FOLLICULAR DEVELOPMENT IN RABBITS IMMUNIZED TO TESTOSTERONE

FOLLICULAR DEVELOPMENT IN RABBITS AFTER ACTIVE IMMUNIZATION TO TESTOSTERONE

By

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ABSTRACT

The role of testosterone in follicular development and ovulation has been investigated by immunizing female rabbits to testosterone-3-bovine serum albumin (T-3-BSA). The intent of this procedure was to render any endogenously produced testosterone inactive by means of its high affinity binding to specific antibody.

In order to determine the time course of the effects of this procedure three groups of 12 - 6 control, 6 experimental - immaturely immunized rabbits were sacrificed at 5, 8 and 11 weeks after immunization. The effects observed at 11 weeks were compared with a group of 10 - 6 control, 4 experimental - maturely immunized rabbits sacrificed after the same length of immunization. In addition a group of 11 - 5 control, 6 experimental - immaturely immunized animals were mated to a proven male 14 weeks after immunization.

Animals were boosted regularly and the antiserum titer followed weekly. Blood samples were taken weekly for the determination of FSH and LH levels and the total and percent bound testosterone and estradiol. After sacrifice the ovaries and uteri were removed for histology. Follicular development was examined and the maximum follicular diameter $\geq$ 1.0 mm and $\geq$ 1.5 mm was recorded. In mated animals the number of corpora lutea per ovary were counted.

Significant antiserum titers to testosterone were

observed in all experimental rabbits by 5 weeks of immunization. Over the 14 weeks of the experiment values ranged from 1:700 to 1:52,000.

In control rabbits serum testosterone values were less than 0.5 ng/ml while values in experimental rabbits rose steadily over the length of the experiment (group values ranged from 0.6 ± 0.1 ng/ml to 2.1 ± 0.9 ng/ml, 2.2 ± 0.7 ng/ml to 5.7 ± 0.8ng/ml and 4.4 ± 1.2 ng/ml to 6.8 ± 1.2 ng/ml at 5,8 and 11 weeks respectively). Testosterone binding in control animals remained in the 93% to 95% range throughout the experiment while in experimental animals testosterone binding increased significantly to approximately 99% at 5 weeks of immunization and remained constant thereafter.

Estradiol values in control rabbits remained relatively constant throughout the ewperiment (group range : 50.7 ± 5.4 pg/ml to 81.8 ± 4.9 pg/ml). A consistent and significant increase in estradiol occurred in experimental animals (group values ranged from 69.5 ± 15.4 pg/ml to 88.5 ± 8.7 pg/ml, 74.5 ± 9.8 pg/ml to 118.5 ± 13.1 pg/ml and 81.6 ± 6.9 pg/ml to 157 ± 30.3 pg/ml at 5,8 and 11 weeks after immunization). Prior to immunization estradiol binding in experimental rabbits was not different from control values (range : 83% to 88%). There was a significant increase in estradiol binding by 5 weeks of immunization in the experimental groups (range: 89.4% to 94.5%). By 11 weeks estradiol binding increased to as much as 97.9%.

Follicular development was abnormal in T-3-BSA immunized rabbits. The ovaries contained numerous large cystic and

hemorrhagic follicles. At 8 weeks experimental ovaries contained more follicles ≥ 1.0 mm (27.2 ± 3.1 versus 15.3 ± 2.9, $p < 0.01$) and ≥ 1.5 mm (5.83 ± 1.7 versus 0.42 ± 0.2, $p < 0.005$). At 11 weeks there were more follicles ≥ 1.5 mm in both immature (5.0 ± 0.9 versus 0.92 ± 0.31, $p < 0.001$) and mature (7.25 ± 0.92 versus 4.17 ± 0.74, $p < 0.05$) experimental ovaries. Increased vascularization, some thecal cell hypertrophy, and marked interstitial cell hypertrophy were characteristic of the experimental ovaries. There was also a significant increase in the number of ovulations in the experimental rabbits (8.0 ± 2.1 versus 4.4 ± 1.9, $p < 0.005$).

Immunization of female rabbits to testosterone is a useful tool for the study of hormone interactions in the regulation of follicular development and ovulation. In addition it may serve as a useful model for the study of the processes involved in the development of cystic ovaries.

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ABBREVIATIONS AND TRIVIAL NAMES

The following abbreviations and trivial names were used in this thesis:

BSA	Bovine serum albumin.
T-[illegible]-BSA	Testosterone-3-oxime-bovine serum albumin.
Testosterone	17 hydroxyandrost-4-en-3-one.
[illegible]	Tetra-1,3,5(10)-triene-3,17 -diol.
DES	Diethylstilbestrol.
LH	Luteinizing hormone.
hLH	Human luteinizing hormone.
FSH	Follicle stimulating hormone.
hFSH	Human follicle stimulating hormone.
PMSG	Pregnant mares serum gonadotrophin.
PMS	Pregnant mares serum.
IFR	Immature female rats.
HIFR	Hypophysectomized immature female rats.
ml, µl	Milliliter, microliter.
g,mg,ng,pg	Gram, milli-, nano-, picogram.
°C	Degrees centegrade.
N	Normal.
PBS	Phosphate buffered saline.
EDTA	Ethylenediamine-tetracetic acid.
cpm	Counts per minute.
mCi	Millicurie.
MCR	Metabolic clearance rate.

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INTRODUCTION

The development of a model which accurately reflects female reproductive function is an extremely difficult and to date mostly ineffective exercise. The difficulty lies in unravelling an apparently complex time-dependent interaction among a number of hormones whose responsibility is the regulation of reproductive function. However, despite this complexity there have been some very interesting discoveries in this field which are bound to lead to integrative theories of how this process works.

In past years perhaps the most researched field within reproduction has been concerned with the feedback relationships between the reproductive hormones; eg. the negative feedback effect of sex steroids on gonadotrophin release. The discovery of the neuroendocrine control of the pituitary and its importance in reproductive function has also been extensively studied. Recently, greater emphasis has been placed on intra-ovarian mechanism of control and the molecular events involved; that is the synergistic relationship between hormones which elevates the one hormone one action concept to a level of multiple hormone interaction.

This thesis is concerned with the role of one hormone (testosterone) and its interaction with estradiol and the gonadotrophins in the regulation of follicular development, atresia and ovulation. This traditionally male hormone has been found to be intimately involved in the regulation of the female reproductive system. The introduction to this thesis will attempt to put current knowledge concerning follicular dynamics in perspective while emphasizing the role which androgens may play in this process.

STRUCTURE OF THE FOLLICLE - THE FUNCTIONAL UNIT

Understanding the structure of the ovary is a necessary prerequisite to any attempt at determining the processes involved in follicular development, ovulation and atresia. It is through this structure that the regulatory hormones exert their action, both by alterations in the structure itself and in the interaction among cell types. The most productive way to view follicular development is to consider the follicle as a fuctional unit consisting of the following components (Figure 1): (1) oocyte, (2) antrum, (3) membrana granulosa, (4) basement membrane, (5) theca interna, (6) theca externa, and (7) the vascular and lymph network. The influence of the gonadotrophins, steroids, and other substances on these components and the resulting interaction among them ultimately determines to what stage a follicle will grow and whether it will go on to ovulate or become atretic.

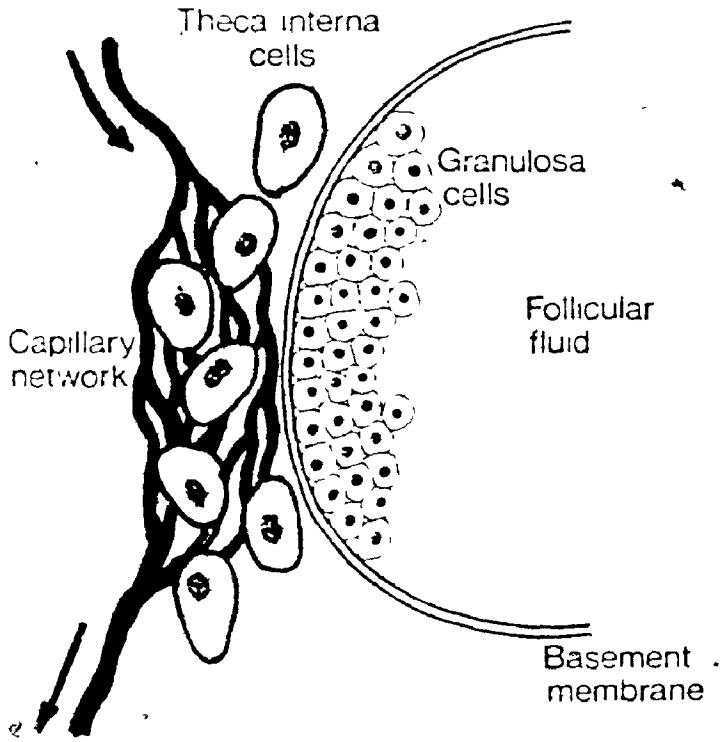


FIGURE 3 : The structure of a portion of an antral follicle wall showing the blood supply and the primary cell types.

Each follicle may be assigned to one of three developmental stages (Lipner, 1973): (1) primordial follicle - lying close to the surface epithelium of the ovary, composed of an oocyte and a single layer of granulosa cells surrounded by a few fibroblasts and lacking an independent blood supply, (2) primary follicle - containing several layers of granulosa cells, a well defined basement membrane and theca interna, and an independent blood supply, (3) secondary follicle - characterized by the formation of an antrum and an increase in density of the inner vascular wreath. If the follicle is proceeding to ovulation it is designated as preovulatory and is characterized by a rapid increase in size associated with hyperemia, granulosa cell proliferation, thecal hypertrophy and vascular engorgement (Lipner, 1973; Bjersing et al., 1974 a-e). Alternatively the follicle may undergo atresia at any stage of development. This atresia is characterized by degenerative changes in the granulosa cells and ovum, ultimately leading to resorption of the follicle (Asami, 1920).

VASCULAR SUPPLY.

For some time now the unique nature of the vascular supply to the ovary has been known (see Burr et al., 1951). Only recently though has there been a concerted effort to determine the role of the vascular supply in ovarian function. In fact there is now considerable evidence indicating that

the vascular network surrounding the follicle is an important mediator of its function.

Burr et al. (1951) provided a detailed description of the vascular network of the ovary; the essential details of which will be reviewed here. Most notably each growing follicle receives its own independent blood supply. An outer vascular wreath found within the theca externa of each follicle is derived from coiled arteries found in the medulla. The vessels of this wreath are characterized by their large lumen and usually single layer of muscle cells. These vessels give rise to an inner capillary wreath lying against the basement membrane of the follicle. Venous drainage is by means of large veins - the vessels of which are composed of a single layer of endothelial cells - forming a similar outer vascular wreath.

Looking at the vascular structure of the follicle it is very clear that this structure must play a functional role in the regulation of follicular development and ovulation. In fact follicular fluid pressure has been found to be intimately related to capillary pressure of the follicle. Blandau et al. (1963) found that in the rat the two pressures were equal and that this relationship did not change during ovulation. Espey et al. (1963) found that a concurrent change in intrafollicular pressure occurred when peripheral

arterial pressure was altered. These and other studies have led to the generally accepted theory that follicular fluid is a transudate of the blood and that the rapid increase in follicular fluid as ovulation approaches is related to vascular changes in pressure and permeability (Burr et al., 1951; Reynolds, 1973).

It remains to be defined precisely what control mechanisms are involved in the regulation of these vascular changes. There is considerable evidence to suggest that steroids are intimately connected with this process. Wolff et al. (1967) found that various steroids were able to change the capillary structure of the vagina in different ways; estrogen inducing formation of pores in subepithelial vaginal capillaries, and progesterone protecting the endothelium against the effects of estrogen. Microvascular changes in pre-ovulatory follicles show a similar pattern; the vessels become increasingly engorged and increasingly permeable (associated with an increase in the number of micropores) while concurrent to this the follicles rapidly increase in size (Bjersing et al., 1975 a). Reynolds (1973) suggests that the mechanism of this hyperemia may be attributed to local release of histamine-like substances and that estrogen is capable of mobilizing histamine in tissues by accumulation of eosinophilic leucocytes. Bjersing et al. (1974 e) have demonstrated that the increase in number of micropores

in the rabbit follicle occurs about 4 hours after an ovulating dose of hCG and that this may be causally or casually related to the peak level of one or more of testosterone, estrogen and progesterone which occurs about 3 hours post-hCG.

Not only must vascular permeability be considered in the process of follicular development but also alterations in blood flow have been found to be very important to ovarian function. Moor et al. (1975 b) have studied intraovarian blood flow in sheep in the preovulatory period and found that, while stromal flow remained constant, there was a tenfold decrease in luteal flow and a significant increase in follicular flow. Also LH, but not FSH, resulted in a 25 - 50% increase in ovarian blood flow within 30 minutes and a 60% increase in follicular flow within twenty minutes. Niswender et al. (1976) also demonstrated a clear association between LH and blood flow in the ewe and suggest that alteration of blood flow by LH may be extremely important to ovarian function.

There appear to be no studies which look at vascular development and its control in relation to growing and non-ovulatory follicles. Nor is it known what relationship vascular changes have to follicular atresia. Certainly regulation of vascular function must be considered an important component of any model of ovarian function.

GONADOTROPHINS AND THECAL-GRANULOSA INTERACTIONS.

Before looking at the regulation of follicular development it is necessary to briefly summarize what is known about the control and production of steroids by the various ovarian compartments; considerable controversy has existed concerning the source and control of these hormones. For some time it was thought that the theca interna was responsible for estrogen and luteinized granulosa cells for progesterone production.

Progesterone secretion has been observed in both thecal and granulosa compartments. For example progesterone secretion has been demonstrated when rabbit thecal cells were incubated in medium (YoungLai, 1972 b) and also when rat preantral granulosa cells were incubated with testosterone (Lucky et al., 1977).

Interestingly, the ovarian follicle has been found to produce considerable quantities of androgens. Incubation with LH, in vitro, of rabbit follicles (YoungLai, 1974 , 1975) or pre-ovulatory hamster (Makris et al., 1975) and bovine (Fortune et al., 1977 a) thecal cells resulted in high androgen production. Similarly, Louvet et al. (1975) have provided in vivo evidence for ovarian androgen stimulation by LH and hCG (but not FSH) in hypophysectomized immature female rats (HIFR).

Recently, some clarification of the site and control of estrogen production has been provided. Moon et al. (1975), incubating HIFR ovaries in organ culture, found that in the presence of testosterone, highly purified FSH caused a marked increase in estradiol secretion; up to 900% of that observed for FSH alone. This suggested that testosterone was being used as a substrate for FSH controlled aromatization to estrogen. Dorrington et al. (1975) then cultured isolated granulosa cells from HIFR and found that: (1) estradiol was secreted in the presence of highly purified FSH and testosterone, (2) that alone neither FSH nor testosterone was effective, and (3) highly purified LH did not stimulate estradiol secretion. Armstrong et al. (1976 a) have provided in vivo confirmation of these observations by administering highly purified FSH to HIFR with or without testosterone or dihydrotestosterone (DHT; non-aromatizable androgen). They found a marked increase in ovarian estradiol when FSH and testosterone, but not FSH alone or FSH plus DHT, were administered. Highly purified LH increased ovarian testosterone, but did not change estradiol content.

Thus a "two-cell-type two-gonadotrophin control of estrogen synthesis" (Fortune et al., 1977 b; Armstrong et al., 1976 b) has been proposed. Thecal cells, under the control of LH produce testosterone which is then transported to the granulosa cells where, under the control of FSH, aromatization

to estrogen occurs. This theory is supported by observations of similar relationships in the hamster (Makris et al., 1975), cow (Fortune et al., 1977 a), sheep (Baird, 1977), and human ovaries (Batta et al., 1977; Fowler et al., 1977).

FOLLICULAR DEVELOPMENT

There is presently considerable disagreement concerning the mechanism(s) controlling the initiation and development of follicles. Despite this uncertainty there is little doubt that follicular development is a continuous process requiring a defined amount of time (Schwartz et.al., 1972) for complete maturation to occur. Peters (1976) has suggested that "follicle initiation and development is a continuum on which the surges of FSH and LH are superimposed to the cyclic event of ovulation."

Early developmental events in the follicle include growth of the oocyte with active RNA synthesis, formation of the zona pellucida from granulosa cells, multiplication of the granulosa cells, organization and differentiation of the theca and vascularization (Peters, 1976). Identifiable thecal cells are already present in follicles with a growing oocyte and only one layer of granulosa cells; by the early pre-antral stage the theca is well defined (Peters, 1976).

Peters has presented evidence which suggests that

neither gonadotrophins nor estrogens are required for the initiation of follicular growth. For example administration of exogenous gonadotrophin or estradiol benzoate to 3 day old mice failed to increase the number of follicles in the beginning stages of development (Peters, 1976). Baker et al. (1976), however, suggest that there may be a critical period in the mouse, which occurs around the time of birth, when gonadotrophins are required to initiate follicular growth. They found that if, prior to birth, hamster ovaries are explanted and maintained in organ culture the oocyte will undergo meiosis to the diplotene stage but no follicular growth occurs. However, if the explants are treated with FSH and LH follicular growth occurs (Baker et al., 1973, 1976).

The number of follicles which begin to develop appears to be related to both the total number of primordial follicles (Krarup et al., 1969) and to some 'factor' released by large antral follicles (Peters, 1976). If testosterone propionate is given to five day old mice there is a 50% reduction in the number of primordial follicles which causes an immediate decrease in the number of follicles which start to grow (Peters, 1976). Also, between two and three weeks of age there is a rapid reduction in the number of follicles which begin to grow. This is associated with the occurrence of large antral follicles since if fluid from these follicles is injected into

neonatal mice growth initiation is markedly reduced in the first week (Peters et al., 1973).

Gonadotrophins were also thought not to be involved in pre-antral follicular growth, however, recent evidence disputes this theory. Gonadotrophin receptors have been found in 5-15 day old rats (Peluso et al., 1976) and administration of testosterone in five day old rats depresses gonadotrophin levels and results in a decrease in the number of pre-antral follicles (Uilenbroek et al., 1976). Eshkol et al. (1971/72) using antisera to gonadotrophins, demonstrated that gonadotrophins were required in the pre-antral period. Besides abnormalities in granulosa cell organization and basement membrane integrity they found that the vascular system of immunized mice developed abnormally and that thecal cells were poorly differentiated. Because the rat anti-gonadotrophin antiserum did not cross-react with hFSH or hLH they were able to reconstitute gonadotrophin control. They found that while FSH restored follicular growth the thecal deficiency and proper vascularization were only restored with both FSH and LH. Purandare et al. (1976) also administered antisera to FSH and LH to 5 day old mice and found that this treatment severely inhibited follicular development and antrum formation at 14 days of age. He also suggests that LH, more than FSH, is responsible for development of thecal cells and the vascular system.

