the ovary (46).

LH enhances the synthesis of both 20α-ol and progesterone by rabbit ovarian tissue in vitro (21, 22). Furthermore, injections of HCG, LH, FSH, or PMS into estrous rabbits causes increased synthesis of 20α-ol by the rabbit ovary in vivo but injections of prolactin and ACTH do not (45). But all four tropins which stimulate 20α-ol synthesis are thought to do so through some LH contamination or inherent LH property. The site of LH action to cause increased synthesis of 20α-ol is the ovarian interstitial tissue (45).

During the period of maximum 20α-ol synthesis, cholesterol stores in the ovary are depleted (47, 48). Hilliard (47) demonstrated that prolactin is necessary for cholesterol storage in ovarian interstitial tissue of hypophysectomized rabbits. If prolactin is not available, 20α-ol secretion is diminished, probably due to lack of cholesterol precursor. This suggests a requirement for prolactin released from the pituitary at the time of mating.

In addition to synthesis of 20α-ol, the rat ovary may also produce estrogen in response to LH (33). At least

both LH (54) and estrogen (72) can cause implantation in rats. Since rat and rabbit ovaries respond similarly to LH regarding progestin secretion (43, 46), the rabbit ovary may produce estrogen in response to LH stimulation as do rat ovaries.

These results suggest that several of the rapid endocrine changes which normally are triggered by mating in rabbits may be simulated by injection of gonadotropins such as LH or HCG.

In Vitro Capacitation

On the assumption that knowledge of sperm capacitation would allow improvement or control of fertility of animals, several researchers have attempted *in vitro* capacitation. The first report of *in vitro* capacitation, in 1958, was accomplished by incubating sperm with uterine endometrial strips at room temperature (60). But, the test system used to assay for capacitation was weak and lacked controls. Thus, partial or incomplete capacitation appeared positive. Other researchers (39, 64) have been unable to obtain capacitation *in vitro* with similar incubations of sperm.

Later, rabbit sperm were at least partially capacitated *in vitro* by incubating them at 37° C in uterine fluid or with β-amylase in phosphate buffered Locke's solution (53). β-amylase catalyzes the successive hydrolysis of the second α- 1,4 glyosidic bond from the free nonreducing

ends of glucose chains, releasing maltose units. This suggests that sperm capacitation involves the enzymatic alteration of a carbohydrate containing macromolecule from seminal plasma which coats sperm (53). Although other laboratories have not been able to capacitate sperm with β-amylase *in vitro* (64), these data suggest that one or more enzymes may be involved in this process.

Theories on Capacitation

Many theories on the mechanism of capacitation have been proposed. Austin originally hypothesized that an enzyme on the sperm digests a path through the zona pellucida (3). And capacitation, he theorized, was the removal of an enzyme inhibitor. Later, he modified his theory to include "ripening" or shedding of the acrosome and exposure of the perforatorium as a part of the process (5, 6, 7). But changes in the acrosome of rabbit sperm have not been demonstrated after capacitation (2).

As evidence accumulates it appears that capacitation is not an all or none phenomenon (10, 30, 59), but a sequence of events. Dzuik (30) demonstrated that capacitation is both qualitative and quantitative. Using buck rabbits genetically different for coat color, he showed that sperm that reside in the uterus of a rabbit for a longer period of time have a competitive fertilization advantage.

Some workers hypothesize that sperm must be in direct contact with uterine endometrial cells to be capacitated (64). This conclusion is based upon their observation that sperm incubated in ligated rabbit uteri with accumulated uterine fluid were not capacitated. But this intimate contact between sperm and the endometrium does not seem to be absolutely necessary because Kirton and Hafs (53) achieved at least partial capacitation of sperm in uteri which had been ligated for two weeks and contained about 4 ml fluid (65). Furthermore, it is possible to at least partially capacitate sperm in vitro in uterine fluid (53).

Contact between sperm and ova, as well as the ability of sperm to penetrate the zona pellucida, are enhanced by capacitation (8). Disintegration of the acrosome cap of the sperm occurs by fussion between the plasma membrane of the ova and the outer membrane of the acrosome cap. As the ovum is penetrated by the sperm, the inner acrosomal membrane is the first part of the cell that interacts with the zone pellucida (8).

The leucocytic response of the rabbit uterus depends on the hormonal status of the female (55). Thus, the estrous rabbit uterus responds to the presence of foreign bodies including sperm with an infiltration of leucocytes into the uterine tissue and lumen (16, 55), but this response is greatly retarded in progestational females. Recently Chang (16) suggested that capacitation of sperm

may be closely related to proteolytic enzymes in the uterus and to the phagocytic reaction.

Many contributions to the understanding of capacitation have been made since the recognition of the necessity for sperm capacitation 17 years ago. In spite of concentrated research endeavors, sperm capacitation is a biological phenomenon that is still not fully elucidated. The complex biological change known as sperm capacitation is recognized, but the micromorphological and biochemical processes associated with capacitation are not understood.

MATERIALS AND METHODS

Details of experimental designs will be given at appropriate locations under Results and Discussion below. More general methods are listed here.

Experimental Animals

Three experiments were designed to study the influence of gonadotropins and ovarian hormones on sperm capacitation in the rabbit uterus. Initially, rabbits were injected with Human Chorionic Gonadotropin (HCG)* or NIH Bovine Leutinizing Hormone (NIH-LH-B_5) to determine their influences on sperm capacitation. In the second experiment rabbits were injected with the progestin, 20α-hydroxy-pregn-4-en-3-one**, to see if this was a regulator of capacitation. And in the third experiment it was determined if these gonadotropins exerted their influence on sperm capacitation by virtue of their regulation of progestin secretion by the ovary.

Mature estrous rabbits of mixed breeding weighing 3 to 5 kilograms were used. All rabbits were caged individually for at least 1 month before being assigned

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*Squibb Chorionic Gonadotropin, E. R. Squibb and Sons, Inc., New York.

**Obtained through the courtesy of Dr. Kenneth T. Kirton, The Upjohn Co., Kalamazoo, Michigan.

to an experiment. They were housed at 18° C with 14 hours of light daily and fed ad libitum.

Preparation of Semen

Semen was obtained from a total of ten fertile rabbits with artificial vaginae as described by Gregoire et al. (35). To standardize ejaculation frequency, all rabbits were ejaculated 3 to 7 days prior to experimental use. In any particular experiment semen samples, of sufficient quality for use in inseminations, (at least 50% motile sperm and void of urine) from two to four rabbits were pooled and sperm concentration was determined with a hemacytometer. The seminal plasma was separated from the sperm by centrifugation (1000 x g) for 10 minutes. After aspirating the supernatant seminal plasma, the sperm were resuspended at a concentration of 500 million per ml in Locke's solution. To reduce possible uterine infection due to direct uterine insemination with large quantities of semen, 100 µg of dihydro streptomycin were added to each ml of reconstituted semen. Precautions were taken to prevent cold shock to the sperm throughout the processing of semen.

Incubator Rabbits

Treatments designed to alter capacitation were administered to incubator rabbits because, as will become more apparent in description of test rabbits below, sperm

develop the capacity to fertilize ova while residing in the uterus of an incubator rabbit. In an attempt to provide relatively homogeneous incubator rabbits, only mature virgin rabbits were used for this purpose. While rabbits were under general anesthesia (ether), both uterine horns were exposed via a mid-ventral incission and assessed for maturity. Only those rabbits appearing normal and in estrus were used. With a syringe and a 26 gauge needle, 90 to 200 million sperm were deposited in each uterine horn. Inflation of the uterus was minimized by limiting the volume of semen to a maximum of 0.2 ml. In the first experiment, the incubator rabbits were then injected intravenously with 0, 50, 75, 100, or 300 IU of HCG. And other rabbits were injected with 100, 250, 500, 1000, or 2000 µg of NIH LH-S1 equivalent as NIH LH-B5. Replication of gonadotropin treatments was performed on three days, one rabbit on each treatment on each day.

A second experiment was designed to determine if 20α-ol influences sperm capacitation as gonadotropin does. Incubator rabbits, either intact or ovariectomized at the time of sperm deposition, were injected subcutaneously with this progestin. Assuming that 20α-ol is absorbed similarly to estradiol (52), we attempted to duplicate the levels of 20α-ol found in rabbits after mating (46). 20α-ol was dissolved in sesame oil at a concentration of 4 mg per ml. Four mg was injected at the time sperm were deposited in

the uterus. Another 4 mg of 20α-ol was injected one hour later followed by an additional 2 mg 2 hours after the start of the incubation period. The procedures used to prepare sperm for the gonadotropin studies above were also used in these experiments.

