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SEXUAL RECEPTIVITY AND FERTILITY OF ANDROGEN INDUCED PERSISTENT ESTROUS RATS

by

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The world is round, and the place which may seem like the end may be only the beginning.

Ivy Baker Priest

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ABSTRACT

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The effect of neonatally administered testosterone propionate (TP) on sexual receptivity and fertility was studied. Dosages of 25 µg and ten µg were used and were administered s.c. in peanut oil on the fifth day of life. Vaginal smears of the animals receiving 25 µg were begun at the age of 61 days and indicated that these animals exhibited modified estrous cycles. By 90 days of age, most of the animals in both dosage groups were in constant vaginal estrus. These prolonged periods of vaginal estrus were often interrupted only by mating.

At 97 days of age 40 females which received ten µg of TP were caged individually with males for six nights and 40% mated. At this same dose, out of 128 exposures, 42% of the females bred when exposed to males for one night. The latter group received 15 I.U. of chorionic gonadotropin (HCG) on the day of mating. In females receiving 25 µg of TP, out of 481 exposures, 33.6% of the animals mated. Eighty-nine per cent of the females which copulated had a vaginal smear consisting primarily of leukocytes the following morning.

Mating did not induce ovulation but ovulation did result after the administration of 15 I.U. of HCG. Priming with two mg of

progesterone two days prior to caging with the male did not induce ovulation, nor did it enhance ovulation after mating.

The progesterone and 20 alpha-hydroxy-pregn-4-en-3-one content of ovaries removed from females which had ovulated with HCG was determined. Ovaries were taken for five days post-mating. The 20 alpha content showed a decrease from day one through day five while the progesterone content went from a peak on day one to a non-detectable level on day two. There was a slight increase from day three through day five.

Thirty-five animals from each dosage group were given 15 I.U. of HCG on the day they were placed with the male. These 35 animals were then subdivided into three groups: (I) ten animals received two mg of progesterone per day for 20 days; (II) ten animals received four mg of progesterone for 18 days; (III) fifteen animals received no progesterone replacement therapy. There were no pregnancies in either of the two dosage groups and thus progesterone replacement therapy had no effect on fertility.

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INTRODUCTION

It is well established that the administration of testosterone propionate (TP) to five-day old female rats results in persistent vaginal cornification and permanent sterility when given in milligram doses (Barraclough, 1961). Behavioral estrus is lost in these animals despite the fact that they display vaginal estrus. These animals are anovulatory and will not receive the male (Barraclough and Gorski, 1962).

At lower doses (five-ten μg), however, reports vary as to mating behavior, ovarian function and fertility. Rats pretreated with ten μg of TP, while receptive to the male, exhibited bizarre patterns of receptivity as well as the same anovulatory persistent estrous syndrome as the animals receiving the larger doses (Barraclough and Gorski, 1962). A further study at the ten μg level confirmed these patterns of receptivity (Ericsson and Baker, 1966). In this latter study ovulation and pregnancy resulted leading the authors to suggest that perhaps the act of mating provided sufficient stimulus to trigger the release of hypothalamic factors which induced ovulation.

The present study was undertaken to investigate the receptivity patterns of rats receiving ten and 25 μg of TP; to study the effect of mating on the persistent vaginal estrus; to

determine if ovulation, either spontaneous or induced by mating, will occur and to observe the effect of progesterone replacement therapy on fertility.

REVIEW OF LITERATURE

The work of Moore and Price (1932) was the first to give a definitive explanation of a feedback mechanism between the pituitary and the gonads. They theorized that the cyclic ovarian and uterine changes observed in animals were due to an interplay between these two organs and the anterior lobe of the pituitary gland. They formulated the classic feedback mechanism in which the ovarian follicle grows and secretes estrogen while under the influence of follicle-stimulating hormone (FSH). This rising estrogen titer in the systemic circulation reaches the anterior lobe inhibiting further secretion of FSH, and induces the release of luteinizing hormone (LH) causing the mature follicle to rupture. The resulting high levels of progesterone released by the corpus luteum then inhibit LH secretion. This classical mechanism of inter-related control is still widely used today in elementary biology and physiology texts.

Soon after this, however, it was seen that this proposed mechanism presented only an incomplete picture of the actual chain of events. The central nervous system also participates in this feedback mechanism interposed between the ovary and the adenohypophysis (Hohlweg and Junkmann, 1932). These same

investigators also theorized that the portion of the central nervous system involved here was the hypothalamus. The hypothalamus was later shown to exert its effect via neurohumoral agents which travel by way of the portal system of the hypothalamo-hypophysial unit (Harris, 1937). It is interesting to note that the possibility of neural involvement was recognized by Haighton in 1796 in his work with rabbits. He demonstrated that ovulation was triggered by an external stimulus (copulation) and that the ovary responded nine hours later.

Pfeiffer (1936) was the first to investigate the effects resulting from gonadectomy and gonadal transplants in neonatal rats. The main results of his work are summarized by Harris (1964):

1. Male rats castrated at birth and transplanted with an ovary when adult, had the capacity to form corpora lutea in this ovarian tissue.
2. Female rats ovariectomized at birth and implanted with an ovary when adult showed normal estrous cycles and corpus luteum formation.
3. Male rats in which the testes were transplanted into the neck region at birth, and which were implanted with an ovary when adult showed no capacity to form corpora lutea in the ovarian tissue.