Before further discussing the role of gonadotrophins in the regulation of follicular development it is necessary to place the function of estrogens and androgens in this process in some perspective. In order to show that steroids have a role in follicular development it is necessary to establish that follicular cells are capable of responding, presumably by possessing specific intra-cellular receptors, to their presence.

There is substantial evidence that granulosa cells possess receptors for estradiol. Autoradiography of the ovary has identified granulosa cells as a target of estradiol (Stumpf, 1969). Richards (1975) has demonstrated specific receptors for estrogen in the nuclei of granulosa cells isolated from ovaries of HIFR. The presence of estrogen receptors is in accord with observations that administration of estrogen in vivo stimulates granulosa cell proliferation and prevents atresia in HIFR (Payne et al., 1958; Harman et al., 1975). Richards et al. (1976a) also showed that in HIFR an increase in nuclear estrogen receptor content was associated with follicular growth while a decrease was indicative of no growth, or of luteinization.

Equally as interesting as the discovery of estradiol receptors in granulosa cells, and of particular interest to this thesis, is the recent discovery of the nuclear translo-

cation of testosterone in estrogen primed HIFR (Schreiber et al., 1976c) and the identification of a specific testosterone binding protein (receptor?) in the cytosol of these animals (Schreiber et al., 1976 a,b). In vivo evidence suggests that testosterone may be involved in the control of follicular development. It is known that estrogen given to HIFR causes an increase in ovarian weight; due largely to hyperplasia of granulosa cells (Payne et al., 1958). Louvet et al. (1975) have demonstrated that if small amounts of hCG, but not FSH, are given to estrogen primed HIFR there is a decrease in ovarian weight which is reversible in a dose dependent manner if an anti-androgen (either antiserum to testosterone or cyproterone) is administered at the same time. Associated with the prevention of the estrogen-induced ovarian weight gain is a decrease in binding of ^{3}H-testosterone in granulosa cells and a concurrent increase in the concentration of ovarian androgens (Schreiber et al., 1976 a).

It is worth noting at this time that increasing evidence points to an interrelated synergism - involving LH regulation of testosterone production in thecal cells and FSH regulation of estradiol production in granulosa cells - operating within the follicular unit to control the processes of follicular development. It is the involvement of testosterone and the role of the theca in this process which is now finally coming under critical investigation.

There are numerous studies demonstrating that gonadotrophins are required for antral formation and subsequent follicular maturation. For example, it is known that if immature female rats are given an injection of pregnant mares serum gonadotrophin (PMSG) pre-ovulatory follicles develop within 48 hours and that this is associated with an increase in the number of FSH receptors found in granulosa cells (Peluso et al., 1977). Peters et al.(1975) reports that if PMSG is given to 21 day old mice (a time when follicle atresia is at a maximum) there is no increase in the number of large follicles, but there is a very definite increase in the number of healthy versus atretic follicles. Also if antisera to FSH is given to a pro-oestrus hamster the subsequent ovulation is prevented - even if exogenous LH is given - due presumably to the absence of large ovulable follicles (Rani et al., 1977). As the follicle matures there is also an increase in granulosa LH receptors (Kammerman et al., 1975) which appear to be related to the follicle's ability to ovulate and luteinize (Richards, 1976 a). Subsequently ovulatory doses of LH (and FSH) induce pre-ovulatory follicles to luteinize which is accompanied by a decrease in LH and FSH and an increase in prolactin receptors (Rao et al., 1977).

Richards and her collaborators are doing some very exciting work concerned with the interrelationships between estradiol, FSH and LH in follicular development (see Richards

et al., 1976a for an excellent review). They have found that in HIFR, under the influence of estradiol (E_2) granulosa cells go through successive changes which are characterized by specific responses to FSH. Initially estradiol acts synergistically with FSH to increase and maintain FSH receptors while at a later period estradiol promotes the ability of FSH to increase LH receptors (Richards et al., 1976 b). In contrast if LH is administered to estradiol primed HIFR there is a marked decrease in FSH, LH and estradiol receptors (Richards, 1975; Richards et al., 1976 b).

The complexity of this system is clearly illustrated by the presence of apparently three different types of hormone regulated controls of receptor content in the differentiation of granulosa cells: "autoregulation, a process by which hormones (E_2, FSH, LH) affect the content (increase or decrease) of their own receptors; coordinated regulation, a process by which steroid (E_2) and a protein hormone (FSH) interact to affect the content of receptor for the same or a different protein hormone (LH); and heteroregulation, a process by which one hormone (LH) affects the content of receptor for an entirely different hormone or hormones (FSH, E_2) " (Richards et al., 1976 b).

There would appear to be a series of interconnected time-dependent events, apparently related to the development

of mechanisms for estrogen synthesis (Richards et al., 1976 a) which determine whether the follicle will respond appropriately to the gonadotrophin surge and subsequently ovulate (Lunenfeld et al., 1976). A major deficiency at this time is knowledge concerning the role of thecal cells and the testosterone produced by them in this process. While it is known that LH or hCG bind to thecal cells there is no information concerning the control of these receptors. Richards et al. (1976 a) point out that it is very possible that local inductive factors interact between granulosa and thecal cells to control follicular development.

OVULATION

In determining the role of steroids in ovulation it is necessary to distinguish between extra-ovarian (e.g. increasing serum estradiol leading to gonadotrophin surge) and intra-ovarian effects (e.g. involvement in the physical and biochemical changes within the follicle leading to ovulation).

While there is still some question concerning the requirement of FSH in the ovulatory process there is no doubt that a LH surge is required for ovulation to occur. Ovulation is prevented if antiserum to LH is given to female rats prior to the expected surge of LH (Laurence et al.,1971/72; Schwartz et al., 1975).

In addition, the LH surge, and therefore ovulation, is blocked if antiserum to estradiol is administered to spontaneous ovulators such as the primate (Sundaram et al., 1973; Ferin et al., 1969), ewe (Caldwell et al., 1970) and rat (Neill et al., 1971). That the extra-ovarian effect of estradiol is responsible for the LH surge in these animals is demonstrated by the observation that administration of diethylstilbestrol (DES) to estradiol immunized animals restores the LH surge and subsequent ovulation (Ferin et al., 1969; Caldwell et al., 1970; Neill et al., 1971). In the hen administration of antisera to testosterone and progesterone but not estradiol, will block ovulation; presumably by preventing their induction of the LH surge (Furr et al., 1975). In induced ovulators, such as the rabbit, the situation is somewhat different. Under normal conditions, while estrogen is required to make the animals receptive, the actual ovulatory surge of LH is produced by the mating stimulus (Schwartz et al., 1972).

A theory connecting intra-ovarian steroids with the complex morphological and biochemical changes of the follicle in the ovulatory process has been proposed (Bjersing et al., 1975 b; Wu et al., 1977). The theory is based on the observation of marked differences between intra-follicular and peripheral concentrations of these steroids. For example, at 3 hours after mating in the rabbit, follicular fluid estradiol

reaches a concentration of about 270 pg/ml while peripheral values change very little after mating; remaining at about 40pg/ml (YoungLai, 1972 a). Rabbit follicular fluid (YoungLai, 1972 a) and ovarian vein (Hilliard et al., 1971) steroids increase dramatically by 2-3 hours after mating and then decline to very low levels prior to ovulation. Mills et al. (1973), studying in vitro steroidogenesis from ^{14}C-acetate in the rabbit follicle at various times after mating found a similar follicular response. All three steroids, progesterone, estrogen and androgen, appear to follow a similar pattern. Having established the presence of these hormones it remains to determine what function they serve in ovulation and whether or not this function differs from their role in follicular development.

Evidence that steroids are in fact involved in intra-ovarian aspects of ovulation was obtained by Lipner and Greep (1971). Using PMS primed immature female rats (IFR) administration of LH to these animals could be blocked by inhibitors of steroidogenesis. They suggested that it was actually the LH induced steroidogenesis which was, in turn, responsible for ovulation. These results were supported and extended by Takahashi et al.(1974) who found that progesterone was capable of restoring ovulation in rats hypophysectomized at 1550 hours on the day of pro-estrus. Very recent evidence suggests that in fact androgens may be intimately involved in this

process. Mori et al. (1977), using the same animal model as Lipner and Greep (PMS-IFR-hCG) found that antiserum to testosterone, given at the same time as the hCG, was able to drastically reduce the number of observed ovulations. They also found that, while antiserum to progesterone was able to prevent the ovulation normally occurring after hCG, if testosterone or DHT was given in addition ovulation was restored.

There is considerable evidence, both direct and indirect, suggesting specific functions for steroids in inducing ovulation. Lipner (1973) has summarized the evidence implicating progesterone in the initiation of an increased ovarian blood flow and hyperemia; probably the result of histamine release from local mast cells. Prostaglandins are known to be required in the ovulatory process and there is some evidence that steroids may be involved in the regulation of their synthesis (Le Maire et al., 1975). Espey (1971) suggests that the decomposition of the dense connective tissue in the theca externa and tunica albuginea is the result of steroid stimulation of fibroblasts to secrete proteolytic enzymes. Steroids may also increase synthesis of lytic enzymes (Bjersing et al., 1974 b) and induce a dose dependent labilization of lysosomal membranes with resulting enzyme leakage. Also, estrogen and progestogen may inhibit collagenase activity (Bjersing et al., 1974 d). Bjersing suggests that the fall in steroids in the last few hours before ovu-

lation might result in increased collagenase activity and help to effect follicle rupture. Finally, estrogen is capable of restoring the number of annular nexuses in immature female rabbits; the number of annular nexuses reach a peak at 8 hours after hCG and then decrease; estrogen follows a similar but earlier pattern (Bjersing et al., 1974 c).

There is also evidence to suggest that androgens may modulate progesterone production. Schomberg et al. (1976) using a monolayer culture of porcine granulosa cells found that androgens stimulated progesterone production. This observation was supported by similar results obtained by Lucky et al. (1977) using cultured granulosa cells from HIFR. This relationship is not clear however as Erickson et al. (1975) did not find increased progesterone production when rabbit follicles were treated with androgen.

It is clear then that steroids have a major, if not principal role in the intra-ovarian changes occurring after the LH surge and leading to ovulation. Exactly what steroids are involved in which processes is not established; however, all three classes of ovarian hormones (estrogen, progestogen, and androgens) have been implicated. Le Maire et al. (1975) make the important point that a dual function may exist for steroids; "first, the initial increase of steroidogenesis, triggered by LH in the follicle, may be responsible for the

initiation of further biochemical processes leading to ovulation and, second, the withdrawal of steroids as ovulation approaches may also play a role in the ovulatory process".

ATRESIA

Atresia of follicles is the single most common event in the ovary; follicles which begin to develop must either subsequently ovulate or undergo atresia. The term atresia is used to denote all processes by which an ovarian follicle loses its integrity and is disposed of (Byskov, 1976). It is important to realize that the mechanism controlling atresia may be very different according to the developmental stage of the follicle. For example around the time of birth many small follicles become atretic by extrusion through the surface epithelium of the ovary (Peters, 1969).

Morphologically atresia has been characterized according to the relative size (small, medium or large) of the follicle and differentiated according to the particular compartment that atretic processes are first observed. In small follicles the oocyte is usually the first to show degenerative changes (pyknosis, increasing density of zona and invasion of oocyte by granulosa cells) followed by atretic changes in the granulosa (Asami, 1920; Franchi et al., 1962). In medium and large follicles atresia usually occurs first in granulosa cells (pyknosis, lipid

deposits, decrease in mitotic figures) and soon after in the oocyte and thecal layers (Asami, 1920; Byskov, 1976). While medium sized follicles become atretic without luteinizing many large follicles do go through stages of luteinization (Byskov, 1976). Medium and large follicles undergoing atresia have also been known to complete the first meitotic division with extrusion of the first polar body (Zamboni, 1972; Thibault et al., 1975). Eventually the atretic follicles collapse and migration of connective tissue cells and capillaries from the theca occur; at which time the tissue is considered to be interstitial (Asami, 1920).

Atresia appears to involve the interaction of components of the whole follicular unit. This conclusion is derived from the observation that only 5% of the total number of follicles in the rat ovary which show the beginnings of atresia are found in small follicles having a poorly differentiated theca (Byskov, 1976). Similarly, in the rabbit the greatest number of atretic follicles is in the 400-500 μm range, a size at which the theca is well developed (Nicosia et al., 1975). Also in the human the rate of atresia increases as follicle size increases (Himelstein-Braw et al., 1976).

That gonadotrophins and steroids are involved in the control of follicular atresia is clear; the mechanism of their involvement is another matter. The fate of a

follicle appears to rest on the temporal interaction between the gonadotrophins and the estrogen - androgen balance within the developing follicle.

Early work by Payne and Runser (1958) demonstrated that androgens were capable of antagonizing the well known affect of estrogen on ovarian weight gain and thereby increase the incidence of atresia. Interestingly, administration of hCG (probably acting on thecal cells) had the same effect as testosterone (Payne et al., 1958; Harman et al., 1975). As mentioned earlier antiserum to testosterone inhibits the reduction in ovarian weight gain in HIFR given estrogen plus hCG (Louvet et al., 1975).

Receptor studies lend further support to the theory of gonadotrophin-steroid control of atresia. Richards et al. (1976 b) has shown that follicular atresia is associated with a concurrent decrease in receptor for the gonadotrophins and estradiol in rat granulosa cells. If LH is given to estradiol or FSH primed HIFR their follicles will undergo atresia (Richards et al., 1976 b). In the estrogen-primed HIFR LH induces a marked reduction in granulosa estradiol receptor (Richards, 1975). Since the number of LH receptors on granulosa cells are very low with this treatment Richards suggests that LH may stimulate production of a product from thecal cells (androgens ?) which may mediate the initiation of

atresia and result in the reduction of estradiol receptors. In the presence of estradiol and FSH, LH will cause luteinization of the follicle (Richards et al., 1976 a).

Follicular atresia appears, therefore, to be dependent on a balance between androgens and estrogens - increasing androgens resulting in atresia while increasing estrogen resulting in follicular growth - this balance being dependent on the follicles' developmental competence to be responsive to the synergistic interaction of FSH and estrogen on follicular development (Richards et al., 1976 a; Lunenfeld et al., 1976). This synergism may at the same time be dependent on the development of the "competence" of granulosa cells to aromatize androgens to estrogen. Recent evidence indicates that a non-aromatizable testosterone metabolite, DHT, can inhibit aromatase (Armstrong et al., 1976 a) and that this may be responsible for the atresia of non-competent follicles.

REPRODUCTION IN THE FEMALE RABBIT

The research for this thesis has used the rabbit as a model for follicular development and ovulation. There are some specific features of the rabbit reproductive system which will be briefly reviewed as they will be important to the interpretation of the results to be presented.

The rabbit is an induced ovulator; as such it effec-

tively remains in continuous estrus until it is mated (Eckstein et al., 1960). The mating stimulus triggers an almost immediate release of LH (Dufy-Barbe et al., 1973; Hilliard et al., 1975) which will stimulate 'mature' follicles to ovulate approximately ten hours later. While this series of events is generally true it must be noted that ovulation does not always follow mating while at other times it appears to occur simply after sexual excitement without coitus (Eckstein et al., 1960). Laboratory rabbits, while showing some seasonal variation, will usually mate at all times of the year (Eckstein et al., 1960).

Oogenesis in the rabbit is somewhat unique in that meiosis does not begin until after birth and is not completed until the end of the third week of life (Teplitz et al., 1963). By the eighth week ovaries contain an abundance of small and medium sized follicles, some of which are atretic (Asami, 1920). As would be expected follicles do not survive indefinitely in the estrous rabbit and appear to have follicular maturation cycles of 7-10 days (Hill et al., 1933). Hammond (1925) has noted that follicular development in ovaries of the same animal often vary considerably; he attributes this to a disproportionate blood supply to the ovaries.

Atresia in the rabbit is morphologically similar to that described earlier. In large and medium sized follicles

atretic changes are first noted in the granulosa cells (e.g. fatty degeneration, pyknosis) eventually leading to invasion of the granulosa by connective tissue cells and capillaries from the theca and then changes in the oocyte (Asami, 1920). In small follicles atresia appears first in the oocyte and then in the granulosa and thecal cells (Asami, 1920). Most follicles appear to develop to medium size, with formation of the antrum before undergoing atresia; the greatest number of atretic follicles were found in the 400 μm to 500 μm diameter size (Nicosia et al., 1975). Hammond (1925) has described the presence of hemorrhagic follicles in some ovaries and suggests that "with these blood follicles the preliminary process of ovulation has taken place, but the follicle being 'over mature', and possibly being in an early stage of absorption, rupture does not occur and the blood vessels of the theca break down".

PURPOSE OF THIS RESEARCH

This introduction has attempted to indicate the degree of complexity we are dealing with in the study of ovarian function and to put in some perspective the unique role that steroids, and in particular androgens, appear to have in this process. Until recently very little has been known about the functional significance of androgens in the female reproductive system. It is clear

however, that as our knowledge increases in this regard, we are finding that androgens are indeed an integral part of the control mechanisms of ovarian function.

Research to be presented in this thesis has been concerned with the role of androgens in the reproductive function of the female rabbit. There are two reasons for choosing to study the rabbit: first, the rabbit follicle is known to produce very large amounts of androgens (Hilliard et al., 1974; YoungLai, 1975), and second, the rabbit is an induced ovulator and therefore a clearer distinction may be made between intra-ovarian and extra-ovarian effects of androgens.

The approach taken in this thesis has been to study the development of reproductive function in the absence of functional testosterone. This has been accomplished by the active immunization of the animals to testosterone. As indicated in the introduction this procedure has been used extensively for gonadotrophins and a number of steroids and is based on the theory that the binding of endogenously produced testosterone to specific antibodies will reduce the free biologically active fraction of the hormone thereby rendering it unavailable to target tissue (Nieschlag et al., 1974 a).

Broadly speaking, our hypothesis is that testosterone is required for regulation of normal ovarian function. Therefore immunization to testosterone will disrupt reproductive function in the rabbit. More specifically we have been interested in answering the following questions:

1. Does immunization to testosterone affect follicular development in immature virgin rabbits and if so when are the changes first observed?
2. What effect does immunization to testosterone have on the total and percentage bound plasma testosterone and estradiol?
3. Is the effect of immunization to testosterone different when animals are immunized as immature or mature animals?
4. Upon reaching maturity will immaturely immunized rabbits mate and if so ovulate?
5. If the answer to '4' is positive is there any observable differences in ovulation between immunized and control animals?
6. What effect does immunization to testosterone have on the uterus?

EXPERIMENTAL DESIGN, MATERIALS AND METHODS

EXPERIMENTAL DESIGN

As stated in the introduction this project has used immunization to testosterone as the mechanism for neutralizing the endogenously produced hormone. Control animals were immunized with bovine serum albumin (BSA) while experimental animals received testosterone-3-oxime-BSA (T-3-BSA).