Sperm were recovered from all incubator rabbits after 0, 3.5, 7, or 10 hours residence in the uterus. Rabbits were anestized and reopened along the midline. Hemostats were placed immediately at the tubouterine junctions and cervices of both uterine horns. One ml of Locke's solution was injected into each uterine horn with a syringe and an 18 gauge needle. After manually distributing the Locke's solution along the length of each uterine horn, fluid containing sperm was aspirated using a pre-warmed syringe. Sperm were deposited into centrifuge tubes maintained at 37° C and sperm motility and concentration were estimated. Sperm were assayed for capacitation after concentrating the sperm to 25 million per ml by centrifugation and resuspension in Locke's solution. An average of 5.3 per cent (range of 0.4 to 30.0 per cent) of the sperm deposited were recovered from incubator rabbits but no association could be established between treatment and recovery rate. Motile sperm were observed in every sample and sperm heads were frequently attached to leucocytes. This low recovery may be accounted for by one of four factors: (1) sperm may move through

the cervix and are lost into the vagina (71); (2) sperm possibly migrate up the oviducts and into the body cavity; (3) sperm are phagocytized (44, 55); or (4) sperm are not recovered from the uterus by the flushing method used.

Sperm Capacitation Test Rabbits

The assay for capacitation of sperm recovered from incubator rabbits amounted to measuring sperm fertility after they were inseminated into test rabbits after the time of ovulation. The criterion for fertility was cleaved ova. Because it was advantageous to obtain many ova from each test rabbit, test rabbits were superovulated by a modification of the method of Kennelly and Foote (51). Each morning and evening for three days, test rabbits were injected subcutaneously with 0.4 mg of Armour FSH* dissolved in saline. About 12 hours after the last injection of FSH, an intravenous injection of 75 IU of HCG was given to induce ovulation.

Although superovulated ova are obtained by modifying the hormone levels in a rabbit, they develop into normal fetuses when transferred to the oviducts of normal early pseudopregnant rabbits (57). Rabbits normally ovulate 10.5 to 13 hours after the injection of HCG (41). Fertility is greatly decreased from inseminations after 10 hours (2). The reason for this decrease in fertility is,

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*Armour Follicle Stimulating Hormore--Pituitary.

as explained in the review of literature, caused by the requirement for sperm capacitation before the end of the fertile life of the ova. As will be described in the results section, when superovulated test rabbits were inseminated at various intervals after HCG injection, a similar decrease in fertility was observed from inseminations performed beyond 10 hours after HCG injection. But the decrease in fertility occured about 1 hour later than Adams and Chang (2) observed with normal ovulations. These results suggested that the appropriate time to inseminate sperm to be assayed for capacitation would be 12.5 to 13 hours after the ovulatory inducing injection of HCG, because ova recovered from test rabbits would not be cleaved unless sperm were capacitated before insemination.

Thus, about 12.5 to 13 hours after the ovulating injection of HCG, the superovulated test rabbits were anestized with ether and a mid-line incision was made to expose both uterine horns. To test their ability to fertilize ova, 0.32 to 2.52 million sperm were deposited near the tubouterine junction of each uterine horn. After the incision was closed test rabbits were returned to individual cages. Approximately 26 hours after these surgical inseminations, the test rabbits were killed by cervical dislocation. The reproductive tracts were quickly removed and oviducts were dissected free of excess fat. Then a blunt 16 gauge needle was inserted into the

fimbriated ovarian end. Ova were collected from the tubouterine end in depression slides as warm saline was flushed through the oviducts. Ova were observed for cleavage with a dissecting microscope (x 35). When division of ova could not be clearly ascertained at low magnification, phase contrast or dark field illumination were used (x 250).

Collection of Ovarian-Venous Blood

As will be shown in the results and discussion sections, comparison of the results from the experiments on capacitation in gonadotropin injected rabbits with some previously published data led to the suggestion that the gonadotropins were acting on sperm capacitation in the uterus through ovarian steroids. This suggestion motivated the following experiment on progestin levels in ovarian venous effluent. For this part of the study, mature estrous female rabbits weighing 4 to 5 kg were used. They were injected with 75 or 300 IU of HCG or with 100 or 1000 µg of NIH LH at predetermined intervals before or after surgery. Anaesthesia was induced with approximately 25 mg of pentobarbital* per kg body weight. The stage of anaesthesia was regulated empirically by amount of injected pentobarbital and by intravenous injection of mikedimide**.

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*Nembutal Sodium, Abbott Laboratories, North Chicago, Ill.

**"Methetharimide Parlam," Parlam Division, Ormont Drug and Chem. Co. Inc., Englewood, N.J.

Through a ventral mid-line incision, an ovarian artery and vein were located. The collateral venous circulation was ligated, thus all blood leaving the ovary passed through the ovarian vein. About 5 cm from the ovary, the ovarian vein was isolated. Then 500 USP of heparin per kg body weight was injected into the ear vein. A 4-cm section of Intramedic polyethylene tubing* with an outside diameter of 0.038 inches was inserted into a small nick in the ovarian vein. A 25-cm length of heparinized tubing with an inside diameter of 0.034 inches had been previously slipped over one end of the smaller tube. Experience showed that replacing the greater proportion of the length of the smaller tube with the larger tube materially increased blood flow rate. After ligating the vein around the cannula, the viscera were replaced and the abdominal incision was closed around the catheter which led out into a calibrated conical 12-ml centrifuge tube. A hemostat at the tip of the cannula controlled blood flow. Ten-milliliter blood samples were collected hourly. The blood flow rate was usually 0.5 to 2 ml per minute. Blood samples were not retained if flow rate was less than 0.15 ml per minute. Following collection in centrifuge tubes, the blood samples were chilled. About 6000 dpm of progesterone-^{14}C and of 20α-hydroxy-preg-4-en-3-one-^{14}C

*Intramedic Polyethylene tubing, Clay-Adams, Inc., New York.

were added to each sample before centrifugation to allow calculation of extraction efficiency of these progestins from whole blood. After the blood samples were centrifuged for 15 minutes at 10,000 x g, the plasma was decanted and stored at -16° C.

Isolation and Identification of Progestins

Following thawing, 5-ml plasma samples were extracted three times with two volumes of diethyl ether. The ether was evaporated under nitrogen and the residue was dissolved in benzene: methylene chloride (1:1) and spotted on silica gel thin layer chromatography plates. After two-dimentional chromatography in hexane:ethyl acetate (5:2) and methylene chloride: diethyl ether (5:2), progesterone and 20α-ol spots were visualized with ultraviolet light. The areas on the silica gel plates containing these two progestins were scraped into 12-ml centrifuge tubes. Then the progestins were eluted from the silica gel with a double washing of ethyl acetate. For further purification the progestins were spotted on Eastman silica gel thin layer chromatography plates* and chromatographed in isopropyl ether:ethyl acetate (5:2). After elution from the silica get with absolute ethanol, progestin was quantified by absorption at 240 mμ in a Beckman model Dk-2A

*Eastman Chromagram sheet 6060, Distillation Products Industries, Rochester, N.Y.

recording spectrophotometer. Extraction efficiency was determined by measuring the radioactivity present in an aliquot of the sample used to quantify mass. Mass of progesterone or 20α-ol was corrected for losses of radioactivity.

RESULTS AND DISCUSSION

Section I: Methodological Experiments

Superovulation of Test Rabbits

At the outset of this research, it was desirable to improve the assay for degree of capacitation of sperm recovered from treated incubator rabbits. In this assay, as described above, sperm fertility was measured in test rabbits which were inseminated after the time of ovulation. The criterion used to assess fertility was the percentage of cleaved ova recovered from oviducts. Thus it was advantageous to obtain as many ova as possible from each test rabbit. Consequently, the objective of this first experiment was to develop a method to use superovulated rabbits as test rabbits to assay treated sperm for degree of capacitation.

In an effort to find the level of injected hormone and the interval between injections which maximized ova numbers, variants on the superovulation method of Kennelly and Foote (51) were tested on the mixed large breed rabbits (3 to 5 kg body weight) in our colony. The reported method involved injection of 0.5 mg Armour FSH twice daily for three days to stimulate follicular growth and injection of ovulating hormone 12 hours after the last

injection of FSH. But preliminary tests showed that this injection scheme was not satisfactory for our rabbits.