In order to determine the time course of the effects of this procedure three groups of 12 - 6 control,6 experimental - (referred in text as group 1,2 or 3) immaturely immunized rabbits were sacrificed at 5,8, and 11 weeks after immunization. The effects observed at 11 weeks were compared with a group of 10 - 6 control, 4 experimental - maturely immunized rabbits sacrificed after the same length of immunization. In addition a group of 11 - 5 control, 6 experimental - immaturely immunized animals were mated to a proven stud at 14 weeks after immunization.

Animals were boosted regularly and the antiserum titer followed weekly. Blood samples were taken weekly for the determination of FSH and LH levels and the total and percent bound testosterone and estradiol. At sacrifice the ovaries and uteri were removed, photographed and fixed

for histology.

SYNTHESIS OF TESTOSTERONE-3-OXIME BOVINE SERUM ALBUMIN

A non lyophylized solution of testosterone-3-oxime-bovine serum albumin (T-3-BSA) (Figure 2) was synthesized according to the methods of Erlanger et al. (1957, 1959) and Lieberman et al. (1959) as detailed below.

A. Testosterone-3-(o-carboxymethyl)oxime

A solution of 5.0 gm (17.4 mmoles) of testosterone and 5.0 gm (39.3 mmoles) of (o-carboxymethyl)-hydroxylamine in 250 ml of ethanol and 21 ml of 2N sodium hydroxide (NaOH) was heated at a reflux for 3 hours. The solution was then reduced to a small volume in a vacuum and then diluted with 3 times its volume of water. The pH was then adjusted to 8 with the addition of dilute NaOH. Unreacted testosterone was removed by diethylether extraction and the aqueous layer acidified with concentrated hydrochloric acid. The testosterone-oxime was then extracted into ethyl acetate and allowed to dry over anhydrous sodium sulfate overnight. The ethyl acetate was then removed in a vacuum leaving a crystalline residue.

The residue was recrystallized by adding small portions of warm ethyl acetate while stirring and heating in a water bath. This was continued until all of the residue

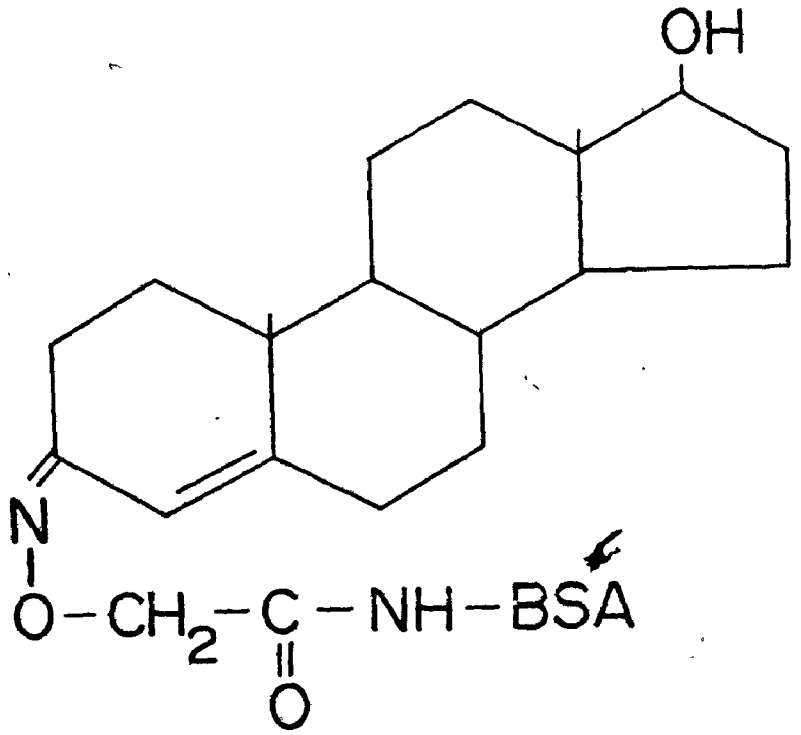


FIGURE 2 : Molecule of testosterone-3-oxime-bovine serum albumin. Attachment of testosterone to BSA renders the hapten antigenic.

was in solution and the solution was clear. While still warm the solution was filtered and then allowed to sit undisturbed until it reached room temperature. It was then left at 4°C overnight to allow further precipitation. The solution was then poured into a drying cup and washed with cold ethyl acetate. When dry the white powdery crystals were weighed and found to be 3.7 g or a yield of 74%. The melting point was 179-183°C which was in agreement with that of Erlanger et al. (1959).

B. Testosterone-3-oxime-bovine serum albumin

To 30 ml of dioxane 1.1 gm (3.05 mmoles) of testosterone-3-oxime and 0.75 ml (0.6 g, 3.05 mmoles) of tris-n-butylamine was added and allowed to cool to 10°C. To this solution 0.4 ml (0.45 g, 3.05 mmoles) of isobutyl chlorocarbonate was added and the reaction allowed to proceed for 20 minutes at 4°C.

The above mixture was then added, in one portion, to a well stirred and cooled solution of 4.2 gm (0.06 mmole) of BSA in 220 ml of 1:1 water:dioxane and 4.2 ml of NaOH. Gas evolution was observed. After 1 hour 2 ml of NaOH was added. The solution was then stirred and cooled for 4 hours. The solution was dialyzed against running water for 18 hours and then brought to pH 4.5 with HCl.

The solution, estimated to contain approximately 4 gm of T-3-BSA, was diluted to 100 μg/ml in normal saline and stored at - 40°C until required for immunization.

BOVINE SERUM ALBUMIN

Bovine serum albumin (BSA) was diluted in normal saline to 100μ g/ml and then stored at - 40°C until required for immunization.

ANIMALS, IMMUNIZATION AND BLOOD COLLECTION

The 47 immature female New Zealand White rabbits were obtained at the same time from a local breeder. They were approximately 8 weeks old and had a mean and standard error weight of 1.4 ± 0.2 g . The 10 mature, non virgin, females were obtained from the above breeder. Their mean and standard error weight was 3.3 ± 0.7g. The animals were housed individually and kept on a 12/12 hour light-dark cycle. Food and water were available ad libitum.

In order to equalize body weights between and within groups the rabbits were divided into four weight classes and then randomly assigned to the four groups. There was no significant difference in body weight between groups.

Each rabbit was immunized with either 1 ml of BSA (controls) or 1 ml of T-3-BSA (experimentals) emulsified

in 1 ml of Freund's complete adjuvant. Five innoculation sites were used : 1 subcutaneously in the dorsal neck area and 4 intra-muscularly; one in each of the gluteus muscles and one in the distal end of each tricept. Booster injections (1 ml) of either BSA or T-3-BSA were given the third week after immunization and at successive two week intervals.

Each week blood (3 ml) was collected from the median ear artery, stored on ice while allowing it to clot, and then spun at 3,000 rpm for 15 minutes. The plasma was removed and stored at -20°C. Samples from all animals within the same group were collected on the morning of the same day.

ANTISERUM SPECIFITY AND MONITORING

The cross reactivity of the antiserum was determined by construction of a dose response curve for testosterone and for the steroids under investigation in doses ranging from 10 pg to 100 ng according to the method of Abraham (1975). A constant amount of ^{3}H-testosterone (5,000 cpm) was added to all incubations. Bound and free steroid were separated by dextran-coated charcoal. The percent cross-reactivity was determined by calculating the amount of testosterone required to displace 50% of the radioactivity divided by the amount of cross-reacting steroid required, multiplied by 100. Table 1 gives the antiserum cross-reactivity for a representative animal.

STEROID	% CROSS REACTIVITY
TESTOSTERONE	100
DIHYDROTESTOSTERONE	55
ANDROSTENEDIONE	0.6
DEHYDROEPIANDROSTERONE	< 0.1
ESTRADIOL - 17β	< 0.1
PROGESTERONE	< 0.1

TABLE 1 : Cross reaction of steroids with antiserum to testosterone cross reactivity is defined as : A/B x 100 where A is the mass of testosterone (pg) required to displace 50% of ^{3}H-testosterone bound to A 1:10,000 dilution of antiserum , B is the mass of steroid (pg) required to displace 50% of ^{3}H-testosterone under the same conditions as A.

Antibody production was monitored weekly according to the method of Nieschlag et al.(1973). Serial dilutions of serum were made and duplicate 100 µl samples were incubated for 2 hours at 4°C with 100 µl of ^{3}H-testosterone (10,000 cpm). The titer was defined as the final serum dilution which bound 50% of the ^{3}H-testosterone.

UNMATED ANIMALS

On the day prior to sacrifice the animals were weighed and the final blood sample taken. An independent person then assigned each rabbit a coded number which was used as identification for all subsequent procedures. Animals within a group were randomly selected and sacrificed with a single injection of sodium pentobarbital.

The ovaries, uterine tubes and uterus were immediately removed and placed in a container on ice. Within 30 minutes the ovaries and uterus were cleared of extraneous tissue, weighed to the nearest 0.1 mg, photographed and fixed in Davidson's fixative.

MATED ANIMALS

Each female animal was placed in the cage of a proven male and observed for successful mating over a 10 minutes period. For those animals in which mating occurred a 3 ml blood sample was taken at 1,2,4,6 and

24 hours after the first mating. At the end of the 24 hours the animals were sacrificed and the same procedures performed as in the non-mated groups except that the number of ovulation sites were recorded. Also the uterine tubes were tied at one end and the interior flushed with 3 ml of normal saline. Using a disecting microscope the number of zygotes present were recorded.

Those animals which did not mate on the first attempt were given three more chances over a two day period. Those which mated were handled in the same manner as above while those which did not were sacrificed at the end of the fourth attempted mating.

BOUND TESTOSTERONE AND ESTRADIOL

The percentage of bound testosterone and estradiol was determined by equilibrium dialysis according to the methods of Forest et al. (1968) and Clarke et al. (1971). 1 ml of plasma (1:5 dilution with normal saline) was placed in a cellophane dialyzing tube (7" long by ¼" diameter). The tube was secured with double knots and then placed in a 25 ml erlenmyer flask containing approximately 10,000 cpm of ^{3}H-testosterone or ^{3}H-estradiol in 10 ml of normal saline. The flask was covered and incubated at 37°C for 18 hours in a shaking Dubnoff water bath.

Duplicate 200 µl samples of the inside and outside solution were added to 1 ml of NCS tissue solubilizer, mixed and allowed to sit for 30 minutes. 10 ml of scintillation cocktail was added and the samples counted for 5 minutes.

The percentage of hormone bound was calculated as:

$$\% \text{ bound} = 1 - \frac{(\text{c.p.m. outside-background})}{(\text{c.p.m. inside-background})}$$

RADIOIMMUNOASSAYS

Total serum testosterone was measured by radioimmunoassay (RIA). Due to the poor extractability of testosterone from serum containing antibody to this hormone 100 mg of guanidine-HCL was added to 100 µl of serum (Csapo et al., 1975). This was allowed to sit for 30 minutes prior to double extraction with diethylether. There was a 90% recovery with this procedure without any effect on the RIA. The dried sample was dissolved in 100 µl of Buffer A, incubated for 30 minutes at room temperature with 100 µl of antiserum to testosterone, and then incubated for 2 hours at $4^{o}C$ with 100 µl of ^{3}H-testosterone (10,000 cpm) in Buffer A. Dextran-coated charcoal was used to separate bound from free hormone. The assay had a sensitivity of 40 pg. See Table 2 for quality control values.

Total serum estradiol was also measured by RIA.

The sample (200 µl) was extracted with 3 ml of diethyl-ether, evaporated to dryness and then dissolved in 100 µl of Buffer A. It was then allowed to sit for 30 minutes at room temperature after the addition of 100 µl of highly specific antiserum prepared against estradiol-17β-O-carboxymethyloxime-BSA (Wright et al., 1973) and then incubated at 4°C for 1 hour with 100 µl of estradiol 6,7-^{3}H (10,000 cpm) before separating bound from free hormone with dextran-coated charcoal. The assay had a sensitivity of 3.0 pg. Quality control values are given in Table 2.

Serum LH was measured using a heterologous double antibody radioimmunoassay (Moor et al., 1975 a). Highly purified ovine LH (LER 1056 C2, from Dr. L.E. Reichert, Jr.) was used for iodination. An impure rabbit pituitary LH-FSH-TSH preparation (WP360A rabbit LH-FSH-TSH, from Dr. A.F. Parlow, NIAMDD) was used as standard. The standards ranged from 0.6 ng to 60 ng. Antiserum to rabbit LH was raised in the guinea pig and the precipitating antibody was goat anti-guinea pig gamma globulin. Duplicate 100 µl samples were incubated at 5°C with 50 µl or antiserum (1:45,000 dilution in 2% normal guinea pig serum-PBS-0.05 MEDTA), 50 µl tracer (10,000 cpm in 2% BSA-PBS) added the following day, and 100 µl of precipitating antibody (1:15 dilution in PBS) added on the fourth day. On the following day the tubes were centrifuged at 3000 rpm, the supernatant aspirated, and the

precipitate counted. The sensitivity of the assay was 1 ng.

Serum FSH was measured using a radioimmunoassay kit supplied by Dr. A.F. Parlow (NIAMDD). Rabbit FSH (AFP-538-C) was used for iodination and preparation of standards. The standards ranged from 0.05 ng to 5 ng. Guinea pig anti-rabbit FSH antiserum (AFP-27-1) was used at a final dilution of 1:300,000 and precipitated with goat anti-rabbit gamma globulin. The assay procedure was essentially identical to that used for LH except that 50 µl of serum was used. The sensitivity of the assay was about 0.3 ng.

TISSUE PREPARATION AND HISTOLOGY

After fixation for 24 hours in Davidson's fixative tissue was stored in 70% alcohol. The tissue was cleared by immersion in successive concentrations of alcohol, then terpineol, and finally terpineol-wax. It was then embedded in wax and 5 micron sections cut. The complete ovary of each animal was sectioned but only every fifth section was retained for histology. One slide of continuous sections was made from each uterus. All slides were stained with hematoxylin and eosin.

ANALYSIS OF TISSUE

Using a calibrated microfilm viewer maximum follicular diameters (measured from basement membrane) were deter-

mined and follicles then assigned to one of five classes: 1.0 to 1.5 mm, 1.5 to 2.0 mm, 2.0 to 2.5 mm 2.5 to 3 mm and greater than 3 mm. General qualitative histological features were also noted; including presence of hemorrhagic follicles, basement membrane integrity, number and health of granulosa cells, nature of theca interna and interstitial cells and the vascular supply to follicles. In mated animals the number of corpora lutea were counted.

Uterine sections were analyzed for qualitative changes in surface epithelium, endometrium and myometrium.

QUALITY CONTROL AND STATISTICAL ANALYSIS

Serum from experimental animals was pooled and used for quality control in assays for total and percent bound testosterone and estradiol. The mean, standard error and coefficient of variation of anlysis of these samples is listed in Table 2.

In most cases data are expressed as the mean and standard error of the mean. For tests of significance"t" values were obtained using the paired"t"test for comparison of values at different sampling times within the same animal and the unpaired"t"test for comparison of control versus experimental values within a sampling period (Dixon et al., 1969). The acceptable level of significance was taken as 0.05.

	WITHIN ASSAY			BETWEEN ASSAYS		
	MEAN	S.E.	C.V.	MEAN	S.E.	C.V.
TESTOSTERONE n = 10,10	4.0	0.1	4.1	3.7	0.2	14.2
ESTRADIOL n = 10,7	88.5	3.0	10.6	79.3	5.4	17.6
% BOUND TESTOSTERONE n = 7,8	99.2	0.1	0.2	99.1	0.1	0.2
% BOUND ESTRADIOL n = 13,12	96.2	0.2	0.2	95.3	0.2	0.9

TABLE 2 : Quality control values for pooled serum from experimental animals.

S.E., plus or minus the standard error of the mean; C.V., coefficient of variation (%); n, the number of sample for within and between assays.

MATERIALS

For the synthesis of T-3-BSA the following reagents were used : (0-carboxymethyl) hydroxylamine from Aldrich Chem. Co., Milwaukee, Wis.; bovine serum albumin (BSA) from Sigma Chem., St. Louis, Mo.; dioxane, tris-n-butylamine, and iso-butyl-chlorocarbonate from B.D.H. Chemicals, Toronto, Ont.; all other chemicals were reagent grade.

Freund's complete adjuvant was obtained from Grand Island Biological Co., Grand Island, New York.

The rabbits were sacrificed with 3 ml Sodium Pentobarcitol injection (65 mg/ml) obtained from MTC Pharmaceuticals, Hamilton, Ont. Davidson's fixative consisted of formalin, 95% alcohol, glacial acetic acid and distilled water in the ratio of 2:3:.5:1:3.5. Alpha terpineol was obtained from Fisher Scientific Co., Fair Lawn, N.J. while haematoxylin and eosin were purchased from BDH Chemicals, Toronto.

The following steroids were purchased from Steroids Inc., Whinton, N.H. : testosterone (17β hydroxyandrost-4-en-3-one); estradiol (estra-1,3,5(10)-triene-3,17β-diol); dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); androstenedione (androst-4-ene-3,17-dione); progesterone (pregn-4-ene-3,20-dione); dehydroepiandrosterone (3β-hydroxyandrost-

5-en-17-one).

Testosterone [1,2,6,7-^{3}H(N)](1.0mCi) and estradiol, [6,7-^{3}H(n)](0.25 mCi) were obtained from New England Nuclear, Boston, Mass.

NCS tissue solubilizer and the PPO (2,5-diphenyloxazole) used to make the scintillation cocktail (5 g/l toluene) were obtained from Amersham/Searle Corp., Don Mills, Ontario.

Testosterone antiserum for the RIA was obtained from one of the immunized animals. A dilution of antiserum which bound 50% of 10,000 cpm of ^{3}H-testosterone was used.

Guanidine-HCl was purchased from Sigma Chem. Co., St. Louis, M.O. Buffer A consisted of 2 g each of sodium azide and gelatin, 18 g. of sodium chloride, 32.7 of $Na_2HPO_4.7H_2O$ and 10.76 g. of $NaH_2PO_4.H_2O$ made up to 2 liters with distilled water and adjusted to pH 6.8.

Dextran-coated charcoal consisted of 1.25 g. of charcoal, 0.125g of dextran T-70 (purchased from Fisher Scientific Co. Fair Lawn, N.J.) in 500 ml of Buffer A.

RESULTS

ANTIBODY PRODUCTION

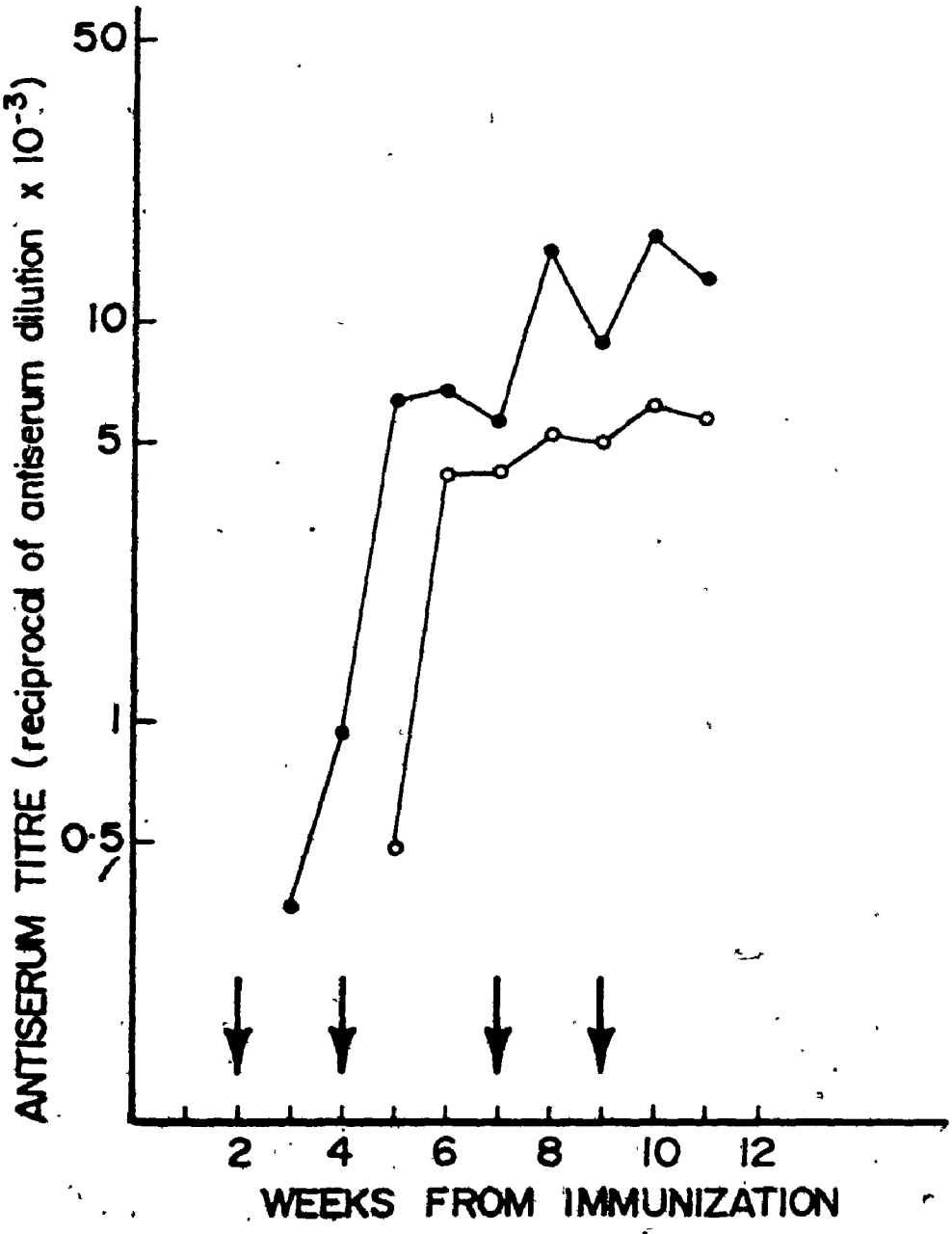
Antiserum titers to testosterone were observed in all rabbits immunized to T-3-BSA. Figure 3 illustrates a typical antibody response in one immaturely and one maturely immunized rabbit. In the immature animal the titer was detectable by the third week, increased sharply up to about the 5th week and then, apparently in response to booster injections, rose more slowly up to the end of the sampling period. In the mature animal a significant titer was not obtained until the 5th week, however, from then on the response of the mature animal followed a similar pattern to that of the immature animal.

While the pattern of response of each animal was similar there was considerable variability in the final titer attained. Table 3 provides the median and range of the titers for each group of animals. It can be seen that the highest titers appear at about the 8th week of immunization, however, the range is considerable in all groups at all times. BSA-immunized control animals had no detectable titer at any time during the study.

UNMATED: IMMUNIZED AS IMMATURE ANIMALS

Total Androgen and Percentage of Bound Testosterone

FIGURE 3 : Representive time course of antiserum titer in rabbits immunized to testosterone-3-BSA as immature (●) or mature (o) animals. ↓ signifies time of booster injection.


ANTISERUM TITRE (reciprocal of antiserum dilution x 10⁻³)
50
10
5
1
0·5
2
4
6
8
10
12
WEEKS FROM IMMUNIZATION

GROUP	WEEKS FROM IMMUNIZATION			
	5	8	11	14
1 IMMATURE n = 6	7,700 3,200-10,800	------	------	------
2 IMMATURE n = 6	4,000 3,000-14,000	20,600 14,200-52,000	------	------
3 IMMATURE n = 6	1,470 700- 4,500	8,800 3,400-18,000	9,100 3,900-16,200	------
4 IMMATURE n = 5	4,280 1,660- 6,400	10,750 3,080-15,000	7,500 2,350-12,800	7,700 2,400-23,000
5 MATURE n = 4	3,800 900-10,800	4,350 2,750- 7,800	5,300 3,200- 8,000	------

TABLE 3 : Antiserum titer (median and range for each group) in rabbits immunized to testosterone-3-BSA as immature or mature animals. Booster injections were given at 3,5,7,9,11 and 13 weeks.

Results to be presented in this and the following section may be found in Figures 4 - 6 inclusive. Unless otherwise indicated levels of significance are between consecutive sampling periods within control or experimental animals. Values represent the mean and standard error of the mean.

In BSA-immunized controls serum androgen was never greater than about 0.5 ng/ml and with the exception of group 1, did not differ significantly during the course of the experiment. Although group 1 controls showed a significant rise at 5 weeks the mean did not exceed 0.5 ng/ml. The mean percentage of bound ^{3}H-testosterone in these animals ranges from 92.2± 0.4 % to 95.4 ± 0.2 %. There was no significant change in the binding at any interval in any group.

Prior to immunization with T-3-BSA the experimental animals showed no significant difference from control animals either in total androgen or the percentage of bound testosterone. Within 5 weeks of immunization to T-3-BSA a significant ($p < 0.05$) rise in serum androgens was noted for all groups; values being 1.81 ± 0.48 ng/ml, 2.14 ± 0.87 ng/ml, and 0.63 ± 0.13 ng/ml in groups 1,2 and 3 respectively. These values continued to rise significantly at each sampling interval. While total androgen did not generally exceed 10 ng/ml two animals in group 2 were found to have extremely high (54.9 ng/ml and 28.4 ng/ml) serum androgen levels. Also by

FIGURE 4 : Histograms (mean ± S.E) showing total serum androgen (A) and estrogen (E) and percent bound ^{3}H-testosterone (T-B) and ^{3}H-estradiol-17 β (E_2-B) in group 1 rabbits prior to immunization (pre-immun.) and 5 weeks after immunization to BSA (□ , control, n=6) or T-3-BSA (▨ or ■ , experimental, n=6).

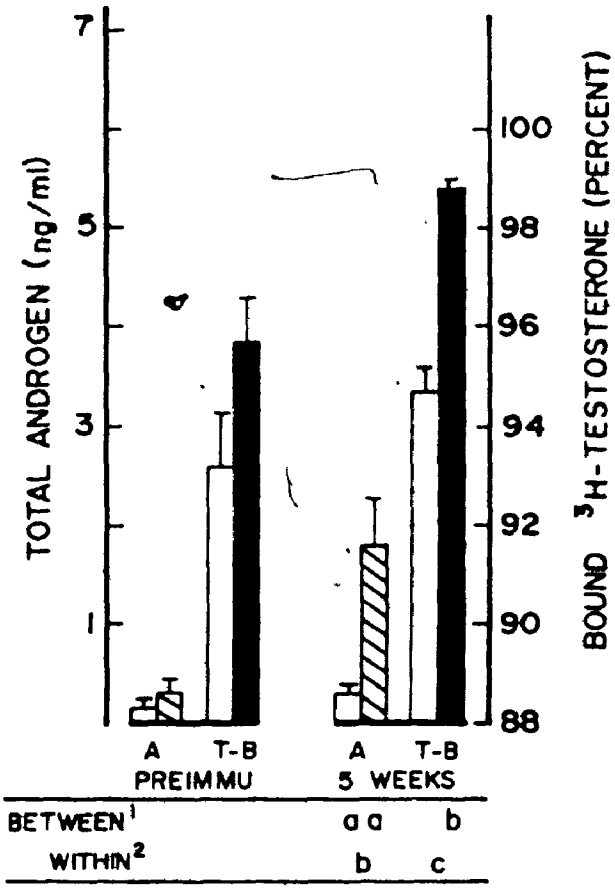
1. Comparison of control or experimental values between consecutive weeks. Statistics by paired "t" test.

2. Comparison of control to experimental values within a week. Statistics by unpaired "t" test.

a : $p < 0.05$.

b : $p < 0.01$.

c : $p < 0.001$.


TOTAL ANDROGEN (ng/ml)
7
5
3
1
BOUND ³H-TESTOSTERONE (PERCENT)
100
98
96
94
92
90
88
A
T-B
A
T-B
PREIMMU
5 WEEKS
BETWEEN¹
a a
b
WITHIN²
b
c

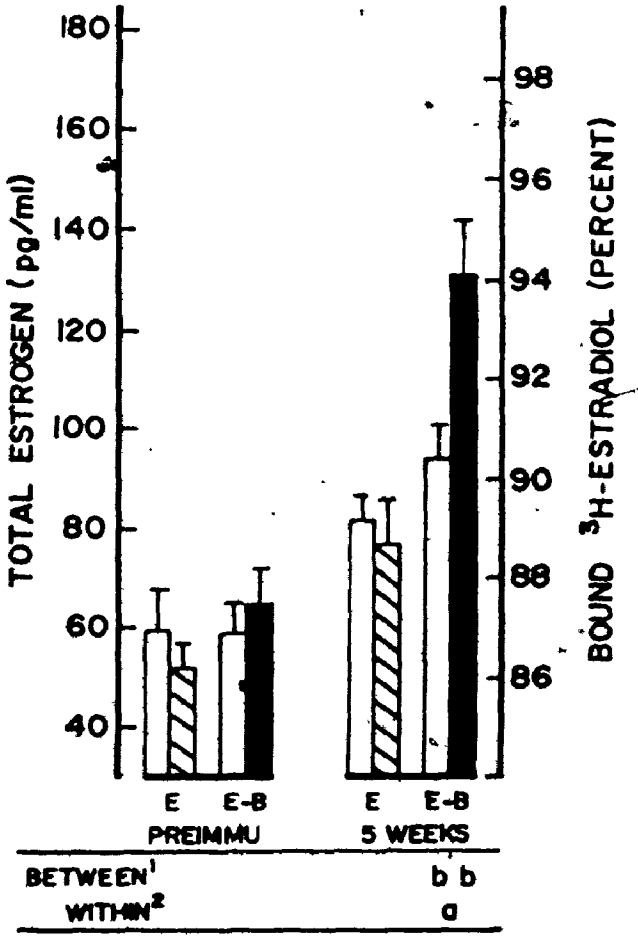

TOTAL ESTROGEN (pg/ml)
180
160
140
120
100
80
60
40
BOUND ³H-ESTRADIOL (PERCENT)
98
96
94
92
90
88
86
E
E-B
E
E-B
PREIMMU
5 WEEKS
BETWEEN¹
b b
WITHIN²
a

FIGURE 5 : Histograms (mean ± S.E) showing total serum androgen (A) and estrogen (E) and percent bound ^{3}H-testosterone (T-B) and ^{3}H-estradiol (E_2-B) in group 2 rabbits prior to immunization (pre-immun.) and 5 and 8 weeks after immunization to BSA (□ ,controls, n=6) or T-3-BSA (▨ or ■ , experimental, n=6).

1. Comparison of control or experimental values between consecutive weeks. Statistics by paired "t" test.

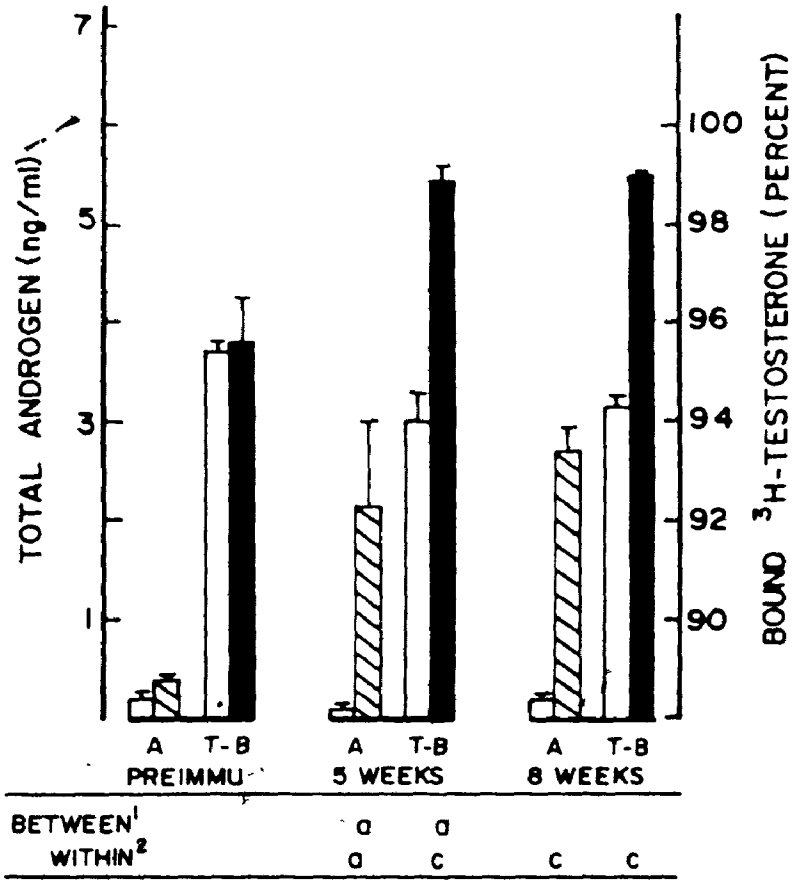
2. Comparison of control to experimental values within a week. Statistics by unpaired "t" test.

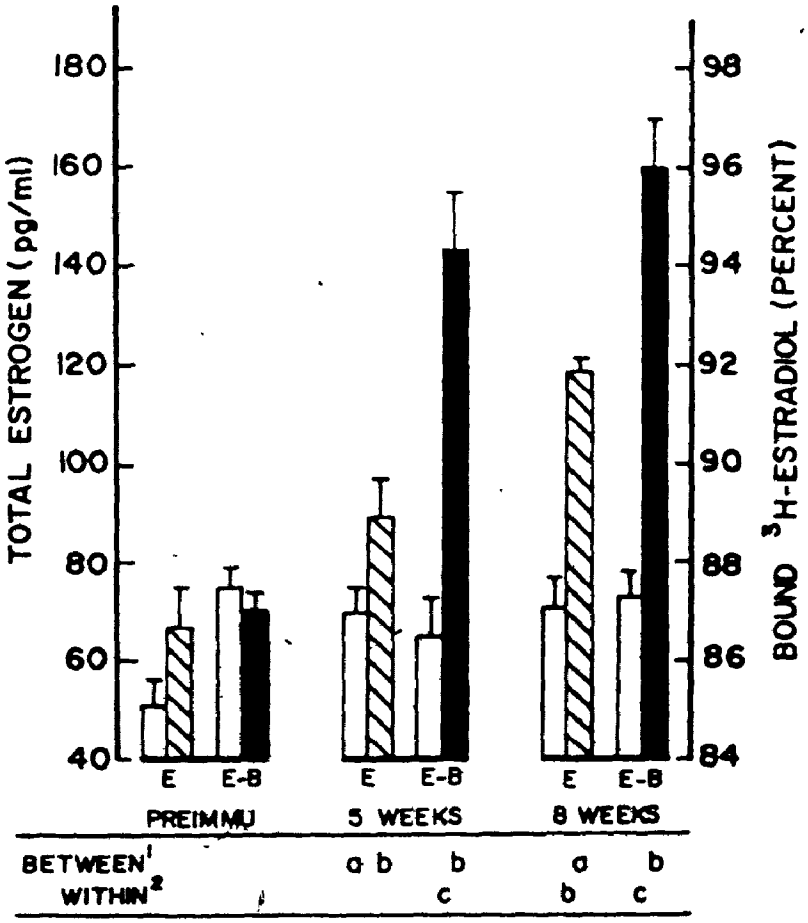
a : $p < 0.05$.

b : $p < 0.01$.

c : $p < 0.001$.

* Two animals with very high values (54.9 ng/ml, 28.4 ng/ml) excluded from calculation. See text for discussion.


TOTAL ANDROGEN (ng/ml)
7
5
3
1
BOUND ^{3}H-TESTOSTERONE (PERCENT)
100
98
96
94
92
90
A
T-B
PREIMMU
5 WEEKS
8 WEEKS
BETWEEN[1]
WITHIN[2]
a
a
a
c
c
c


TOTAL ESTROGEN (pg/ml)
180
160
140
120
100
80
60
40
BOUND ^{3}H-ESTRADIOL (PERCENT)
98
96
94
92
90
88
86
84
E
E-B
PREIMMU
5 WEEKS
8 WEEKS
BETWEEN[1]
WITHIN[2]
a b
b
c
a
b
b
c

IGURE 6 : Histograms (mean ± S.E) showing total serum androgen (A) and estrogen (E) and percent bound ^{3}H-testosterone (T-B) and ^{3}H-estradiol (E_2-B) in group 3 rabbits prior to immunization (pre-immun.) to BSA (□ , controls, n=6) or T-3-BSA (▨ or ■ , experimental, n=6).