The results (Table 1) were obtained from 7 different superovulation procedures tested in the course of the capacitation experiments listed below. Preliminary studies revealed that FSH injections for three days yielded many more ova than injections for two days. Consequently only the former are listed in Table 1. The data suggest that too much FSH was as detrimental as too little and reveal that injection of 0.4 mg of Armour FSH twice daily for 3 days resulted in an average of 22.1 ova from 220 rabbits--more than 3 times the number expected without superovulation. Superovulation with 0.3 mg FSH twice daily for three days also appeared satisfactory, with limited numbers of observations. But injections of 0.5 mg FSH resulted in fewer ovulations, in numerous hemoragic follicles, and in variable numbers of 3 to 4 day old corpora lutea at the time the rabbits were killed (36 hours after HCG). These observations suggest that the Armour FSH contained enough LH activity to induce ovulation after the first or second injection and these ovulations resulted in the corpora lutea found at slaughter. Clearly, large differences must exist among strains of rabbits regarding their superovulatory response to FSH, because Kennelly and Foote did not observe corpora lutea after injections of 0.5 mg Armour FSH

TABLE 1.--Rabbit ova recovered after superovulation.

Treatment	Rabbits	Ova[a] per Rabbit	
		Average	Range
	----------	(No.)	----------
0.2 mg FSH,[b] 2X-3 day[c]	2	23.0	6-40
0.3 mg FSH, 2X-3 day	4	35.8	11-54
0.25 mg FSH, 2X-3 day	10	15.2	0-53
0.4 mg FSH, 2X-3 day	220	22.1	0-83
0.5 mg FSH, 2X-3 day	2	6.5	5-8
0.5 mg FSH, 1X-3 day	8	17.1	2-40
0.6 mg FSH, 1X-3 day	33	14.7	0-71
Total	279		
Average		21.0	

[a]Ova flushed from oviducts 36±2 hours after injection of ovulating hormone.

[b]Armour F.S.H. - P. (Follicle Stimulating Hormone - Pituitary).

[c]Frequency and duration of subcutaneous FSH injections (e.g., 2X-3 day was twice daily injection for 3 days).

even though they used smaller rabbits (personal communications).

Preliminary studies on superovulation of Dutch rabbits (2 to 3 kg body weight) in our colony indicate that injection of 0.25 mg of FSH twice daily for 3 days was a satisfactory superovulation method for them. But fewer ova were obtained from Dutch rabbits by any of the treatment combinations tested, than from the larger rabbits. One important conclusion from this experience is that it is necessary to titer the dose of FSH required for superovulating different breeds, strains, and maturity groups of rabbits.

Time of Inseminations of Superovulated Test Rabbits

To determine the appropriate time to inseminate superovulated test rabbits with sperm to be assayed for degree of capacitation, 2.5 million freshly ejaculated, washed sperm were surgically deposited in each uterine horn of superovulated test rabbits at 0, 6, 8, 10, 12, or 14 hours after an ovulating injection of HCG. This experiment was designed similarly to one performed by Adams and Chang (2) using non-superovulated rabbits.

Figure 1 shows the fertility of superovulated and non-superovulated rabbits (Adams and Chang, 2) when inseminated at various intervals after HCG injection. Adams and Chang found that fertility was greatly decreased by

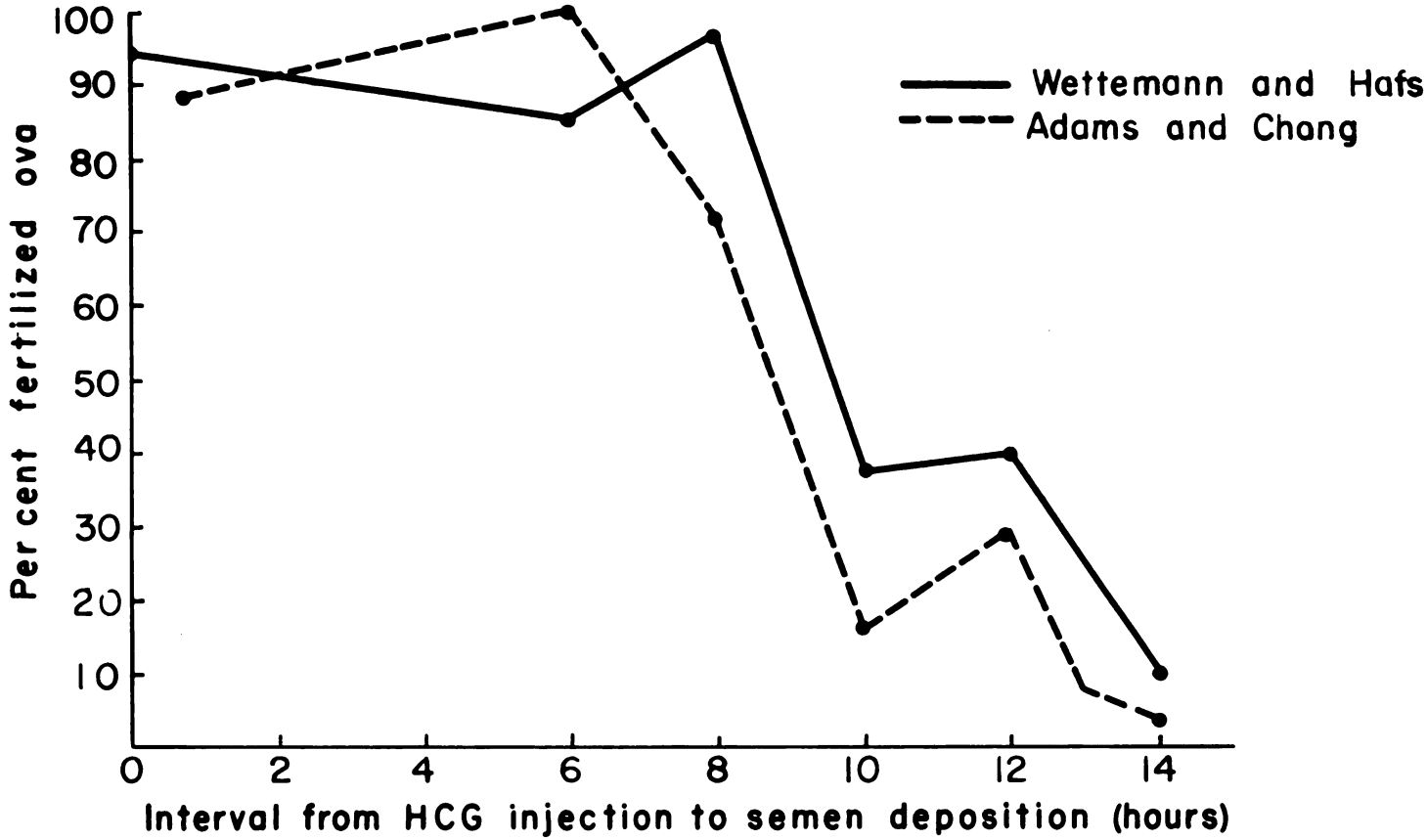

Figure 1.--Fertility of superovulated rabbits(—),[3] relative to normally ovulated rabbits (Adams and Chang)[2], inseminated into the uterus with freshly ejaculated sperm at the time of injection of ovulating hormone.

inseminations 8 hours or later (just before ovulation) after injection of HCG. The reason for this decrease in fertility is because sperm are not capacitated before the end of the fertile life of the ova. When superovulated test rabbits were inseminated at various intervals after HCG injection, a similar decrease in fertility was observed from inseminations performed beyond 8 hours after HCG injections (Table 2). But this decrease in fertility occured about 1 hour later than Adams and Chang observed. Thus it appears that either superovulated rabbits may ovulate about 1 hour later or their ova are fertile for about 1 hour longer.

Because of the low fertility with freshly ejaculated sperm, 12 to 13 hours after injections of HCG was selected as an appropriate time to inseminate superovulated test rabbits with capacitated sperm. Control test rabbits inseminated during capacitation experiments described below confirm the above results. Of 238 ova recovered from 10 test rabbits inseminated with freshly ejaculated sperm 12.5 hours after an ovulatory injection of HCG (negative control treatment), only 17 ova were fertilized. Sperm inseminated at the time of HCG injection (positive control treatment) were fertile as expected; 164 of the 215 ova recovered from 12 rabbits were cleaved. These experiments justified the use of superovulated rabbits as capacitation test rabbits.

TABLE 2.--Fertility of superovulated rabbit ova following uterine insemination[a] at various intervals after HCG injection.

Interval from HCG Injection to insemination	Rabbits	Ova[b]		Ova Fertilized
		Recovered	Fertilized	
(Hours)	----------	(No.)	---------	(%)
0	3	79	74	93.7
6	3	74	63	85.1
8	3	133	129	97.0
10	3	72	27	37.5
12	3	63	25	39.7
14	3	128	13	10.2

[a]Freshly ejaculated and washed sperm surgically injected into the uterus adjacent to tubouterine junction.

[b]Ova flushed from oviducts 36 ± 2 hours after injection of ovulating hormone (HCG).

Methods of Inseminating Test Rabbits

In most capacitation test systems previously reported, test rabbits are inseminated by depositing sperm in the infundibular end of the oviduct. This site is chosen presumably because it theoretically eliminates time otherwise required for sperm transport to site of fertilization. But disturbing the infundibulum prior to the time of ovulation may result in loss of some ova. And if sperm are transported anteriorly in the oviduct, as they normally are, many of the sperm to be tested will be lost into the peritoneal cavity before the time of fertilization.

To devise a method to inseminate rabbits to obtain higher fertility and recover more ova we inseminated rabbits surgically with 2.5 million sperm per uterine horn at the time of HCG injection in one of four ways; (a) the semen was deposited about 1.5 cm deep into the infundibular end of the oviduct, (b) the semen was dropped on the ovary, (c) the semen was injected into the oviduct near the tubouterine junction, or (d) the semen was injected into the uterus near the tubouterine junction. Ova were flushed from the oviducts of these rabbits 36 ± 2 hours after insemination and the results are presented in Table 3.

The very low fertility obtained when sperm were dropped on the ovary was expected, because most sperm are lost in peritoneal fluid. The 9% fertility obtained is reminiscent of the fertility obtained by others from

TABLE 3.--Fertility of rabbit ova following inseminations[a] at the ovary, upper oviduct, lower oviduct, or upper uterus at the time of injection of ovulating hormone.

Site of Insemination	Rabbits	Ova[b]		Ova Fertilized
		Recovered	Fertilized	
	----------	(No.)	----------	(%)
Ovary	3	33	33	9.1
Upper oviduct	4	90	31	34.4
Lower oviduct	5	39	35	89.7
Upper uterus	6	116	108	93.1

[a]Freshly ejaculated and washed sperm.

[b]Ova flushed from oviducts 36±2 hours after injection of ovulating hormone.

intraperitoneal inseminations (36). The low fertility with infundibular inseminations may be due to loss of sperm into the peritoneal cavity before the time of ovulation (about 10.5 hours after insemination) or to less capacitation in the upper oviduct. There was no appreciable difference in fertility when rabbits were inseminated into the uterus or into oviduct near the tubouterine junction. The higher fertility at these locations probably resulted from more rapid sperm capacitation or less sperm loss before ovulation than obtained from inseminations near the ovary. If some sperm are lost into the peritoneal cavity via the oviduct after inseminations near the tubouterine junction, a reserve of sperm probably remains in the uterus or lower oviduct available for transportation to the site of fertilization over a period of time.

Although inseminations at the time of injections of ovulating hormone (about 10.5 to 12 hours prior to ovulation) near the tubouterine junction gave higher fertility than inseminations at the infundibular end of the oviduct at this time, it was also necessary to compare the fertilities when rabbits were inseminated shortly after the time of ovulation. For this trial we chose 5 capacitation treatments and inseminated 2 test rabbits with each semen sample. This trial was part of a larger experiment studying the influence of LH on capacitation and the treatments, which are immaterial to the present purpose, will be described

below. Sperm (0.7 to 2.5 million) were deposited, 12.5 ± 0.2 hours after an ovulating injection, into the infundibular end of each oviduct of one test rabbit and a similar number of sperm were injected into each uterine horn of the other rabbit (Table 4). A paired t test revealed no significant difference in fertility between the two methods of inseminating test rabbits after the time of ovulation. But an average of 39.8 ova was recovered from rabbits inseminated in the uterus and an average of only 10.6 ova from oviductally inseminated rabbits. The difference in the number of ova obtained from the two groups may be due to the number of ovulations, but disturbing the infundibulum near the time of ovulation may have interfered with ova transport and caused some ova to be lost into the peritoneal cavity. Since it is more convenient to inseminate rabbits into the uterus and this method may allow recovery of more ova, I chose this method to inseminate test rabbits in the capacitation experiments below.

Section II: Capacitation Experiments

Sperm Capacitation after Injection of Human Chorionic Gonadotropin (HCG)

Initially 200 to 240 million sperm were incubated for 10 hours in the uteri of incubator rabbits injected with 0, 50, 75, 100, or 300 IU of HCG at the time of insemination. When test rabbits were inseminated with

TABLE 4.--Fertility of ova recovered from rabbits inseminated with capacitated sperm[a] deposited in the upper oviduct or uterus.

Treatment[b]	Oviducal Insemination		Uterine Insemination	
	Ova[c]	Fertility	Ova[c]	Fertility
	(No.)	(%)	(No.)	(%)
1	2	50	57	30
2	3	100	55	100
3	5	60	19	90
4	26	62	25	56
5	17	47	43	93
Average	10.6		39.8	

[a]Sperm recovered after 10 hours uterine incubation.

[b]Treatments described under LH capacitation experiments below.

[c]Ova flushed from oviducts 36±2 hours after injection of ovulating hormone.

0.5 to 2.5 million sperm per uterine horn, 12.5±0.25 hours after an ovulating injection fertility increased from 54.9% for sperm incubated in control estrous rabbits to 85.8% for sperm incubated in rabbits injected with 75 IU of HCG (Table 5). Then fertility declined significantly ($P<.05$) to 30.5% for sperm incubated in rabbits injected with 300 IU of HCG. Except that fertility from sperm incubated in control rabbits was considerably higher, these results are in agreement with data recently published by Soupart (62). The optimal level of HCG (75IU) and mating both enhance the rate of sperm capacitation (2, 64). The significant reduction in fertility with higher levels of HCG (300 IU) may be due to reduced sperm capacitation. It could also be due to very rapid capacitation, because capacitated sperm are thought to have a shorter fertile life (28, 29). Thus if 300 IU of HCG hastened capacitation, the sperm incubated in these rabbits may have been overcapacitated and therefore infertile (presumably nearer death) by the time they were recovered from the incubator rabbits.

To determine which of these two possibilities (reduced capacitation or overcapacitation) may have caused the low fertility observed when sperm were incubated in the uteri of rabbits receiving 300 IU of HCG, 250 million sperm were incubated for 0, 3.5, or 7 hours in the uteri of rabbits injected with 75 or 300 IU of HCG. Sperm

TABLE 5.--Sperm capacitation during 10 hours incubation after injection of HCG.

Incubator Rabbit Treatment[a]	Test Rabbits	Incubator Rabbits	Ova[b]		Ova Fertilized
			Recovered	Fertilized	
	(No.)				(%)
Estrous rabbit	12	6	153	84	54.9 ± 10.1[c]
50 IU HCG	6	3	121	88	72.7 ± 18.8
75 IU HCG	7	3	99	85	85.8 ± 12.9
100 IU HCG	6	3	104	71	68.3 ± 17.1
300 IU HCG	6	3	131	40	30.5 ± 14.9

[a]Sperm recovered after 10 hours uterine incubation in incubator rabbit and inseminated into test rabbits 12.5 hours after injection of ovulating hormone.

[b]Ova flushed from oviducts 36±2 hours after injection of ovulating hormone.

[c]Mean ± among test rabbit standard error.

incubated in the uteri of estrous rabbits or rabbits receiving 75 IU of HCG for 3.5 hours were at least partially capacitated (55.8 and 44.5% fertility, respectively, Table 6). But sperm incubated for 3.5 hours in rabbits receiving the high level of HCG fertilized only 5% of the ova recovered from test rabbits inseminated with 0.8 to 1.2 million sperm per uterine horn, 13.0 ± 0.25 hours after an ovulating injection. Thus it appears that injection of 300 IU of HCG caused reduced capacitation.