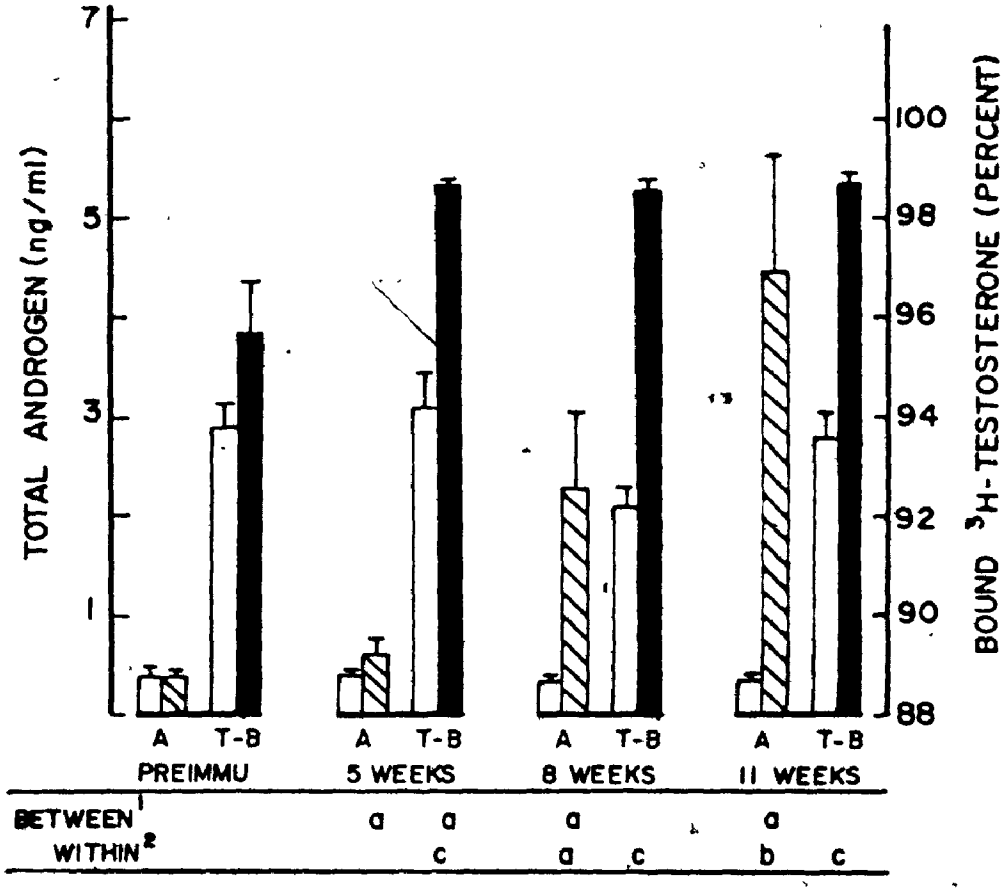
1. Comparison of control or experimental values between consecutive weeks; Statistics by paired "t" test.

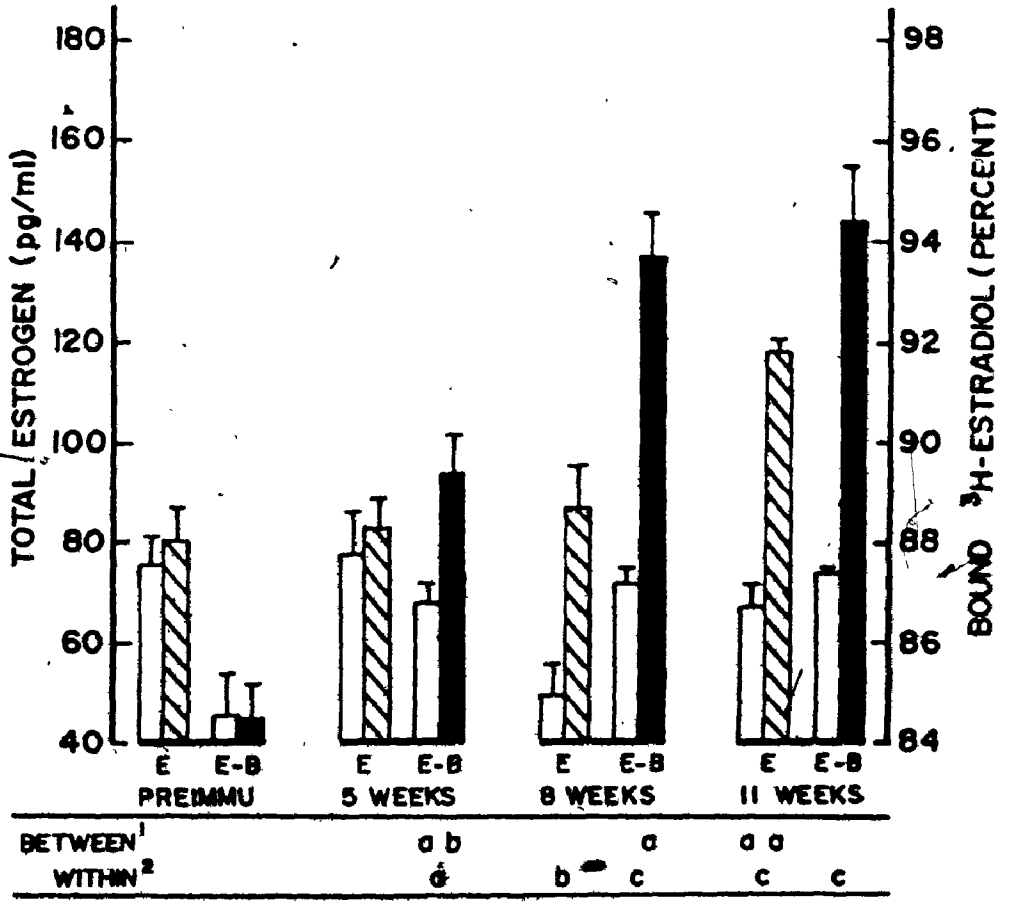
2. Comparison of control to experimental values within a week. Statistics by unpaired "t" test.

a : $p < 0.05$.

b : $p < 0.01$.

c : $p < 0.001$.


TOTAL ANDROGEN (ng/ml)
BOUND ³H-TESTOSTERONE (PERCENT)
7
5
3
1
100
98
96
94
92
90
88
A
T-B
PREIMMU
5 WEEKS
8 WEEKS
11 WEEKS
BETWEEN¹
WITHIN²
a a a a
c a c b c


TOTAL ESTROGEN (pg/ml)
BOUND ³H-ESTRADIOL (PERCENT)
180
160
140
120
100
80
60
40
98
96
94
92
90
88
86
84
E
E-B
PREIMMU
5 WEEKS
8 WEEKS
11 WEEKS
BETWEEN¹
WITHIN²
a b a a a
d b c c c

5 weeks the percentage of bound ^{3}H-testosterone rose significantly (from $p < 0.05$ to $p < 0.01$) in all groups; values being 98.8 ± 0.2 %, 98.9 ± 0.3 %, and 98.7 ± 0.1% in groups 1,2 and 3 respectively. This level of binding was observed at each interval throughout the experiment.

Total Estrogen and Percentage of Bound Estradiol

In control animals serum estrogen values among groups ranged from 49.5 ± 6.8 pg/ml to 81.8 ± 4.9 pg/ml over the length of the experiment. Group 2 animals showed a significant ($p < 0.05$) rise in estrogen between pre-immunization (50.7 ± 5.4 pg/ml) and 5 weeks post-immunization (69.8 ± 5.0 pg/ml) while in group 3 there was a small significant increase between weeks 8 (49.5 ± 6.8 pg/ml) and 11 (67.3 ± 4.5 pg/ml; $p < 0.05$). The pre-immunization ^{3}H-estradiol binding in control animals was 86.9 ± 0.6 %, 87.5 ± 0.4 % and 83.8 ± 0.8 % in groups 1 to 3 respectively. At 5 weeks a significant increased binding for group 1 (90.4 ± 0.7 %, $p < 0.01$) and group 3 (86.8 ± 0.4 %, $p < 0.05$) but not for group 2 (86.5 ± 0.8 %) was found. No significant change was noted after 5 weeks.

Pre-immunization estrogen values in experimental animals did not differ from control values; 52.0 ± 5.0 pg/ml 66.7 ± 8.1 pg/ml and 80.5 ± 7.0 pg/ml in groups 1,2 and 3 respectively. All experimental groups showed increasing

values over time; reaching 76.8 ± 9.6 pg/ml (N.S.) in group 1, 88.5 ± 8.7 pg/ml ($p < 0.01$) and 118.5 ± 13.1 pg/ml ($p < 0.05$) at 5 and 8 weeks respectively in group 2 and 83.2 ± 6.1 pg/ml (N.S.), 87.3 ± 8.5 pg/ml (N.S.) and 118.5 ± 2.5 pg/ml ($p < 0.05$) at 5,8 and 11 weeks respectively in group 3. These values were significantly increased over controls (from $p < 0.01$ to $p < 0.001$) at 8 and 11 weeks.

In experimental animals estradiol binding showed a dramatic increase over time. While pre-immunization values (87.5 ± 0.7 %, 87.5 ± 0.4 % and 83.8 ± 0.7 % in groups 1,2 and 3 respectively) did not differ from controls there was a significant increase in binding by 5 weeks; 94.1 ± 1.1, 94.3 ± 1.2 and 89.4 ± 0.8 ($p < 0.01$) in groups 1,2 and 3 respectively. This binding was considerably higher ($p < 0.05$ to $p < 0.001$) than control values for this time. Estradiol binding continued to rise up to 8 weeks (96.0 ± 1.0, $p < 0.01$ in group 2 and 93.7 ± 0.9, $p < 0.05$ in group 3) and then appeared to level off by 11 weeks (94.4 ± 1.1 in group 3).

Gonadotrophins in Group 1 Animals.

Gonadotrophins were measured at all time intervals, however, except for the first 5 weeks, there were no significant changes in either LH or FSH levels. LH values were approximately 50 ng/ml. After 5 weeks FSH was undetectable.

However, as illustrated by the group 1 animals, while there was no difference between control and experimental values, there was a significant decrease ($p < 0.001$) in FSH from a pre-immunization value of 15.8 ± 2.8 to 3.2 ± 2.2 at 5 weeks after immunization (Table 4).

Ovarian Features

While ovarian weights were not different between control and experimental animals (Table 5) marked alterations in follicular development were apparent. By 5 weeks of immunization there were a number of follicles between 1 mm and 1.5 mm in both control (12.5 ± 3.4) and experimental (17.0 ± 1.0) ovaries (Table 6). This difference was not significant due largely to one control ovary which had 19 follicles in this size range. If this ovary is excluded in the statistical analysis the difference becomes highly significant (control $p < 0.001$). At this age there were few follicles greater than 1.5 mm.

Figure 7 illustrates the general histological features of group 1 ovaries. It is apparent that follicles of various sizes are abundant in both control (A) and experimental (B) animals. While closer examination does not reveal any marked difference between the follicles in these ovaries there does appear to be an increased 'luteinization' of the thecal cells of the experimental animals. This is reflected in a rounding of nuclei and an increase in cytoplasm of these thecal cells

	F S H (ng/ml)		L H (ng/ml)	
	CONTROL	EXPERIMENT.	CONTROL	EXPERIMENT.
PRE-IMMUN.	* 17.4± 3.2	* 14.3± 2.4	60.7± 9.5	103.0± 30.7
1	12.0± 2.8	14.2± 2.1	57.3± 9.3	52.3± 5.2
2	8.2± 3.0	11.6± 2.7	59.7± 8.2	53.0± 6.9
3	3.0± 1.3	5.7± 2.4	40.7± 0.7	41.3± 3.4
4	0.7± 0.5	2.5± 1.1	47.6± 2.2	49.3± 4.1
5	* 1.2± 0.6	* 4.9± 2.9	46.6± 7.5	57.0± 14.0

TABLE 4 : LH and FSH values (mean ± S.E.) for group 1 rabbits immunized to BSA (control) or T-3-BSA (experimental). No significant difference between control and experimental animals at any time.

* Combined FSH values for pre-immunization (15.8 ± 2.8) animals significantly higher ($p < 0.001$) than levels at 5 weeks (3.2 ± 2.2) post-immunization.

GROUP NUMBER	OVARIAN WEIGHT (mg)		UTERINE WEIGHT (g)	
	CONTROL	EXPERIMENT.	CONTROL	EXPERIMENT.
1 (6,6)*	79.9± 7.4	87.3± 4.9	1.55±0.49	1.08±0.14
2 (6,6)*	127.5± 6.5	158.3±10.8	2.20±0.32	1.64±0.28
3 (6,6)*	126.1± 5.3	129.5± 6.9	2.84±0.46	2.36±0.19
5 (6,4)*	189.3±19.8	185.8±19.3	7.88±0.94	4.48±0.82+

TABLE 5 : Ovarian and uterine weight (mean ± S.E.) of rabbits immunized to BSA (control) or T-3-BSA (experimental) as immature (groups 1,2 and 3, sacrificed at 5,8 and 11 weeks post-immunization) or mature (group 5, sacrificed at 11 weeks post-immunization) animals.

* number of animals.

+ $p < 0.05$

GROUP NUMBER	NUMBER OF FOLLICLES GREATER THAN 1.0 mm		NUMBER OF FOLLICLES GREATER THAN 1.5 mm	
	CONTROL	EXPERIMENT.	CONTROL	EXPERIMENT.
1	12.5±3.4	17.0±1.0	0.4±0.4	0.4±0.29
	NS		NS	
2	15.3±2.9	27.2±3.1	0.42±0.29	5.83±1.69
	$p<0.01$		$p<0.005$	
3	12.0±2.7	15.5±2.0	0.92±0.31 +	5.00±0.94
	NS		$p<0.001$	
5	8.25±1.22	10.50±1.22	4.17±0.74 +	7.25±0.92
	NS		$p<0.05$	

TABLE 6 : Mean and standard error of follicles $\geq$ 1.0 mm and $\geq$ 1.5 mm in rabbits immunized to bovine serum albumin (control) or testosterone (experimental as immature (groups 1,2,3; sacrificed at 5,8 and 11 weeks post-immunization) or mature (group 5; sacrificed at 11 weeks post-immunization) animals.

Statistics by "t" test.

+ $p < 0.001$ for group 3 controls vs. group 5 controls.

FIGURE 7 : Ovarian section from a control (A) and experimental (B) animal 5 weeks after immunization. Note the abundance of antral follicles (AF) in both groups.
X10.

A

B

(Figure 8). While larger follicles in both groups show signs of atresia, this appears more prominant in the control ovaries.

By 8 weeks experimental ovaries were found to have numerous large cystic and cystic-hemorrhagic follicles (Figure 9). This was reflected in a significant rise in the number of follicles greater than 1.0 mm and greater than 1.5 mm in experimental (27.3 ± 3.1 and 5.83 ± 1.69 respectively) versus control (15.3 ± 2.9, $p < 0.01$ and 0.42 ± 0.29, $p < 0.005$) ovaries (Table 6). Histologically control ovaries were found to have a larger number of small (1.0mm) antral follicles, a few follicles in the 1.0 mm to 1.5 mm range but, as in group 1 controls, almost no follicles greater than 1.5 mm (Figure 10 A).

Small antral follicles were also common in experimental animals however characteristic to them were the large number of follicles greater than 1.5 mm; some reaching sizes greater than 3.0 mm (Figure 10 B). These large cystic and hemorrhagic follicles found in experimental animals often have few granulosa cells, basement membranes which appear disrupted and thecal layers which appear stretched. The oocyte is in various stages of degeneration as is demonstrated in Figure 11 A which shows the presence of the first polar body in the oocyte of a large (3 mm) hemorrhagic follicle. Follicular growth does not appear to be affected by these

FIGURE 8 : Part of 1 mm follicle in a control (A) and experimental (B) animal 5 weeks after immunization. Note the increased degeneration of granulosa layer (GD) in the control animal and the larger, rounded cells of the theca in the experimental animal (→).

(AN, antrum; TC, thecal cells; GC, granulosa cells).

X256.

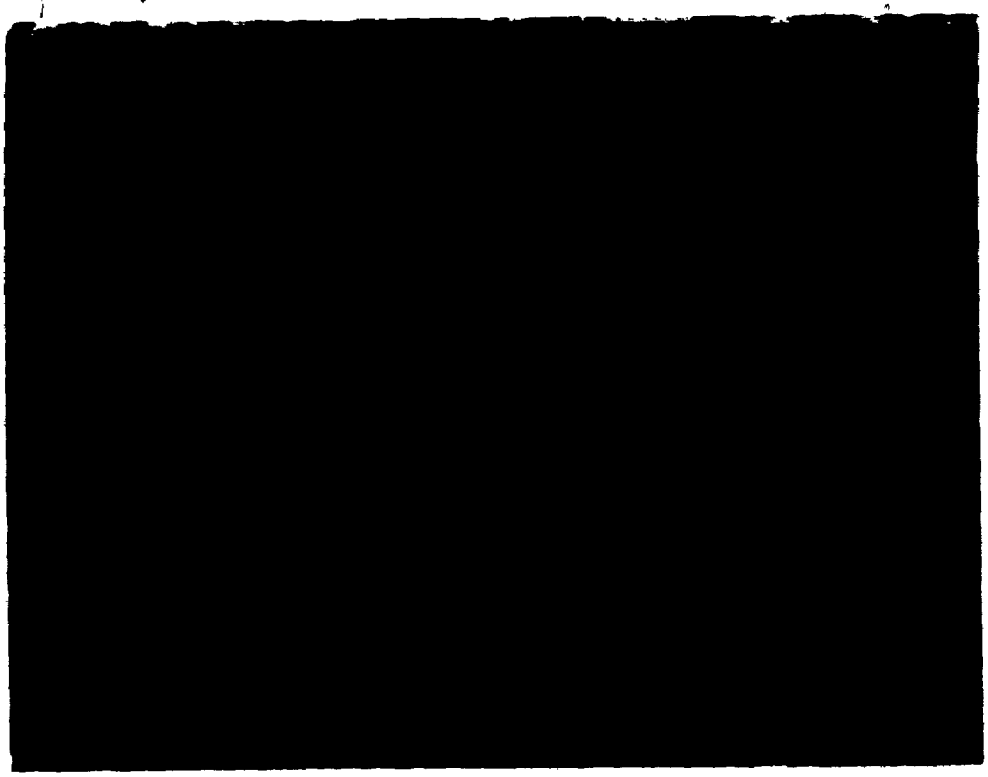
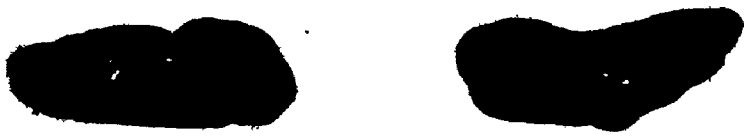


FIGURE 9 : Ovaries from a control (A) and an experimental (B) animal 8 weeks after immunization. Experimental animals have numerous large cystic and cystic hemorrhagic follicles.

A



B

FIGURE 10 : Section through a control (A) and an experimental (B) ovary 8 weeks after immunization. Note the numerous small antral follicles (AF) in the control ovary and the presence of large hemorrhagic follicles (HF) in the experimental ovary.
X10.

FIGURE 11A : First polar body (→) in oocyte of a 3 mm hemorrhagic follicle.
X256.

FIGURE 11B : Mitotic figures (→) in granulosa of a 1.5 mm follicles which is next to a large (3.0 mm) hemorrhagic follicle.
X100.

large cystic and hemorrhagic follicles as is illustrated in Figure 11 B which shows a presumably healthy (mitotic figures in granulosa) follicle next to a large hemorrhagic one.

Figure 12 illustrates what appears to be an increased vascularization associated with the larger follicles of T-3-BSA immunized animals when compared with control follicles of similar size. These vessels appear larger and more engorged than in control animals. In addition the basement membrane in experimental animals is less well defined and often appears disrupted.

The interstitial cells surrounding the larger follicles appear to be distinctly enlarged in the experimental animals (Figures 13 and 14); the nuclear to cytoplasm ratio appears considerably reduced. Also the cells have a more ordered appearance; lying between sheets of elongated connective tissue cells. Interstitial cells further away from the large follicles are similar in appearance to those found in control ovaries.