Unfortunately, this conclusion could not be verified from similar comparisons of sperm after 7 hours of incubation because these three treatments resulted in near zero fertility (bottom three lines, Table 6). Only one reason for this low fertility is plausible to me. Three composite semen samples were used to obtain the data in Table 6--one for 0 hour incubator rabbits, one for 3.5 hour incubator rabbits, and one for 7 hour incubator rabbits. In spite of the precautions taken (compositing semen from two or three bucks, discarding semen samples with low motility, and maintaining relatively constant ejaculation frequency), the data in Table 6 suggest that the composite semen sample used for the 7 hour rabbits may have possessed little fertility at the time of ejaculation or the sperm were damaged during processing. Of course, it was still possible that some unknown but "negative" capacitation

TABLE 6.--Sperm capacitation during 0, 3.5, or 7 hours incubation after injection of HCG.

Incubator Rabbit Treatment[a]	Test Rabbits	Incubator Rabbits	Ova[b]		Ova Fertilized
			Recovered	Fertilized	
	----------(No.)----------				(%)
Estrous rabbit - 0 hours	2	1	41	0	0
Estrous rabbit - 3.5 hours	5	2	111	62	55.8
75 IU HCG - 3.5 hours	6	2	200	89	44.5
300 IU HCG - 3.5 hours	5	2	80	4	5.0
Estrous rabbit - 7 hours	5	2	118	1	0.8
75 IU HCG - 7 hours	4	2	56	0	0
300 IU HCG - 7 hours	6	2	109	2	1.8

[a]Sperm recovered after 0, 3.5, or 7 hours uterine incubation in incubator rabbits and inseminated into test rabbits 13.0 hours after injection of ovulating hormone.

[b]Ova flushed from oviducts 36±2 hours after injection of ovulating hormone.

phenomenon was responsible for the observed lack of fertility of sperm incubated for 7 hours.

Consequently, an experiment was designed to test whether the low fertility obtained from 7-hour incubated sperm (Table 6) was due to some obscure capacitation phenomenon. For this purpose, a single composite semen sample was divided among all incubator rabbit treatments (90 million sperm per rabbit) which consisted of uterine incubation of the sperm for 0, 3.5, 7, or 10 hours after the injection of 75 IU of HCG. Test rabbits were inseminated with 0.1 to 1.0 million sperm per uterine horn 13.0 ± 0.25 hours after an ovulating injection. Unfortunately some of the test rabbits did not respond as well as anticipated to superovulation and, consequently, ova numbers were limited and treatment differences were not significant. There was, however, a trend toward higher fertility after 7 and 10 hours incubation (Table 7). Therefore, the lack of fertility of the 7-hour incubated sperm in the previous experiment (Table 6) was probably an inherent property of that semen sample and not due to any "negative" capacitation phenomenon.

Conclusions that can be drawn from these two experiments are that homologous semen samples should be used for all treatments and experiments should be replicated in time.

TABLE 7.--Sperm capacitation during 0, 3.5, 7, or 10 hours incubation after injection of HCG.

Incubator Rabbit Treatment[a]	Test Rabbits	Incubator Rabbits	Ova[b]		Ova Fertilized
			Recovered	Fertilized	
	(No.)				(%)
Estrous rabbit - 0 hours	6	2	92	33	35.9
75 IU HCG - 3.5 hours	7	2	116	48	41.4
75 IU HCG - 7 hours	7	2	74	58	78.4
75 IU HCG - 10 hours	6	2	76	40	52.6

[a]Sperm recovered after 0, 3.5, 7, or 10 hours uterine incubation and inseminated into test rabbits 13.0 hours after injection of ovulating hormone.

[b]Ova flushed from oviducts 36±2 hours after HCG injection.

Sperm Capacitation after Injection of Luteinizing Hormone (LH)

Aside from their biochemistry, biological differences have not been demonstrated between HCG and LH. In fact these two hormones are usually used interchangeably in reproduction research; for example to induce ovulation. Nevertheless, HCG is not the normal gonadotropin to which the rabbit is exposed after mating. Consequently, it was important to determine if LH affected sperm capacitation similarly to HCG (Table 5).

Incubator rabbits were injected with 0, 100, 250, 500, 1000, or 2000 µg of NIH-LH-S1 (as NIH-LH-B5) when 300 to 400 million sperm were deposited in the uterus to determine the influence of LH on sperm capacitation. One IU of HCG possesses luteotropic activity equivalent to 1 µg of NIH-LH-S1 (61). This experiment was performed simultaneously with the comparable experiment on HCG (Table 5) and the control rabbits were identical in these two sets of data. In contrast to the influence of HCG, sperm capacitation was enhanced by injection of all levels of LH relative to the rate of capacitation in control estrous rabbits (Table 8). About 85% of the ova were cleaved when test rabbits were inseminated with 0.3 to 2.5 million sperm per uterine horn which had been incubated in rabbits injected with LH. These inseminations were performed 12.5 ± 0.25 hours after an ovulating injection. There was

TABLE 8.--Sperm capacitation in incubator rabbits after injection of LH.

Incubator Rabbit Treatment[a]	Test Rabbits	Incubator Rabbits	Ova[b]		Ova Fertilized
			Recovered	Fertilized	
	(No.)				(%)
Estrous rabbit	12	6	153	84	54.9 ± 10.1[c]
100 µg LH	6	3	99	83	83.8 ± 10.6
250 µg LH	6	3	78	68	87.2 ± 15.7
500 µg LH	6	3	174	146	83.9 ± 15.2
1000 µg LH	6	3	150	126	84.0 ± 11.0
2000 µg LH	5	3	111	96	86.5 ± 14.7

[a]Sperm recovered after 10 hours uterine incubation in incubator rabbit and inseminated into test rabbits 12.5 hours after injection of ovulating hormone.

[b]Ova flushed from oviducts 36±2 hours after injection of ovulating hormone.

[c]Mean ± among test rabbit standard error.

no indication of diminished fertility with higher levels of LH, although the high level of LH was equivalent in leuteotropic activity to more than 6 times the high level of HCG.

Recently, Soupart (63) reported that high levels of LH injected into hypophysectomized incubator rabbits inhibited sperm capacitation similarly to high levels of HCG in unoperated rabbits. Possibly, hypophysectomized animals respond differently to LH injections than normal animals. But Soupart's data were very limited, so comparisons should be drawn cautiously.

Comparison of sperm capacitation in rabbits injected with varying levels of HCG (Table 5) with capacitation in rabbits injected with varying levels of LH (Table 8) reveals, for the first time, an important difference in biological responses to these gonadotropins. The data in Table 8 suggest that 100 µg of LH is sufficient to evoke maximal capacitation response, that this level of LH is at or beyond the top of the LH dose-capacitation response curve, and that greater quantities of LH have no additional influence on the capacitation response. In contrast, the data in Table 5, which reveal a negative effect of large doses of HCG on capacitation, suggest either that HCG possesses some intrinsic property different from LH or that the preparation we used contained some contaminant

which had no influence on capacitation when injected at low levels but negatively affected capacitation at high levels.

Sperm Capacitation after Injection of 20α-hydroxy-pregn-4-en-3-one (20α-ol)

The results of the above experiments demonstrate that injections of ovulating hormones increase the rate of sperm capacitation in the rabbit uterus, as mating had been reported to do (2, 64). This result indicates that some rapid endocrine changes which normally occur after mating, such as the increased synthesis of 20α-ol by the ovary, also may be initiated by injection of gonadotropins. A preliminary investigation demonstrated that injection of HCG would increase the synthesis of 20α-ol. Since HCG enhances 20α-ol synthesis and sperm capacitation, 20α-ol may be causally associated with capacitation.

In an attempt to simulate the preovulatory blood levels of 20α-ol, incubator rabbits were injected subcutaneously with a total of 10 mg of this progestin in graded doses. The incubator rabbits were ovariectomized at the time of insemination (330 million sperm), to eliminate endogenous 20α-ol and injections at 1-hour intervals of 4, 4, and 2 mg of 20α-ol were begun immediately in one group of rabbits. After 10 hours incubation in the uteri of these rabbits, the sperm were inseminated into test rabbits at 13 ± 0.25 hours after an ovulating injection of HCG. There was no difference in fertility of sperm

recovered from acutely ovariectomized incubator rabbits or rabbits that were acutely ovariectomized and injected with 20α-ol (Table 9). Both treatments resulted in nearly complete sperm capacitation.

In another trial, 250 million sperm were incubated in estrous rabbits, estrous rabbits injected with a total of 10 mg of 20α-ol, or acutely ovariectomized rabbits. Sperm recovered after 10 hours residence in these incubator rabbits were used to inseminate test rabbits 12.25 ± 0.25 hours after ovulating injection of HCG. There was no significant difference in fertility among these three treatments (Table 9).

At first glance, these results suggest that injections of 20α-ol have no influence on sperm capacitation in the uterus. But another possibility was that the test system for degree of capacitation, namely fertility of inseminations into test rabbits at 12 to 13 hours after injections with ovulating hormone, was not sufficiently sensitive to detect small differences in degree of capacitation. One possible means of improving the sensitivity of this test was to inseminate the test rabbits later than had been done (12 to 13 hours after injection of ovulating hormone) in all previous experiments. The basis for this time for inseminations of test rabbits was given under "Time of Insemination of Superovulated Test Rabbits" above.

TABLE 9.--Sperm capacitation in incubator rabbits after injection of 20α-hydroxy-pregn-4-en-3-one (20α-ol).

Incubator Rabbit Treatment[a]	Test Rabbit	Incubator Rabbit	Time of Insemination[b]	Ova[c]		Ova Fertilized
				Recovered	Fertilized	
	(No.)					(%)
Ovariectomized rabbit	6	3	13.0	200	176	88.0
Ovariectomized rabbit and 20α-ol	7	3	13.0	249	217	87.1
Estrous rabbit	8	2	12.25	96	77	80.2
Estrous rabbit and 20α-ol	5	2	12.25	44	37	84.1
Ovariectomized rabbit	8	2	12.25	160	139	86.9
Estrous rabbit	2	1	13.0	8	7	87.5
	2	1	14.0	32	31	96.9
	3	1	15.0	65	32	49.2
Estrous rabbit and 20α-ol	2	1	13.0	19	15	78.9
	2	1	14.0	42	40	95.2
	2	1	15.0	59	42	71.2

[a]Sperm recovered after 10 hours uterine incubation in incubator rabbits.

[b]Hours after injection of ovulating hormone.

[c]Ova flushed from oviducts 36±2 hours after injection of ovulating hormone.

It was based upon data on freshly ejaculated sperm which were relatively infertile when inseminated.

As outlined earlier, freshly ejaculated sperm presumably require about 6 hours for capacitation before they can fertilize ova. Fully capacitated sperm presumably can fertilize ova immediately. But if some treatment resulted in partial capacitation, then the sperm would require something less than 6 hours exposure to the test rabbits reproductive tract before they could fertilize ova. Inseminations of test rabbits at 12 to 13 hours after injections of ovulating hormone would allow at least 2 hours and possibly as much as 5 hours exposure of the sperm to the uteri and oviducts of the test rabbit before the end of the fertile life of the ova--assuming that ovulation occurs at 11 hours after injection of ovulating hormone and that ova are fertilizable for 4 to 6 hours. This reasoning suggested that inseminations of test rabbits at 15 hours (or more) after injections of ovulating hormone would be a more sensitive test for sperm capacitation than inseminations at 12 to 13 hours. This modification would presumably allow less and in some cases no additional time for sperm capacitation in the test rabbits, beyond that they had achieved before insemination into the test rabbits.

The next experiment was designed to examine this modification of the test system. Four hundred million sperm were incubated for 10 hours in estrous rabbits or in

estrous rabbits injected with a total of 10 mg of 20α-ol and 1.6 million sperm recovered from the incubator rabbits were inseminated into each uterine horn of test rabbits at 13, 14, or 15 hours after injections of ovulating hormone.

Although it was not significant, higher fertility was observed when test rabbits were inseminated 13 or 14 hours after an ovulating injection (regardless of sperm treatment) than when rabbits were inseminated 15 hours after an ovulating injection (Table 9). The depression in fertility of inseminations at 15 hours was presumably caused by insufficient time for partially capacitated sperm to become fully capacitated in the test rabbit's tract before the ova were infertile.

Incubation of sperm in uteri of rabbits which had been injected with 20α-ol did not materially alter fertility relative to that of sperm incubated in estrous control rabbits--at least when inseminations of test rabbits were performed at 13 or 14 hours after injection of ovulating hormone. The 20α-ol injections apparently improved fertility of inseminations at 15 hours (49.2% versus 71.2%) but with the limited numbers of ova, this difference was not statistically significant.

These limited observations on time of inseminations of test rabbits and on influence of 20α-ol apparently warrant replication, but the data in Table 9 suggest that

increased 20α-ol secretion may not be responsible for the marked fertility improvement observed after injection of LH or HCG. However, blood levels of 20α-ol after injection of this progestin were not determined and consequently we cannot be certain that it was absorbed in quantities equivalent to those known to be secreted by the ovary after mating.

Section III: Progestins in Ovarian Venous Blood

Samples of ovarian venous blood were collected hourly, after a total of 26 estrous rabbits were injected with 75 or 300 IU of HCG or with 100 or 1000 µg of NIH-LH-S1 (as NIH-LH-B5). Blood was collected from individual rabbits for an average of 3.1 hours with a range of 0.75 to 7.0 hours. Ovarian veins were cannulated just prior to or up to 6 hours after HCG or LH injection. This allowed collection of blood samples from some rabbits beginning before gonadotropin injection and continuing in others until 8 hours after injection. Progesterone and 20α-ol were quantified in each plasma sample.

Secretion of 20α-ol by the ovary was not influenced significantly by level or kind of injected gonadotropin (Tables 10 and 11). Consequently the 20α-ol secretion rate for the 4 treatments are combined and presented with standard errors in Figure 2. Secretion of 20α-ol averaged 30 µg per gram of ovary per hour prior to gonadotropin

TABLE 10.--Secretion of 20α-hydroxy-pregn-4-en-3-one[a] after injection of HCG.

Hours after injection	Level of HCG		
	75 IU	300 IU	Avg
0	--------- 30 ----------		30
1	697	1,072	979
2	1,398	1,231	1,331
3	1,081	764	923
4	789	1,362	1,035
5	803	990	878
6	292	789	541
7	513	292	347
8	-	112	112

[a] µg per gm ovary per hour.

TABLE 11.--Secretion of 20α-hydroxy-pregn-4-en-3-one[a] after injection of LH.

Hours after injection	Level of LH		
	100 µg	1000 µg	Avg
0	---------- 30 ----------		30
1	1,589	588	1,089
2	1,078	2,943	2,321
3	701	1,354	1,093
4	523	1,050	874
5	530	624	590
6	199	946	647
7	247	339	302
8	0	223	149

[a] µg per gm ovary per hour.