By 11 weeks of immunization there was no longer a significant difference between control and experimental animals in the number of follicles greater than 1.0 mm, however there is a [illegible] of [illegible]es

FIGURE 12 : Portion of a large follicle in a control ovary (A) and a cystic follicle in an experimental ovary (B). Note the presence of large blood engorged vessels (→) in the thecal layer (TC) and a basement membrane (BM) which is indistinct and disrupted in comparison to the control follicle.

(AN, antrum; GC, granulosa cells).
X205.

[redacted]

[redacted]

FIGURE 13 : Interstitial cells (IC) surrounding large follicles in control (A) and experimental (B) animals. Note the larger and more ordered arrangement of interstitial cells in the experimental animals; particularly nearer to the large cystic follicle (→).

(AF, antral follicle; CF, cystic follicle). X64

[redacted]

FIGURE 14 : Higher magnification of Figure 13. Note the larger size and organized appearance of interstitial cells next to thecal cells (TC) in experimental (B) versus control (A) ovaries.

(AN, antrum; GC, granulosa cells).
X256.

1.5 mm in the experimental ovaries ($p < 0.05$) (Table 6). The follicles in these animals did not exceed 2.5 mm and, while the histological features were similar to that of group 2 experimental animals, very few of these follicles were hemorrhagic.

UNMATED : IMMUNIZED AS MATURE ANIMALS

Total Androgen and Percent Bound Testosterone

Androgen values in the maturely immunized control animals did not differ from those of immaturely immunized animals; never exceeding 0.5 ng/ml (Figure 15). Again pre-immunization experimental values did not differ from controls. While considerable variability existed in T-3-BSA immunized animals there was no significant increase in androgen values in the total group until 8 weeks post-immunization (5.77 ± 0.8 ng/ml, $p < 0.01$). Similar to the immaturely immunized animals testosterone binding (93.2 ± 0.8 % at 11 weeks) in control animals did not change over the course of the experiment. In experimental animals testosterone binding was significantly increased (98.8 ± 0.3 %, $p < 0.05$) by 5 weeks and did not change thereafter.

Total Estrogen and Percent Bound Estradiol

Control estrogen values ranged from 65.7 ± 13.1 pg/ml

FIGURE 15: Histograms (mean + S.E) showing total serum androgen (A) and estrogen (E) and percent bound ^{3}H-testosterone (T-B) and ^{3}H-estradiol (E_2-B) in group 5 rabbits prior to immunization (pre-immun.) and at 5,8 and 11 weeks after immunization to BSA (□ ,controls, n=6) or T-3-BSA (▧ or ■ , experimental, n=4).

1. Comparison of control or experimental values between consecutive weeks. Statistics by paired"t"test.

2. Comparison of control to experimental values within a week. Statistics by unpaired"t"test.

a : $p < 0.05$.

b : $p < 0.01$.

c : $p < 0.001$.

rence in estradiol values between sample times within animals or between immature control rabbits. In a manner similar to the immature T-3-BSA immunized animals estrogen values in the mature experimental animals increased slowly from 63.3 ± 7.8 pg/ml at pre-immunization to 78.6 ± 11.5 pg/ml at 8 weeks and then rapidly between 8 and 11 weeks to 157.5 ± 30.3 pg/ml ($p < 0.05$). Control estrogen binding was similar to immature controls (88.7 ± 1.1 % at 5 weeks) while experimental values showed the same dramatic increase in binding by 5 weeks of immunization (95.9 ± 0.7, $p < 0.01$) and a further increase at 8 weeks (97.4 ± 0.4, $p < 0.05$).

Ovarian Features

Ovarian weights did not differ between control and experimental animals (Table 5) however, as with the immature animals, abnormalities in follicular development were apparent with the T-3-BSA immunized animals. There was no difference in the number of follicles greater than 1.0 mm but a significant increase in the number of follicles greater than 1.5 mm in experimental (7.25 ± 0.92) versus control (4.17 ± 0.74, $p < 0.05$) animals. At the same time there was a significant increase in the number of follicles greater than 1.5 mm between control rabbits in group 3 (0.92 ± 0.31) and control animals in group 5 (4.17 ± 0.74, $p < 0.001$); the majority of group 5 follicles being in the 1.5 mm to 2.0 mm range (Table 6).

Histologically the experimental ovaries appeared similar to those of group 2 animals. There were numerous cystic and cystic-hemorrhagic follicles; many reaching sizes greater than 2.5 mm. The non-hemorrhagic cystic follicles were as large or larger than the hemorrhagic ones. Similar to observation in group 1 animals the interstitial cells surrounding the large cystic follicles of experimental animals are markedly hypertrophied. This association can be seen in Figure 16 where hypertrophy of interstitial cells between two cystic and one hemorrhagic follicle is apparent. At higher magnification this distinction is clearly demonstrated (Figure 17).

Uterine Features

Uterine weights in control animals (7.88 ± 0.94 g) were significantly greater than experimental animals (4.48 ± 0.82, $p < 0.05$) (Table 5). To some extent this is reflected in histological sections (Figure 18) where an increase in amount of endometrium and the extent of glandular development is apparent.

MATED IMMATURELY IMMUNIZED ANIMALS

Androgen and ^{3}H-Testosterone Binding Prior to Mating

Serum androgen levels in experimental animals were significantly elevated by 8 weeks of immunization (2.94 ± 0.4 ng/ml versus 0.32 ± 0.07 ng/ml, $p < 0.001$) and continued to rise up to the end of the sampling period; reaching a con-

FIGURE 16A : Interstitial cells (IC) between two antral follicles (AF) in a control ovary.

FIGURE 16B : Hypertrophied interstitial cells between a large hemorrhagic follicle (HF) and two large cystic follicles (CF) in an experimental ovary.

X64.


AF

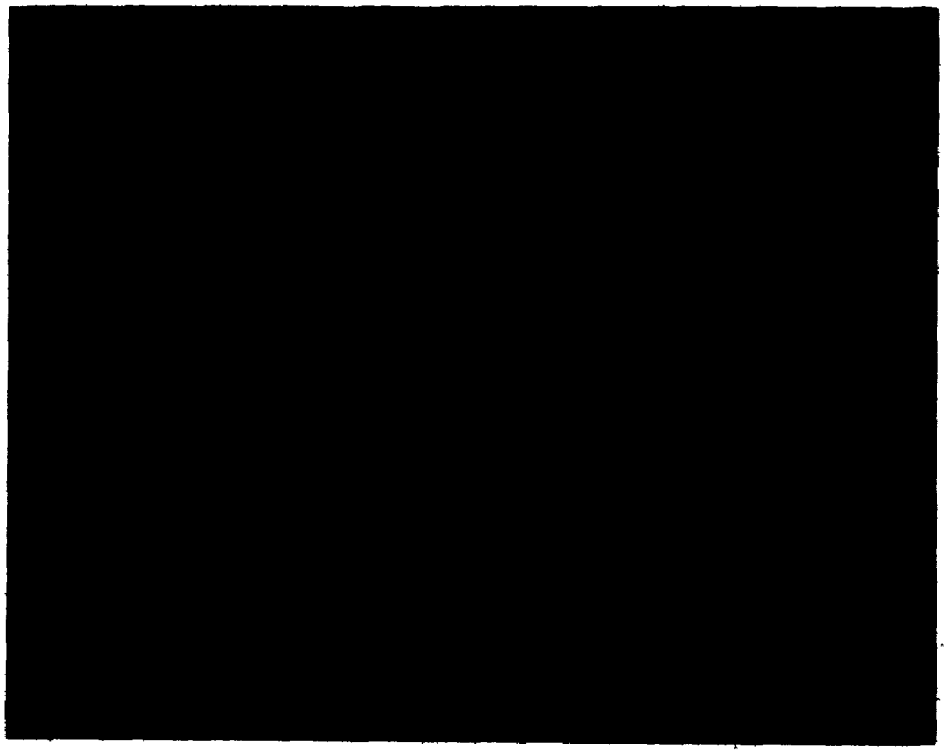


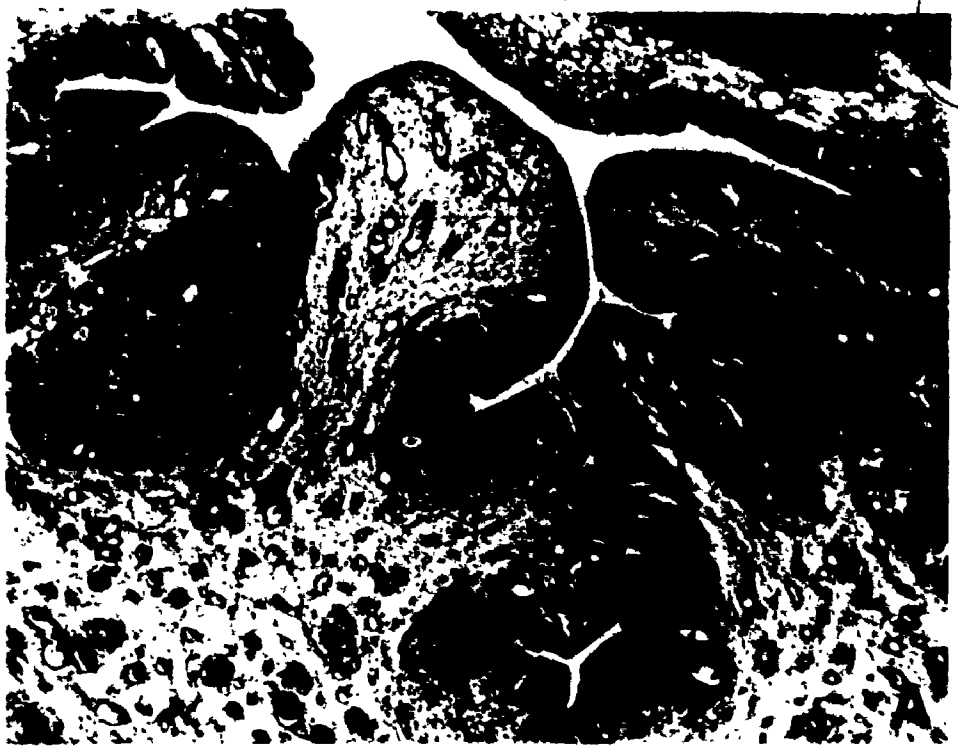
FIGURE 17 : Interstitial cells (IC) surrounding large follicles in the same control (A) and experimental (B) ovary as seen in Figure 16. Note the hypertrophy of cells in experimental animals.

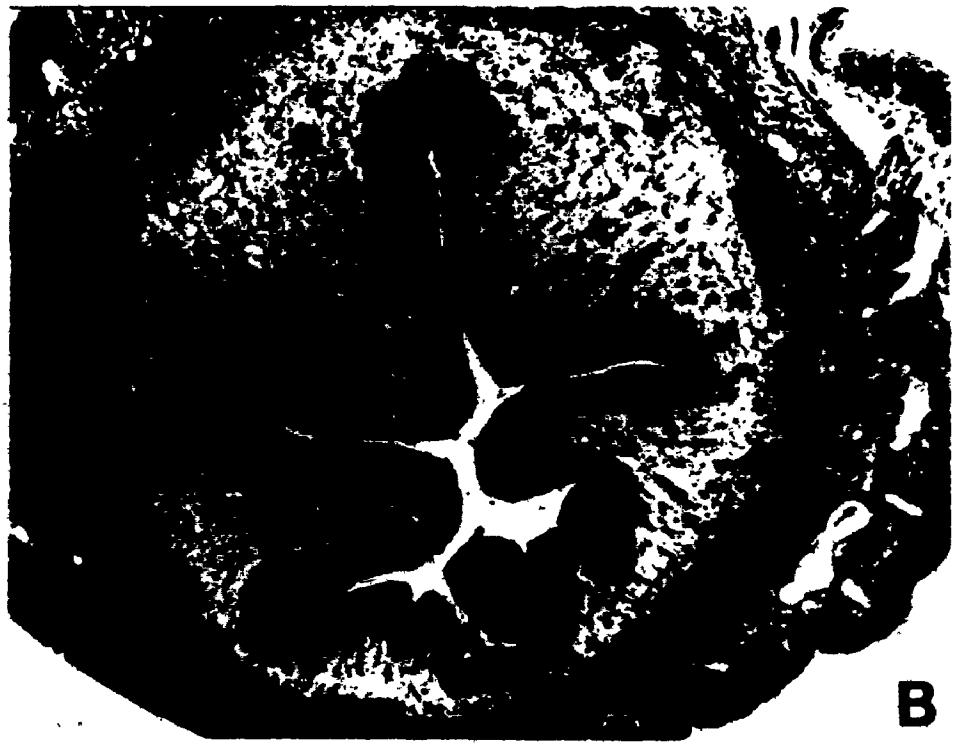
(AF, antral follicle ; CF, cystic follicle). X256.

[redacted]

[redacted]

FIGURE 18 : Uterine section in a control (A) and experimental (B) animal from group 5. Note the increase in amount of endometrium and the extent of glandular development.
X10.




B

centration of 4.64 ± 1.22 ng/ml at 14 weeks (Figure 19). Control androgen values did not change over the sampling period. The percentage of ^{3}H-testosterone bound was significantly elevated by 5 weeks (98.7 ± 0.1 versus 96.0 ± 0.6 at pre-immunization, $p < 0.01$) and did not change from that time onward. ^{3}H-testosterone binding in control animals remained at the 93-95% level (Figure 19).

Estrogen and ^{3}H-Estradiol Binding Prior to Mating

In T-3-BSA immunized animals estradiol values rose steadily throughout the sampling period reaching statistical significance between the 11th and 14th weeks (81.6 ± 6.9 pg/ml and 99.0 ± 7.0 pg/ml respectively, $p < 0.05$) (Figure 20). These values were not significantly different from controls at their respective time periods. Control ^{3}H-estradiol binding remained at the 85-86% level over the course of the experiment while experimental animals again showed a significant rise in binding from 83.3 ± 1.0 % prior to immunization to 91.8 ± 0.4 % ($p < 0.001$) after 5 weeks and 94.5 ± 0.5 % ($p < 0.05$) after 8 weeks of immunization. No further increase occurred after 8 weeks (Figure 20).

Effects of Mating

Of the 5 control and 6 experimental rabbits in group 4 there were 4 successful matings in control and 5 in experimental animals. Mating most often occurred on the first attempt

FIGURE 19 : Histogram (mean ± S.E) showing total serum androgen (A) and percent bound ^{3}H-testosterone (T-B) in group 4 rabbits prior to immunization (pre-immun.) and at 5,8,11 and 14 weeks after immunization to BSA (□ , controls, n=5) or T-3-BSA (▧ or □ , experimental, n=6).

1. Comparison of control or experimental values between consecutive weeks. Statistics by paired "t" test.

2. Comparison of control to experimental values within a week. Statistics by unpaired "t" test.

a : $p < 0.05$.

b : $p < 0.01$.

c : $p < 0.001$.

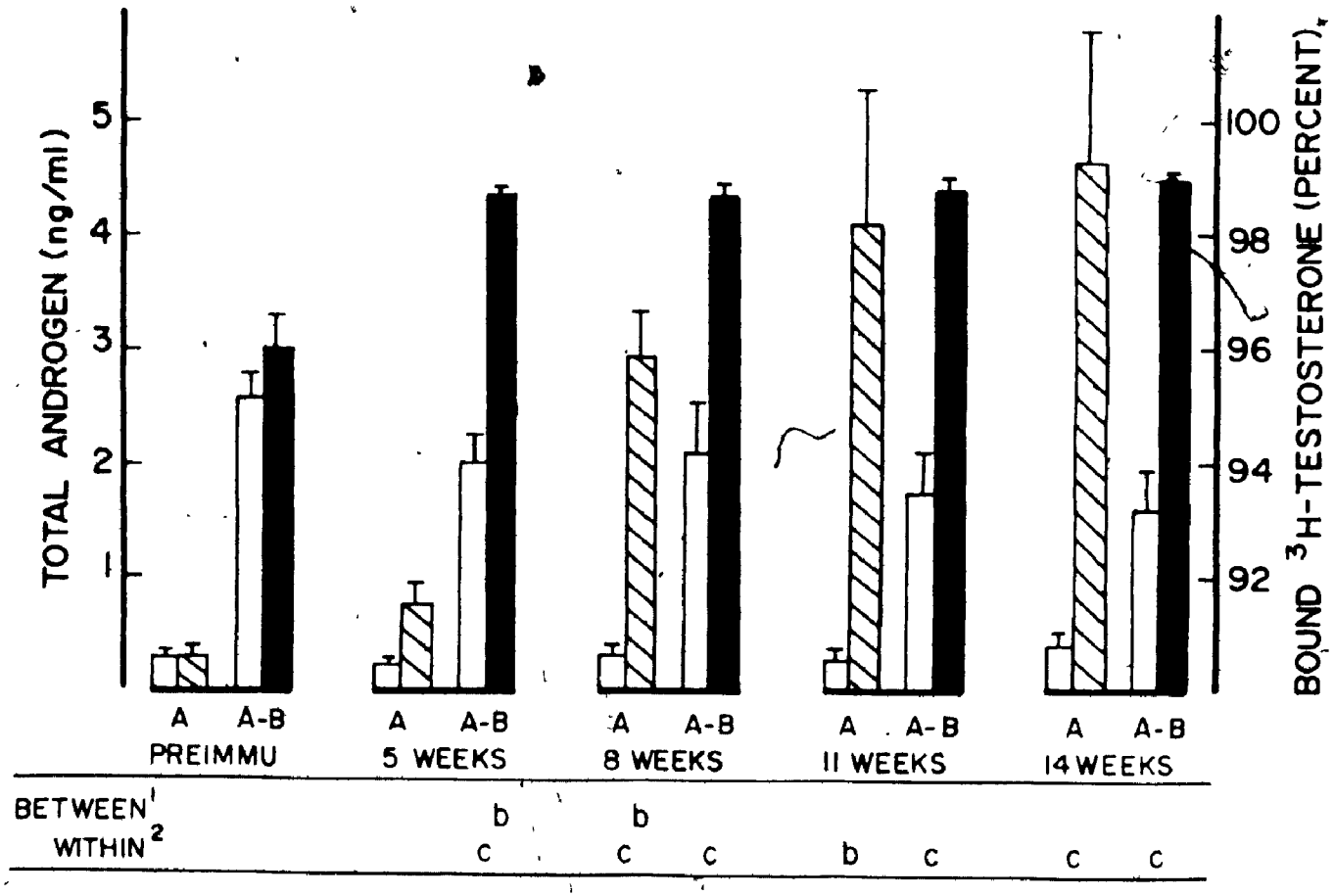

TOTAL ANDROGEN (ng/ml)
5
4
3
2
1
BOUND ^{3}H-TESTOSTERONE (PERCENT)
100
98
96
94
92
A
A-B
PREIMMU
5 WEEKS
8 WEEKS
11 WEEKS
14 WEEKS
BETWEEN[1]
WITHIN[2]
b
c

FIGURE 20 : Histogram (mean + S.E) showing total serum estrogen (E) and percent bound ^{3}H-estradiol (E_2-B) in group 4 rabbits prior to immunization (pre-immun.) and at 5,8,11 and 14 weeks after immunization to BSA (□ , controls, n=5) or T-3-BSA (▨ or □ , experimental, n=6).