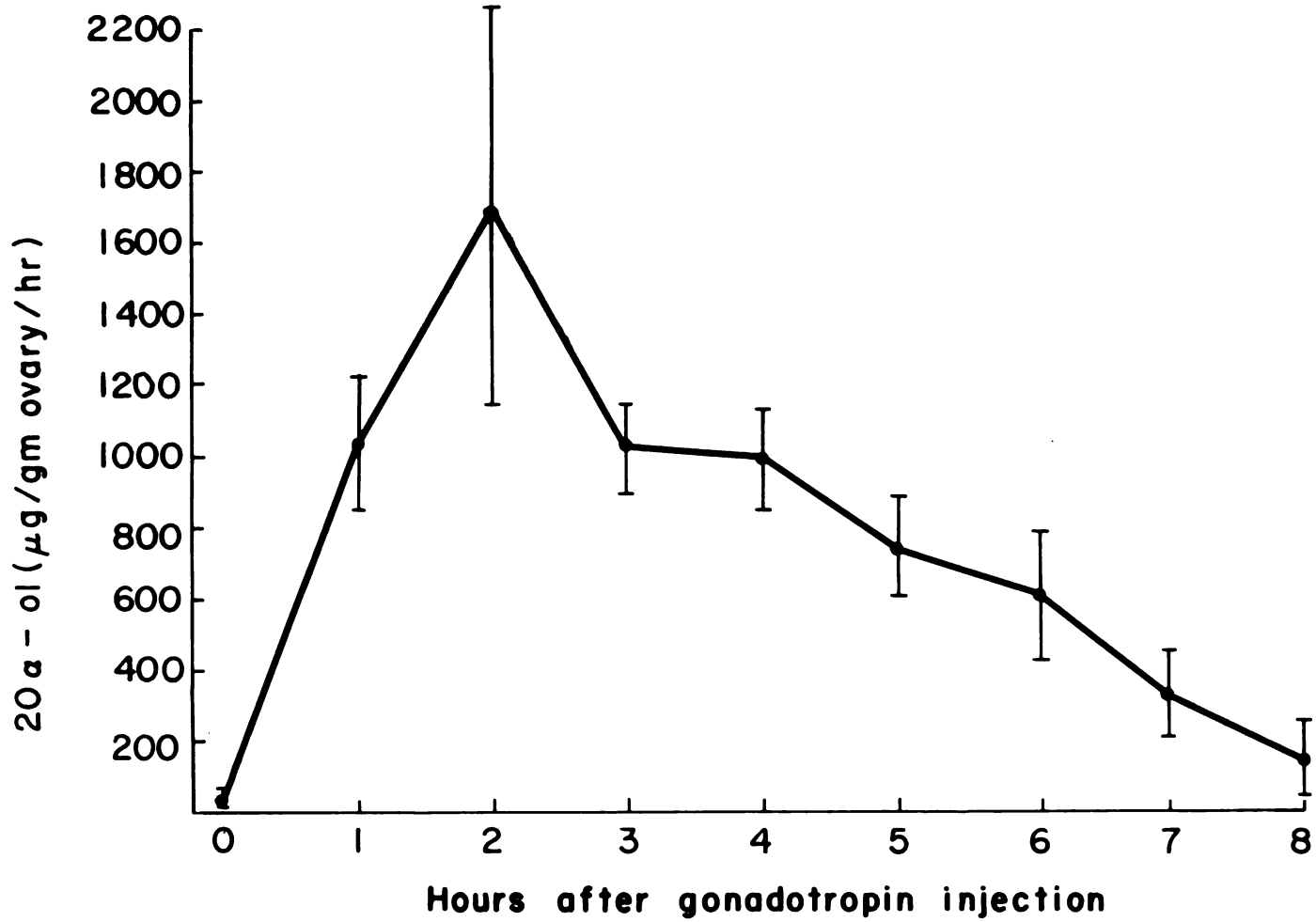

Figure 2.--Average secretion of 20α-hydroxy-pregn-4-en-3-one after injection of gonadotropin.

injection. The maximum rate of secretion was about 1700 µg per gram of ovary per hour and occurred about 2 hours after gonadotropin injection. It returned to pre-injection levels by about 8 hours after gonadotropin injection. This secretion pattern is similar in every respect to that obtained by Hilliard et al. (46) in rabbits after mating. Since there was no difference between level or type of gonadotropin in the rate of 20α-ol secretion, the lowered fertility observed when incubator rabbits were injected with high levels of HCG (Table 5) was not caused by abnormal secretion of 20α-ol. Another conclusion warranted by the data in Tables 10 and 11 is that either there is no dose-response relationship between level of injected gonadotropin and level of secreted 20α-ol, or all levels of injected gonadotropin were beyond the effective dose-response range.

Levels of progesterone in the same samples of ovarian venous blood were only 3 to 4 per cent as large as levels of 20α-ol (Tables 12 and 13). But the secretion pattern of progesterone was similar to that for 20α-ol with the exception that the high levels of gonadotropin tended to result in greater progesterone secretion than low levels of gonadotropin (Figure 3), especially for LH (Table 13). Progesterone was undetectable in the plasma prior to injection of gonadotropin and increased to a maximum of about 50 µg per gram of ovary per hour within 2 hours after the injection

TABLE 12.--Secretion of progesterone[a] after injection of HCG.

Hours after injection	Level of HCG		
	75 IU	300 IU	Avg
0	---------- 0 ----------		0
1	3	70	53
2	64	16	45
3	33	58	49
4	29	92	50
5	42	39	32
6	0	1	1
7	0	0	0
8	-	0	0

[a] µg per gm ovary per hour.

TABLE 13.--Secretion of progesterone[a] after injection of LH.

Hours after injection	Level of LH		
	100 µg	1000 µg	Avg
0	---------- 0 ----------		0
1	0	8	4
2	4	104	79
3	0	114	57
4	2	70	43
5	1	20	12
6	0	32	19
7	1	0	0
8	-	1	1

[a] µg per gm ovary per hour.

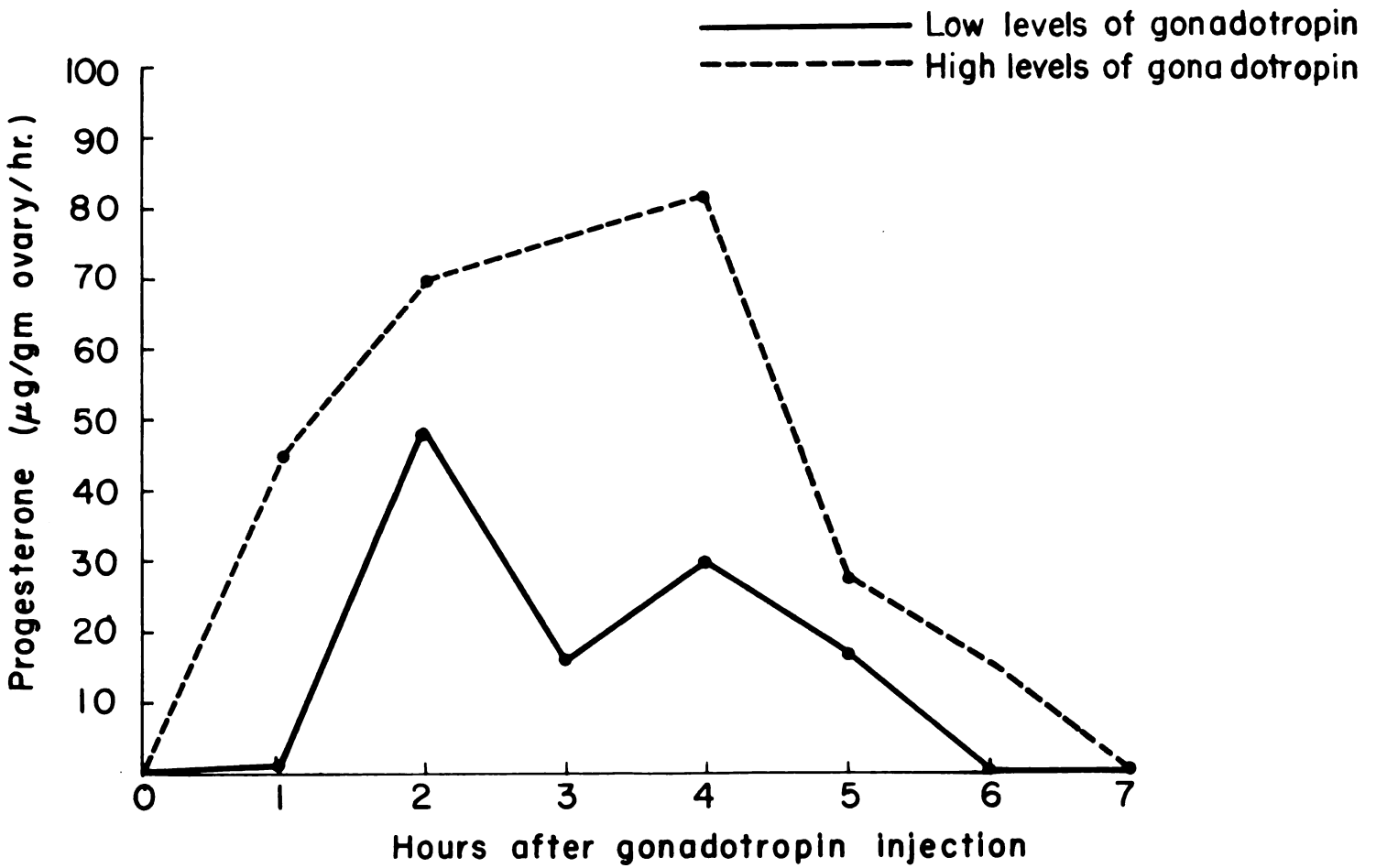

Figure 3.--Average secretion of progesterone after injection of high or low levels of LH or HCG.

of low levels of gonadotropin. High levels of gonadotropin resulted in maximum secretion of about 80 μg per gram of ovary per hour about 4 hours after injection. Basal levels of progesterone secretion were restored by 7 hours after gonadotropin injection. There was a significant difference ($P<0.025$) in the secretion rate of progesterone induced by low levels of LH relative to that for high levels of LH. But this difference had no effect on sperm capacitation (Table 8). And there was no marked difference in progesterone secretion between high levels of LH and high levels of HCG which could be interpreted to cause the difference in sperm capacitation noted earlier (i.e., Table 5 and 8).