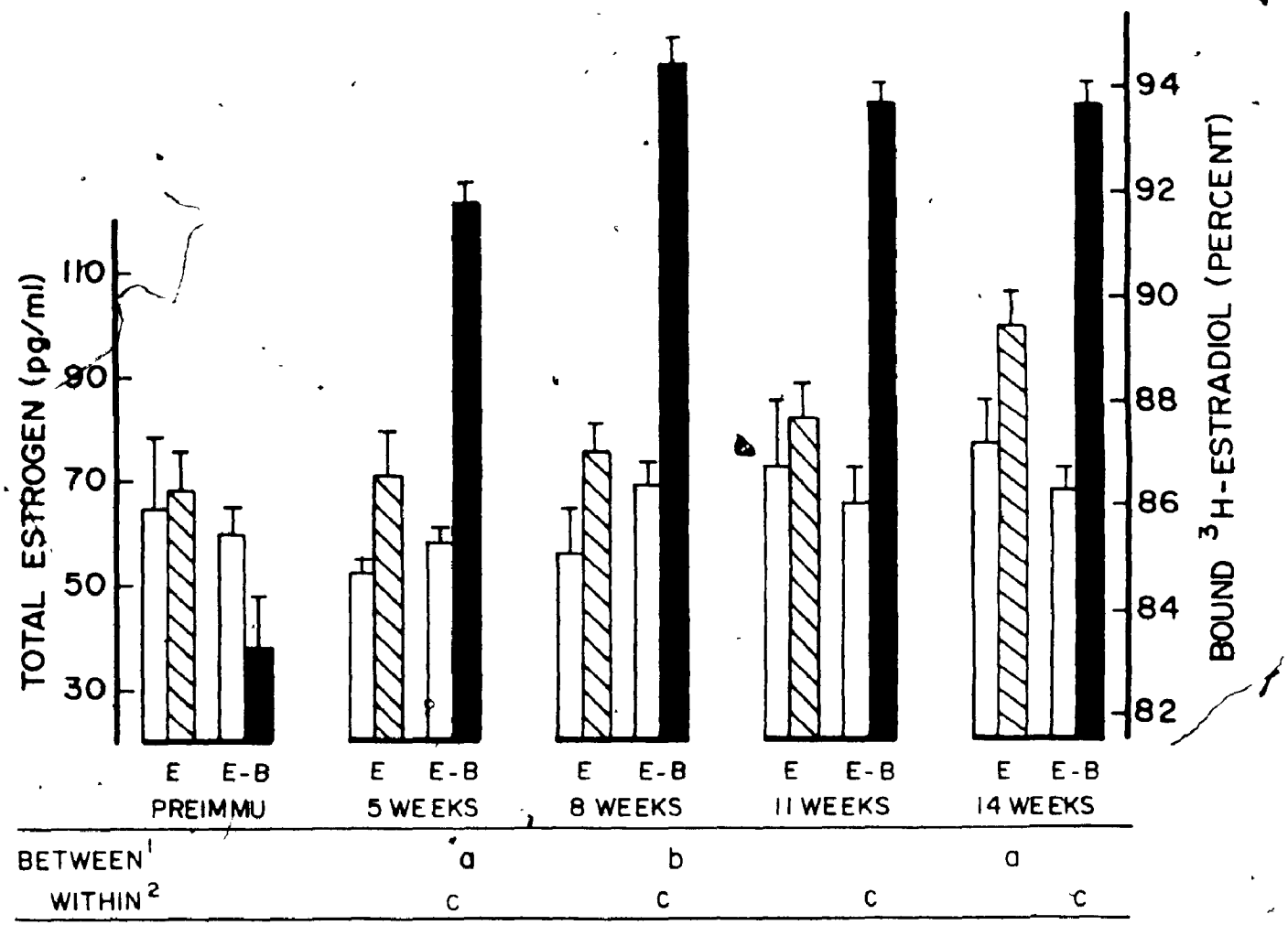
1. Comparison of control or experimental values between consecutive weeks. Statistics by paired"t"test.

2. Comparison of control to experimental values within a week. Statistics by unpaired"t"test.

a : $p < 0.05$.

b : $p < 0.01$.

c : $p < 0.001$.


TOTAL ESTROGEN (pg/ml)
110
90
70
50
30
BOUND ^{3}H-ESTRADIOL (PERCENT)
94
92
90
88
86
84
82
E
E-B
PREIMMU
5 WEEKS
8 WEEKS
11 WEEKS
14 WEEKS
BETWEEN[1]
WITHIN[2]
a
b
a
c
c
c
c

and there was no observable difference in the behaviour of the animals. One control animal was discovered to have mated only at the time of sacrifice (48 hours after first attempt) and therefore, with this animal, only the number of corpora lutea is included in the presentation of the following results.

Steroid Levels After Mating

As Table 7 details serum androgen levels in control animals did not differ from pre-mating values at any time after mating. In experimental animals serum androgen levels rose within the first hour and were significantly greater by 2 hours (4.64 ± 1.23 ng/ml at 0 hours and 6.89 ± 1.78 at 2 hours, $p < 0.05$). After peaking at 2 hours, values steadily declined until at 24 hours androgen levels were significantly lower than pre-mating levels (1.33 ± 0.65 at 24 hours, $p < 0.01$) (Table 7).

Serum estrogen values increased, although not significantly, in control animals 1 hour after mating and decreased thereafter. As noted earlier estrogen levels in experimental animals were significantly higher than controls prior to mating. An increase in estrogen after mating was also noted in these animals however values were not significantly different from controls.

Gonadotrophins in Mated Animals

ANIMAL	SERUM TESTOSTERONE (ng/ml)						SERUM ESTROGEN (pg/ml)					
	0	1	2	4	6	24	0	1	2	4	6	24
CON 21	0.38	0.55	0.57	0.32	0.36	0.24	88	120	103	90	95	83
CON 22	0.24	0.24	0.27	0.24	0.30	0.28	55	127	127	115	103	113
CON 24	0.38	0.05	<0.05	<0.05	<0.05	<0.05	60	113	98	98	180	90
$\bar{x}$ ±S.E.	0.33 ±0.05	0.28 ±0.14	0.30 ±0.20	0.20 ±0.10	0.24 ±0.13	0.19 ±0.10	67.7 ±10.2	120 ± 4.0	109.0 ± 9.0	101.0 ± 7.4	126.0 ±27.0	95.3 ± 9.1
T-3-19	3.00	3.02	3.39	2.20	1.00	0.38	108	138	155	105	93	50
T-3-20	1.99	4.11	4.29	2.53	1.76	0.50	80	80	95	97	85	105
T-3-21	8.60	11.06	12.36	11.00	11.01	3.89	98	103	95	93	70	75
T-3-23	6.34	6.76	9.81	7.51	6.14	1.17	125	170	163	195	147	125
T-3-24	3.26	3.72	4.60	4.26	2.63	0.69	93	168	140	148	140	138
$\bar{x}$ ±S.E.	4.64 ±1.23	5.73 ±1.48	6.89[1] ±1.78	5.50 ±1.67	4.51 ±1.85	1.33[2] ±0.65	100.8[3] ± 7.6	131.8 ±17.8	129.6 ±14.6	127.6 ±19.6	107.0 ±15.4	98.6 ±16.2

TABLE 7 : Serum androgen and estrogen values for group 4 rabbits immunized to BSA (control) or T-3-BSA (experimental) prior to (0) and at 1,2,4,6 and 24 hours after mating to a proven male.

1, $p<0.05$ for 0 versus 2 hours.
2, $p<0.01$ for 0 versus 24 hours.
3, $p<0.05$ for control versus experimental at 0 hours.

Serum LH levels were significantly higher in experimental (64.6 ± 4.4 ng/ml) versus control (40.3 ± 1.3 ng/ml, $p < 0.01$) animals prior to mating. However, the LH surge, in response to the mating stimulus, appeared similar; peak levels being attained 1-2 hours after mating. While 24 hour LH levels in experimental animals (111.0 ± 32.7 ng/ml) appeared elevated over controls (37.7 ± 7.0 ng/ml) there was no statistically significant difference. In experimental animals, however, these LH values were significantly greater than pre-mating values ($p < 0.05$) at both 6 and 24 hours (Table 8).

In both control and experimental animals serum FSH was undetectable prior to mating. In control animals low but detectable levels were noted at 1,2 and 4 hours after mating and then returned to undetectable levels by 6 hours (Table 8). In experimental animals all but 2 animals had undetectable FSH at 1,2,4 and 6 hours after mating. At 24 hours, however, FSH values were significantly elevated over controls (6.8 ± 1.5 versus 1.7 ± 0.7; $p < 0.05$ if FSH values for control animals are taken at the limit of sensitivity of the assay).

Ovarian Features

Morphologically control ovaries demonstrate a few large visible follicles and a number of pinpoint ovulation sites. In experimental animals follicular characteristics

ANIMAL	SERUM LH (ng/ml)						SERUM FSH (ng/ml)					
	0	1	2	4	6	24	0	1	2	4	6	24
CON 21	39	>1250	>1250	>250	56	24	<1.0	1.3	2.2	1.1	<1.0	<1.0
CON 22	39	>1250	>1250	230	67	47	<1.0	3.2	4.2	1.0	<1.0	3.0
CON 24	43	465	>1250	220	95	42	<1.0	1.7	2.0	2.6	<1.0	<1.0
$\bar{x}$±S.E.	40.3 ±1.3	-	-	-	72.7 ±11.6	37.7 ±7.0	-	-	-	-	-	1.7 ±0.7
T-3-19	54	>1250	895	>250	180	90	<1.0	<1.0	2.7	1.0	<1.0	11.6
T-3-20	66	428	420	100	111	29	<1.0	<1.0	2.2	1.0	<1.0	3.8
T-3-21	56	490	605	>250	76	220	<1.0	<1.0	<1.0	<1.0	<1.0	7.1
T-3-23	78	680	>1250	>250	165	141	<1.0	<1.0	<1.0	<1.0	<1.0	8.2
T-3-24	69	>1250	>1250	>250	106	75	<1.0	<1.0	<1.0	<1.0	<1.0	3.4
$\bar{x}$±S.E.	64.6[1] ±4.4	-	-	-	127.6[2] ±19.4	111.0 ±32.7	-	-	-	-	-	6.8[3] ±1.5

TABLE 8 : Serum LH and FSH values for group 4 rabbits immunized to BSA (control) or T-3-BSA (experimental) prior to (0) and at 1,2,4,6 and 24 hours after mating to a proven male.

1, $p<0.01$ for control versus experimental at 0 hours.
2, $p<0.05$ for 0 versus 24 hours.
3, $p<0.05$ for control versus experimental at 24 hours.

were similar to those in group 2 and group 5 animals (Figure 21). There were a number of cystic and cystic hemorrhagic follicles which in some cases appeared to cover the whole ovarian surface. Despite the presence of these large cystic follicles there was an increased number of ovulations in experimental animals. Controls were found to have 4.4 ± 1.9 corpora lutea per ovary while experimental animals had 8.0 ± 2.1 ($p < 0.005$) (Table 9). Ovarian weight in experimental animals was also significantly greater than in control animals (Table 9).

Histologically control ovaries were found to have a number of corpora lutea and a few large follicles while experimental ovaries, in addition to this, contained the characteristic large cystic and hemorrhagic follicles (Figure 22). Corpora lutea in experimental animals appear somewhat smaller and less developed than in control ovaries, however, they appear normal in all other respects; no retained oocytes were found (Figure 23). Besides the numerous large cystic and cystic-hemorrhagic follicles (some of which are greater than 3 mm in diameter) there are a number of follicles in various stages of atresia many of which have luteinized and some of which appear to have collapsed.

ANIMAL	UTERINE WEIGHT (g)	OVARIAN WEIGHT (ng)	NUMBER OF CORPORA LUTEA PER OVARY
CONTROL (n = 3)	4.6±0.6	124±52	4.4±1.9*
EXPERIMENT (n = 5)	4.9±0.6	223±51[1]	8.0±2.1[2]

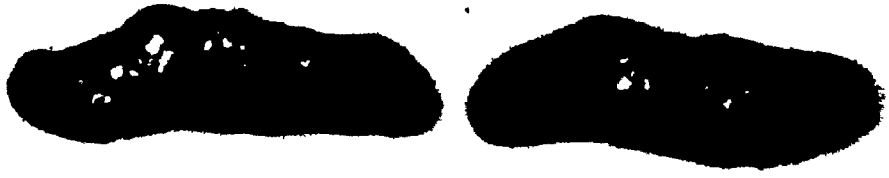
TABLE 9 : Uterine and ovarian weights and number of corpora lutea per ovary in rabbits immunized to BSA (controls, n = 3) or T-3-BSA (experimental, n = 5) and mated 24 hours previously.

1,$p<0.05$

2,$p<0.005$

*,n=4; due to undetected mating.

FIGURE 21 : Morphology of ovaries from a mated control (A) and experimental (B) animal, 14 weeks after immunization. Corpora lutea are visible in control but are masked in experimental animals due to the numerous hemorrhagic follicles.



A

B

FIGURE 22 : Section of ovary from a mated control (A) and experimental (B) ovary 14 weeks after immunization. Note the presence of corpora lutea (CL) in both sections and the large cystic (CF) and hemorrhagic (HF) follicles in the experimental ovary.
X10.

[redacted]

[redacted]

FIGURE 23 : Corpora lutea (CL) in a mated control (A) and experimental (B) animal 14 weeks after immunization and 24 hours after mating. X100.

[redacted]

[redacted]

DISCUSSION

As the results demonstrate immunization of female rabbits to testosterone has a marked and consistent effect on a number of components in the reproductive system. Total and percent bound testosterone and estradiol are altered, follicular development becomes abnormal, and the ovulation rate is increased. The interpretation of what this means in terms of the function of testosterone will be difficult. However, it is useful to ask two major questions : first, to what extent can we be assured that the immunization procedure has effectively prevented the action of testosterone and, second, how do these results fit in with other evidence concerning the role of testosterone in female reproduction?

EFFECTIVENESS OF IMMUNIZATION

Both antibody production, in terms of titer obtained, and the percentage of testosterone bound after immunization of female rabbits to T-3-BSA are similar to that found in the male rabbit (Nieschlag et al., 1973). In the male this was accompanied by atrophy of accessory reproductive glands and a loss of sexual activity; suggesting that testosterone was unavailable to cellular receptors in these tissues. Similarly the 'castration level' rise in LH and Leydig cell hypertrophy also observed are attributable to the effective binding of

testosterone to antibody resulting in loss of negative feedback control (Nieschlag et al., 1974 a).

The difficulty of assessing the effectiveness of this procedure in the female is that there is no established index by which to measure biological neutralization. While equilibrium dialysis has demonstrated that the amount of 'free' hormone in the serum is significantly reduced there is no information concerning whether the amount which remains 'free' is sufficient for certain androgen requiring functions nor does it indicate whether there are compartmental differences (e.g. follicular fluid) in the extent of testosterone binding.

Perhaps the most disturbing finding related to this is the initial rise and then marked decrease of testosterone within 24 hours after mating (Table 8). Wickings et al.(1976), using male rabbits immunized to testosterone, demonstrated a tenfold decrease in the metabolic clearance rate (MCR) of testosterone. Thus part of the reason for the increase in testosterone over the length of immunization is attributed to a decreased metabolic clearance rate of the antibody-bound-testosterone pool (Thorneycroft et al., 1975; Wickings et al., 1976). If the testosterone being produced is tightly bound to antibody a marked decrease in testosterone would not be expected. It is possible that a dramatic increase in tes-

tosterone production - as a result of mating - may saturate antibody binding sites resulting in a decreased binding and increased clearance of the hormone. However, the drop in testosterone values below pre-mating levels suggests that the antibody-bound-testosterone pool is relatively labile and once the production of testosterone by ovarian follicles is stopped - this occurring about 2 hours after mating (Mills et al., 1973) - the clearance of the pool is relatively rapid.

There is some evidence that immunization does not necessarily remove all the biological effects of the hormone. For example while immunization to estradiol prevents its LH-releasing activity there is still some evidence of its activity in vaginal and endometrial tissue (Sundaram et al., 1973). Fairclough et al.(1976) in fact suggest that the high binding affinity of steroid receptors may effectively compete for antibody bound hormone. Also, Thorneycroft et al.(1975) have found that in some animals neutralization of circulating testosterone in immunized males eventually ceased even though there were anti-testosterone titers.

This procedure, therefore, is not as clear cut as originally suggested. Despite these problems the technique is extremely useful to the study of reproductive processes; particularly in the study of feedback relationships and processes which may be governed by the ratio of two hormones;

for example the ratio of estradiol to testosterone which has been suggested to be involved in the regulation of follicular development.

TESTOSTERONE

The increase in testosterone levels in T-3-BSA immunized rabbits was significant in all cases; although the time required for this significant increase varied from 5 to 8 weeks. It is difficult to explain these high levels - in some cases as much as 50 ng/ml - in terms of simply a decrease in metabolic clearance. It is important to know both the source of this hormone and whether its production rate is affected by immunization; either by an increase in the number of follicles secreting the hormone or by an increase in the amount of hormone secreted by individual follicles.

In the case of the male high testosterone values can be primarily attributed to blocking of a negative feedback control mechanism resulting in the characteristic hyperplasia and hypertrophy of Leydig cells (Nieschlag et al., 1974 b). In the female there is no evidence of such a feedback relationship, however, it is possible that similar to the male, there is an increased rate of testosterone production. The results presented here provide some evidence that this may in fact be occurring; both the increase in follicular size

and the apparent hypertrophy of theçal cells may contribute to an increased production rate for testosterone. This is further supported by the observed dramatic decrease in testosterone levels 24 hours after mating. Interestingly, Surve et al. (1976) found a similar dramatic drop in serum progesterone between days 9 and 12 of pseudopregnancy in rabbits immunized to progesterone.

Of the researchers who have immunized females to testosterone only Mori et al.(1977), using the rat as a model, have measured serum testosterone values. They found a threefold increase in testosterone after 17 weeks of immunization. This is not as large as the thirteenfold or greater increase observed in this experiment, however, ovaries from both experiments were characterized by the presence of large cystic follicles.

The testosterone production rate of the ovary in these animals could be determined by measuring the difference between arterial and venous testosterone levels prior to and after immunization to T-3-BSA. At the same time the follicles of various sizes, including cystic and cystic-hemorrhagic follicles, could be incubated *in vitro* to determine the source of the testosterone.

ESTRADIOL BINDING

The increase in estradiol binding after immunization to testosterone presents a further complication to discussion of the effects of this procedure. The increase in binding is significant by 5 weeks of immunization and continues to rise in all groups until the end of the experiment; in some animals reaching almost 98%.