A comparison of the data on capacitation, 20α-ol secretion and progesterone secretion is presented in Table 14. Secretion of 20α-ol during the 7 hours immediately after injection of either gonadotropin increased about 25 fold from that for control estrous rabbits. Progesterone secretion also increased dramatically during this period. Since sperm capacitation and progestin secretion were both enhanced by injection of 75 IU of HCG, or by 100 or 1000 μg of LH, these data suggest that 20α-ol and progesterone may be positively associated with sperm capacitation.

When incubator rabbits were injected with 300 IU of HCG sperm capacitation in the uteri was inhibited. This

TABLE 14.--Sperm capacitation and progestin secretion after gonadotropin injection.

Level of gonadotropin	Fertilized ova	Progestin[a] secretion	
		20α-ol	Progesterone
	(%)		
Estrous control	55	210	Not detectable
75 IU HCG	86	5573	171
300 IU HCG	30	6500	276
100 µg NIH-LH	84	4767	7
1000 µg NIH-LH	84	7849	349

[a] µg per gm ovary per 7 hours.

decreased capacitation was not due to increased synthesis of 20α-ol because the rabbits injected with 300 IU of HCG produced an average of 6500 μg of 20α-ol per gram of ovary during the 7 hours--less than the 7849 μg per gram of ovary produced by rabbits injected with 1000 μg of LH, which enhanced capacitation. And more progesterone was secreted by rabbits injected with 1000 μg of LH than by rabbits injected with 300 IU of HCG (349 μg versus 276 μg). Thus neither excessive 20α-ol nor excessive progesterone appeared to inhibit sperm capacitation when rabbits were injected with high levels of HCG.

Since sperm capacitation is not enhanced in ovariectomized rabbits injected with gonadotropins (15, 63), the enhancement of capacitation by gonadotropins is exerted through the ovary. Capacitation results from the 20α-ol injection experiment presented earlier in this thesis indicate that 20α-ol alone may not enhance capacitation and suggest that other ovarian hormones normally may influence capacitation, possibly by acting synergistically with 20α-ol or progesterone. Although chronically ovariectomized rabbits do not regain their full capacitation ability when injected with estrogen (15, 63), this does not eliminate estrogen as an influential hormone because several weeks after ovariectomy, uteri are atrophic and much less vascular.

It appears that an ovarian hormone, possibly estrogen, is secreted in response to injections of HCG or LH. Estrogen may be synergistic with other ovarian hormones or act alone to promote sperm capacitation. When rabbits are injected with high levels of HCG, capacitation could be inhibited in one of two ways. Either excessive amounts of estrogen could be produced or a contaminant of the HCG may inhibit capacitation. Data in this thesis indicate that tests of these hypotheses may shed considerable light on the endocrine mechanism of sperm capacitation.

Section IV: Suggestions for Studying In Vivo Capacitation

Experience in the course of this research has demonstrated that sperm capacitation is both an interesting and a difficult phenomenon to study. In addition to the major results of this thesis, a lesson is that many precautions must be observed to insure that conclusions drawn are valid and not artifacts of techniques used or of uncontrolled variables.

Wherever possible, composite samples of ejaculated sperm should be divided among all treatments and procedures such as incubation periods, storage in a waterbath, and centrifugation should be standardized to insure that inherent or procedural differences among semen samples are not responsible for observed treatment differences. Another technique which appeared quite useful was addition

of antibiotic (streptomycin) to semen before uterine incubation. This addition, it appeared, reduces the inflammatory response in the oviducts of test rabbits, which often hinders the recovery of ova.

The test for degree of sperm capacitation is another variable in the published literature, which must be carefully controlled. If a faulty or weak test system is used, erroneous results and conclusions may be obtained. The alterations on reported methods tested in this research should improve the efficiency of the capacitation assay. Firstly, insemination of test rabbits into the uterine horns adjacent to the tubouterine junction rather than into the fimbriated ends of the oviducts was more convenient, apparently increased recovery of ovulated ova, and resulted in improved fertility. Secondly, timing of insemination of test rabbits is critical, to insure that sperm are not capacitated to any appreciable extent in the test rabbits. Superovulated test rabbits should not be inseminated earlier than 13 hours after the injection of an ovulatory hormone. The precise time to be recommended remains to be determined, but the data in this thesis suggest that 15 hours or more is preferable. Thirdly, a group of rabbits should be inseminated with freshly ejaculated sperm at the time ovulatory hormone is injected (positive control) in every experiment. The ova recovered from these rabbits should be cleaved, because the inseminated sperm have ample

opportunity to become capacitated before the time of ovulation. Another group of rabbits should be inseminated with freshly ejaculated sperm after the time of ovulation (negative control) to determine that sperm cannot be capacitated before the end of the fertile life of the ova.

Lastly, when an experiment is composed of many treatments, it is advisable to perform all treatments on each day and if possible, replicate all treatments on at least two days.

SUMMARY AND CONCLUSIONS

Rabbit sperm were incubated in uteri of rabbits injected with 0, 50, 75, 100, or 300 IU of HCG to determine if HCG alters the ability of the uterus to capacitate sperm. When incubator rabbits were injected with 75 IU of HCG the fertilizing capacity of sperm incubated in their uteri was increased; 86% of the ova recovered from test rabbits inseminated with these sperm were cleaved, compared to 55% from test rabbits inseminated with sperm incubated in non-injected estrous rabbits. Thus, this level of HCG mimics the effect of mating on sperm capacitation in the rabbit uterus. But when incubator rabbits were injected with 300 IU of HCG at the time sperm were surgically deposited in the uteri, only 41 of 131 ova recovered from the test rabbits inseminated with these sperm were cleaved. Another trial demonstrated that low fertility observed when incubator rabbits were injected with high levels of HCG was caused by failure of sperm capacitation.

In contrast to the influence of HCG, sperm capacitation was enhanced by injection of low or high levels of LH. About 85% of the ova recovered from test rabbits were cleaved when the test rabbits were inseminated with sperm

incubated in uteri of rabbits injected with 100, 250, 500, 1000, or 2000 μg of NIH-LH.

Since mating and injection of gonadotropin enhance capacitation and both also cause increased synthesis of 20α-ol, incubator rabbits were injected with 10 mg of 20α-ol at the time sperm were deposited in the uteri, to determine if 20α-ol enhances sperm capacitation. When test rabbits were inseminated at 13 or 14 hours after an injection of ovulating hormone with sperm incubated in these rabbits, no differences in fertility could be observed. In all treatments fertility was very high, suggesting that test rabbits were inseminated too soon after ovulation and that the test system was not sensitive enough to detect small differences in fertility. In a modified test system, rabbits were inseminated with sperm incubated in control or 20α-ol injected incubator rabbits at 15 hours after an injection of ovulating hormone. Based upon limited observations, there was slightly (21%) but not significantly higher fertility with sperm from 20α-ol injected rabbits. The numbers of ova in this experiment should be increased to be more certain whether or not 20α-ol influences sperm capacitation.

Progesterone and 20α-ol were quantified in ovarian venous blood after injection of gonadotropins to determine if subsequent changes in progestin secretion parallel changes in sperm capacitation. Secretion of 20α-ol by the

ovary was stimulated when rabbits were injected with 75 or 300 IU of HCG, or with 100 or 1000 µg of LH. But there was no difference in 20α-ol secretion due to kind or level of injected gonadotropin. Thus, the inhibition of capacitation observed when incubator rabbits were injected with 300 IU of HCG is not caused by abnormal 20α-ol secretion. This does not eliminate the possibility that 20α-ol may be stimulatory to capacitation, because blood levels of 20α-ol were not monitored in the injected rabbits.

The secretion pattern of progesterone was similar to that for 20α-ol in the same samples of ovarian venous blood, but there was a gonadotropin dose effect on progesterone secretion. Rabbits injected with 75 IU of HCG secreted 171 µg of progesterone during the first 7 hours after injection and rabbits injected with 300 IU of HCG secreted 276 µg of progesterone. Injection of 100 and 1000 µg of LH caused secretion of 7 and 349 µg of progesterone, respectively. Since rabbits injected with 1000 µg of LH secreted more progesterone than rabbits given 300 IU of HCG and 1000 µg of LH enhanced capacitation, progesterone is not the inhibitor of capacitation when rabbits are injected with 300 IU of HCG.

To my knowledge gonadotropins exert their influence only through the gonads. Since 20α-ol and progesterone are not responsible for the reduction in sperm capacitation after injection of 300 IU of HCG, another ovarian hormone is probably involved.

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