A similar effect has been noted by Nieschlag et al. (1974 b) when they immunized male rabbits to testosterone (estrogen binding increased from 81.6 ± 2.5% to 91.6 ± 2,2%). However, they failed to make any account of the reason for this or what significance it had to their interpretation. With the female more caution is required as estradiol is clearly an important steroid in female reproduction; any change in binding would be expected to alter its normal function.

What the estradiol is binding to remains a major question. A number of serum steroid binding globulins have been identified. These proteins appear to be involved in the regulation of the amount of 'free' steroid in the plasma (Westphal, 1971). A specific rabbit testosterone binding protein (R-TeBG), with high affinity and low capacity, has been demonstrated in the plasma of adult male (Rosner et al., 1973) and female (Mahoudeau et al., 1973 b) rabbits. DHT is an effective competitor for R-TeBG while estrone and estradiol

are poor (Mahoudeau et al.,1973 a). There is some contradiction concerning the binding of estradiol in rabbit plasma. Some reports indicate that estradiol does not appear to bind to any plasma proteins but shows a non-specific (low affinity, high capacity) type of binding (Westphal, 1971, p. 356; Rosner et al., 1973; Danzo et al., 1975). These results contradict reports by Mahoudeau (1973a) of estradiol binding both to albumin and a component having the same R_f value as R-TeBG.

There appear to be three possibilities to account for this increased estradiol binding : (1) despite the antiserum having a low cross-reactivity to estradiol (Table 1) the large concentration of antiserum present <u>in vivo</u> may be enough to increase estradiol binding, (2) if the affinity of testosterone for the antibody was greater than its affinity for R-TeBG there may be relatively little testosterone bound to R-TeBG, thereby removing the competition between estradiol and testosterone and resulting in an increase in estradiol binding, (3) assuming the presence of a binding protein for estradiol, the immunization may cause an increased concentration of this protein in the serum. Clearly any combination of these processes may be occurring. However, the dramatic increase in estradiol binding at 5 weeks, associated with maximal binding of testosterone by antibody, strongly implicates the first two processes. Obviously, the nature of this binding must be clarified if immunization to testosterone

is to continue to be used in studying female reproductive processes.

ESTROGEN

The observed significant increase in estrogen 9 weeks after immunization to testosterone does not conform to the hypothesis proposed in the introduction; that is that testosterone is used as a substrate for aromatization to estrogen (Armstrong et al., 1976 b). If this was the case a drop in estradiol production would be expected.

Interpretation of these results runs into the same problems as with testosterone. First of all there is the increased binding of estradiol. This could very easily alter the MCR of estradiol and lead to higher serum levels. Second, as will be discussed shortly, immunization may lead to a decrease in number of atretic follicles and therefore an overall increase in the production rate of estrogen.

A study by Baird (1977) using sheep showed that if antiserum to testosterone is infused into the ovarian artery over a 1 hour period estradiol production does not increase in response to a LH pulse. However, estrogen production could not be completely inhibited. He suggests that while thecal-granulosa cooperation in the aromatization of testosterone to estradiol appears to be important it may

not be the only source of estradiol and that in fact thecal cells may produce some estrogen.

Baird's results raise the possibility that the increased estrogen found in these experiments could in fact still represent a decrease in aromatization of testosterone to estrogen but an increase - perhaps due to both a greater number of follicles secreting 'thecal' estrogen and a decrease in the MCR - in total serum estrogen. As with testosterone this could be assessed by measurement of the MCR and ovarian production rate of estradiol prior to and at various times after immunization to testosterone.

GONADOTROPHINS (GROUP 1)

The gonadotrophin pattern found in the first 6 weeks of group 1 animals is similar to that found in the human female infant (Winter et al., 1975) and neonatal female rat (Goldman et al., 1971; Ojeda et al., 1977). Winter et al.(1975) have shown that the human female neonate shows a striking and prolonged rise in serum FSH levels which gradually decline over a four year period. In addition there is a small LH rise in the immediate neonatal period. In the neonatal female rat both serum LH and FSH are significantly elevated over diestrous adults (Goldman et al., 1971). Ojeda et al.(1977) suggest that, especially in the case of FSH, these high levels may be due to stimulatory action of progesterone and/or andro-

gens on gonadotrophin release from the pituitary.

If the stimulatory action of testosterone on FSH production in the rat was similar to the rabbit then one would expect immunization to testosterone to decrease FSH levels. While no decrease in FSH was observed in this experiment this may have been due to not immunizing the animals soon enough.

In this experiment the significant decrease in FSH values in both control and experimental animals between the first and sixth sample raises the possibility that we are observing in these 8 week old rabbits the tail-end of a similar FSH peak. It may be useful therefore to determine if in fact there is a similar occurrence in the rabbit and if so to question what the significance of this is in terms of follicular development. As demonstrated in this experiment numerous antral follicles are present by the end of the first sampling period. Asami (1920) found an abundance of small and medium follicles by 8 weeks of life in the rabbit. This information, along with the knowledge that in the rabbit meiosis does not begin until after birth and is not completed until the end of the third week of life (Teplitz et al., 1963), suggests a lucrative model for investigation of early follicular development. Possibly a more pronounced or totally different effect on follicular development and

gonadotrophin-steroid interactions would be obtained if neonatal rabbits were immunized to testosterone.

FOLLICULAR DEVELOPMENT

This experiment has demonstrated that immunization of female rabbits to testosterone considerably alters the course of normal follicular development. To summarize the results the following statements can be made : (1) the effect appears gradual and is first demonstrated by an increase in number of large follicles which appear normal as far as the number of granulosa cells and oocyte condition are concerned but perhaps having hypertrophied thecal cells (5 weeks), (2) by 8 weeks there are numerous large cystic and cystic-hemorrhagic follicles which possess few granulosa cells, degenerating oocytes, and marked hypertrophy of interstitial cells surrounding the cystic follicles, (3) from 8 weeks on, all animals have a significantly greater number of follicles 1.5 mm or larger although at 11 weeks few of these are hemorrhagic, (4) there appears to be an increased number of blood engorged vessels visible within the theca of these follicles, and (5) the size of the follicle does not appear to be related to whether or not it is hemorrhagic.

These observations suggest that immunization to testosterone has a protective effect on follicles; instead of becoming atretic they appear to continue to grow. This is

not surprising considering that administration of androgen has an atretogenic effect (Payne et al., 1958). With increasing "growth" - in terms of the size of the follicle - there is a depletion of granulosa cells and an increasing hyperamia leading to follicular fluid accumulation and sometimes hemorrhage. This deficiency in granulosa cells may be the reason for the absence of any significant increase in ovarian weight in these animals despite the presence of these large cystic follicles. Eventually the follicle assumes features which are recognized as being cystic. At least superficially these features are similar to a number of pathological and experimental conditions including polycystic ovary syndrome in humans (DeVane et al., 1975), persistent estrus (Edwards et al., 1977) and immunization to luteinizing hormone releasing hormone (LHRH) (Kerdelhue et al., 1976) or to T-3-BSA in the rat (Hillier et al., 1974), rabbit ovaries treated with inhibitors of prostaglandin synthesis (Grinwich et al., 1972; O'Grady et al., 1972) or antihistamines (Knox et al., 1977, 1972), and in primates immunized to estradiol (Ferin et al., 1974).

Before discussing these relationships in further detail it is necessary to first note the limitations inherent in the design of this experiment. First, no attempt has been made to quantify the number of follicles which are less than 1.0 mm. This is a major deficiency and is due largely to the

to the enormous amount of time required to count every follicle in each ovary of the 57 animals. Obviously what is needed now is a closer look at the total follicle population of a smaller number of animals. Second, there has been no attempt to classify follicles as healthy or atretic. Since most of the atretic follicles found in the rabbit are in the 0.4-0.5 mm range (Nicosia et al., 1975) and the rate of atresia increases with increasing follicular size (Byskov, 1976) it stands to reason that the majority of follicles greater than 1.0 mm would in fact be considered atretic. This distinction is, however, largely subjective and whether for instance the 1.0-1.5 mm follicle shown in Figure 11B with mitotic figures in the granulosa cells is able to ovulate remains unanswered. The third limitation to this design is depth; while there are obvious structural changes occurring in these follicles these changes have only been studied at the light microscopic level. If the sequence of events is to be followed accurately electron micrography will have to be used. Having stated these limitations it is still of value to speculate about what may be occurring in these animals.

There is evidence that both estrogen (Reynolds, 1973) and LH (Szego et al., 1964) cause histamine release in uterine and ovarian tissue respectively. Estrogen is known to have two receptor systems in the rat uterus : one genomic and related to increases in uterine RNA and protein synthesis

while the other eosinophilic and related to such things as water imbibition, increased vascular permeability, and histamine release (Tchernitchin et al., 1975). There is also evidence that progesterone may be involved in stimulating release of histamine from local mast cells within the ovary (Lipner, 1973). This results in an increase in the ovarian fractional blood flow as a result of the hyperemia induced by histamine (Wurtman, 1964) and presumably an accompanying increase in bulk transvascular filtration (Szego et al., 1964).

Most studies concerning histamine action have been related to its role in ovulation. There is no information on the importance of histamine in follicular development nor on its relation to the development of cystic follicles. From a purely speculative point of view, it is possible that in the ovary androgens may have an inhibitory action on histamine release and that immunization to testosterone prevents the inhibition and results in hyperemia; eventually leading to the characteristic cystic follicles.

However, the role of histamine in the production of cystic follicles is by no means clear. For example treatment of rabbits with chlortrimeton, an antihistamine, produces multiple large hemorrhagic follicles and a reduced number of ovulations when an ovulatory dose of hCG is given (Hamada et al., 1977 a; Knox et al., 1977, 1972). At the

same time Lipner et al. (1971) have shown that histamine depleted rat ovaries still become hyperemic and ovulate after administration of hCG.

Similar to the situation with histamine, it is known that steroids are involved in the regulation of prostaglandin synthesis (Le Maire et al., 1975). LH administration to indomethacin (inhibitor of prostaglandin synthesis) treated rabbits produces large cystic and cystic hemorrhagic follicles (Grinwich et al., 1972; O'Grady et al., 1972) and decreased ovulations (Armstrong et al., 1974; Grinwich et al., 1972; O'Grady et al., 1972; Hamada et al., 1977 b). Whether similarities between these animals and those treated with T-3-BSA, as far as the processes involved in the development of cystic follicles is concerned, must be considered.

The importance of understanding the processes involved in follicular growth and how these may go wrong can not be overemphasized. In women a pathological condition known as polycystic ovary is a relatively common clinical problem (Williams, 1974) and is associated with abnormal follicular growth. The hormone profile consists of low FSH, elevated androgen levels (DeVane et al., 1975). Ovarian vein estrogen is very low, however, serum estrogen is elevated due to peripheral aromatization of androstenedione to estrone (Baird et al., 1975). Histologically follicles are found to be cystic, in various

stages of atresia, and often have hyperplastic and luteinized thecal cells (Williams, p. 411, 1974).

There is a need to develop an animal model which could be used to study the processes involved in this condition. The persistent estrous condition in rats (induced by neonatal masculinization of the hypothalamus by androgens), characterized by polycystic ovaries, was originally proposed as a model. However, it was found that their gonadotrophin levels were the reverse of that found in the human condition (Edwards et al., 1977). This was true also for T-3-BSA immunized rats (Hillier et al., 1974).

Will the testosterone immunized rabbit serve as an appropriate model? The cystic ovaries are similar but in the rabbit neither FSH nor LH is altered. However, this does not rule out the possibility that an increased amount of gonadotrophin may be made 'available' to the follicle due to increased ovarian blood flow.

What appears common in these conditions is a hyperstimulation of androgen production associated with polycystic ovaries. What is different between the immunized animals and the persistent estrous and human polycystic conditions is that in the former testosterone is presumably inactive while in the latter two it is not. Clearly then all of the

above models may be made use of in an elucidation of the microenvironmental changes that lead to the overt polycystic condition. It is the transition period that must be studied closely. Use of the testosterone-immunized rabbit model may be useful in this regard as this experiment has identified a relatively narrow period within which the development of cystic follicles can be studied.

The marked hypertrophy of interstitial cells which occurs by 8 weeks of immunization (Figure 14 and 16) suggests that androgens may be involved in regulating interstitial-follicular interactions. The role of interstitial tissue in rabbit ovarian function is still a controversial issue. While it was originally proposed that the 20α dihydroprogesterone, produced by interstitial cells in large quantities after mating was required to enhance and maintain the post-mating gonadotrophin surge (Hilliard et al., 1969) there is now evidence to dispute this claim (Goodman et al., 1976; YoungLai,1977).

Whatever its functional significance is, the interstitial cell hypertrophy observed in this experiment is similar to what occurs after mating and during gestation in the rabbit (Spies et al., 1968). Hilliard et al.(1969) suggest that rabbit interstitial cells act as a storage reservoir for cholesterol esters and that these can be rapidly mobilized

for synthesis of 20α dihydroprogesterone in response to LH stimulation. Spies et al.(1968) suggest that in the rabbit estrogen and prolactin interact synergistically to induce hypertrophy of interstitial cells and to promote synthesis and release of 20α dihydroprogesterone.

There is evidence that incubation of rabbit ovarian slices with prolactin will decrease estradiol and increase androgen production (Powell et al., 1976). At this time the possibility of interaction between testosterone, prolactin and estradiol and their relationship to follicular development and interstitial cell hyperplasia remains purely speculative. This will probably remain so until a RIA for rabbit prolactin is developed. This experiment does suggest that immunization to testosterone may affect this relationship in some manner.

Interstitial cell hyperplasia is known to occur under other experimental and clinical conditions. For example, Kerdelhue et al.(1976) immunized female rats to LHRH and found strikingly similar results to that reported in this experiment; that is the presence of multiple cystic follicles and marked interstitial cell hypertrophy. In addition hyperplasia of interstitial cells is seen in some cases of polycystic ovary in the human (Greenblatt et al., 1976).

OVULATION

Perhaps the most surprising aspect of this experiment is the significant increase in ovulations in the testosterone-immunized rabbits. This occurs despite the presence of numerous cystic ovaries which in some cases appear to encompass the whole surface area of the ovary.

Edwards et al.(1977) have raised an important issue concerning the influence which cystic follicles may have on the growth and death rates of earlier stages. At least superficially these results suggest that follicular development appears to occur relatively independent of surrounding follicles. Certainly a population of follicles is capable of maturing and ovulating in response to a gonadotrophin surge despite the presence of multiple cystic follicles. There may, however, be some space constraint as corpora lutea do appear smaller in these animals. It would be interesting to know if these follicles attain their normal size prior to ovulation.

The principal questions remain, however, why do they ovulate at all and why in greater numbers? This certainly contradicts evidence in the rat where active immunization to testosterone totally prevents ovulation (Hillier et al., 1974) and administration of testosterone antiserum along with an ovulatory dose of hCG severely inhibits ovu-

lation (Mori et al., 1977). This is also contrary to the suggested requirement of testosterone for aromatization to estrogen (Fortune et al., 1977 b; Armstrong et al., 1976 b) and its postulated action in the first few hours after the ovulatory stimulus (Le Maire et al., 1975).

The results do support Peters' (1976) notion of the dissociation between growth events or controls and ovulatory events or controls. This may be especially clear in induced ovulators which are not dependent on estradiol stimulated gonadotrophin release. Under the experimental conditions as defined in this thesis the following mechanism may be proposed. As mentioned earlier the decrease in free testosterone may have a 'saving' effect on follicles resulting in a decreased rate of atresia (not measured directly). At some point in a follicle's growth a 'decision' is made either to (1) not grow any more and become atretic or (2) continue to grow and eventually ovulate. With immunization to testosterone the first decision appears to be blocked at least to some extent. Follicles continue to grow and eventually become cystic. If an ovulating stimulus (i.e. LH) is received then the same growth processes with the associated hyperemia and follicular fluid accumulation appear to occur and the actual ovulatory process is independent of the effects of immunization to testosterone. The increased ovulation rate may then be attributed to both an increase in the number of

available follicles and perhaps an increase in available gonadotrophin as a result of the hyperemia of the ovary. The small but significantly higher concentration of LH prior to mating in these animals may also have contributed to this increased ovulatory rate.

IMMATURE VERSUS MATURE RABBITS

There does not appear to be any marked difference between immunization of immature and mature animals. Both respond to immunization in a similar manner, however, mature animals do appear to have a delayed response in reaching a significant antibody titer and in the resulting increase in serum androgen. The explanation for this is not apparent.

Histologically there appeared to be some differences between mature and immature animals. Most noticeable is the significantly greater number of follicles in the 1.5 to 2.0 mm range found in the mature control animals. These results would suggest that as an animal ages the maximum size which follicles attain increases. The functional significance of this is difficult to determine, however, it is known that follicles are an important source of estrogen during pregnancy. Increased follicular size may contribute to this estrogen requirement. At the same time the increase in follicular size may represent a developmental

change in the mechanism regulating follicular growth.

Two qualifications must be made in the interpretation of this data : First, we do not know the age of the mature animals and, second, there is no record of whether the mature animals had been pregnant in the past. Despite this it appears that in the mature animals exposure of the ovaries to some environmental factor (s) whether it is pregnancy or age-related, results in altered follicular growth.

Further study of this phenomenon could provide significant information concerning the regulatory mechanism of follicular growth. Initial clarification of this process could be obtained by looking at follicular development in virgin females over a longer time span and females mated at defined frequencies over a similar time span.

The experimental ovaries of immature and mature animals did not appear to be significantly different. Both showed the characteristic cystic and hemorrhagic follicles. Interstitial cell hypertrophy did appear to be greater in mature animals. This may be related to the greater amount of interstitial tissue in the experimental animals.

The uterine weight of experimental groups is lower

than controls, however, only the mature animals show a significant decrease. The reason for this decrease is difficult to assess in light of the problems related to estradiol and estradiol binding. The mature uterus may be more responsive to a decreased availability of estradiol as a result of increased serum binding.

SUMMARY

Follicular growth and development is a complex process involving multiple hormone interactions. Considerable evidence now points to testosterone as playing a significant role in this regulatory process. Since the rabbit produces considerable quantities of testosterone and is an induced ovulator (thereby allowing a clearer distinction between extra - and intra - ovarian events) it has been used as a model to study the effects of active immunization to testosterone. Serum levels of total and percent bound testosterone and estradiol, serum gonadotrophins, and follicular development and ovulation were followed for a period of 14 weeks in both immaturely and maturely immunized animals.

Immunization to testosterone was found to dramatically increase serum testosterone and the percentage of bound testosterone. At the same time estradiol and the percentage of bound estradiol also increased significantly. At some time between 5 and 8 weeks after immunization follicular development beca-

me abnormal and was characterized by cystic and hemorrhagic follicles. In addition an increase in the number of ovulations per ovary was observed.

As may have been expected immunization to testosterone does affect other hormone besides testosterone and may in fact not completely inhibit the action of testosterone itself. Despite these difficulties the procedure promises to be extremely useful in the study of follicular growth and the processes involved in development of cystic ovaries.

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