4. Many female rats into which testes were transplanted at birth failed to show any sign of estrous cycles when they became adult, but entered a state of constant vaginal estrus and failed to form corpora lutea in the ovaries.

As a result of these data Pfeiffer (1936) proposed that the hypophysis underwent sexual differentiation which was not due to genetic influences, but rather to the presence or absence of steroids and differentiated sex glands. The animal is born with the ability to secrete gonadotropins in a cyclic fashion but this ability is lost if the hypophysis comes under the influence of the testes. A similar situation exists in the case of the differentiation of the duct system (Mullerian or Wolffian) in the fetus. The duct system has the inherent capacity to develop as a female unless the influence of the testes is exerted, in which case, development follows the male pattern (Burns, 1964). It is worth noting here that while Pfeiffer had the right idea about the process of sexual differentiation, i.e., that it was due to the presence or absence of steroids and differentiated sex glands, he was in error concerning the anatomical structure affected by these steroids. The effect(s) of steroids on sexual differentiation were further studied and refined by a number of workers (Mazer and Mazer, 1939; Bradbury, 1941; Huffman, 1941). These investigators found that the administration of exogenous steroids could replace the cumbersome method

of testicular transplants employed by Pfeiffer. They observed that androgen given to prepubertal female rats would result in sterility.

That it is the central nervous system, rather than the pituitary gland, which undergoes sexual differentiation was demonstrated by the use of CNS blocking drugs such as dibenamine and Nembutal (Everett, Sawyer and Markee, 1949; Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949). These drugs, it was found, could block ovulation by preventing the release of LH in the rat. Further evidence that the pituitary was not the site involved was provided by a number of investigators (Sawyer, Everett and Markee, 1948, 1949; Everett and Sawyer, 1949; Sawyer, Markee and Everett, 1949, 1950; Harris and Jacobsohn, 1952; Martinez and Bittner, 1956). In essence this work demonstrated that transplanting the pituitary to other than the normal location in the body resulted in the loss of adenohypophysial functions. The work also showed that by placing a male pituitary beneath the hypothalamus of a previously hypophysectomized female, one could maintain normal functions in the female, including estrous cycles, pregnancy and lactation.

Strong direct evidence of hypothalamic importance here was provided by Evans (1965). He demonstrated the ability of ovine median eminence extracts (MEE) to reactivate, functionally and cytologically, eight-67 day old renal pituitary autografts. Thus there is evidence that hypothalamic neurohumors act not

only as "releasing" factors but also have tropic effects on many other phases of adenohypophysial function.

The observation that early androgen treatment would result in sterility in the female rats was expanded by demonstrating that a single injection of 1.25 mg of testosterone propionate (TP) induced sterility in five-day old mice (Barraclough and Leathem, 1954). An important aspect of this work is that this androgen syndrome could be induced by a single injection, whereas, previously, administration was prolonged over a period of time and large doses were injected. Barraclough (1961) showed that 1.25 mg of TP injected into five-day old rats induced permanent sterility and resulted in a constant vaginal estrus accompanied by an anovulatory condition.

The possibility existed, however, that this sterility may have been due in part to ovarian malfunction. Jacobsohn (1964) demonstrated that this was not the case by transplanting anovulatory ovaries from TP-treated females to the kidney capsule of recipient ovariectomized female rats. These ovaries were normal in appearance and function and ovulated normal ova. Similar transplants were also employed in which other sites were used. When the ovaries of TP-sterilized females were placed in the eye chamber of a normal ovariectomized rat, normal cycles were maintained complete with ovulation and corpus luteum formation (Harris, 1965). If, however, these ovaries were placed in the eye chamber of a TP-treated rat no ovulation occurred.

In support of the hypothesis that it was the hypothalamus and not the pituitary which was affected by the neonatal administration of TP, the anterior pituitary was removed from such treated females and transplanted into the sella turcica of hypophysectomized animals (Segal and Johnson, 1959). The recipient female experienced normal estrous cycles and even pregnancy. From this and other data it appears certain that it is the central nervous system, and probably the hypothalamus which is responsible for the anovulatory, constant estrus condition observed in the neonatally TP-treated rat. Further evidence that this androgen effect is manifested at a level higher than the pituitary is supplied by the observation that TP-sterilized rats will release sufficient LH to cause ovulation when given an electrical stimulation to the hypothalamus (Gorski and Barraclough, 1962). In females sterilized with ten μg of TP electrical stimulation alone was sufficient to induce ovulation. In females treated with 1250 μg of TP, however, priming with two mg of progesterone two days prior to stimulation was required. This progesterone administration did not cause ovulation but it allowed the pituitary gland to store sufficient LH to cause ovulation when released.

Testosterone propionate is not the only steroid which has been shown to be effective in producing this condition, although data and conclusions differ between certain investigators. Takewaki (1962) states that among other effective compounds are

estrogens, progesterone, desoxycorticosterone, acetate and cholesterol. Gorski (1963) observed that while various doses of estradiol benzoate from 100 μg to one μg will induce sterility in 93.4% and 41.7%, respectively, as will diethylstilbestrol, only androgens will produce true persistent vaginal cornification. Steroids showing primarily anabolic activities also have been shown to induce sterility (Jacobsohn, 1964).

The timing of this administration has been shown to be critical. Barraclough (1961) showed that 1.25 mg of TP administered to two and five-day old female rats resulted in permanent sterility in all cases. Similar injection into ten-day old females resulted in fertility in six out of ten animals while injection into 20-day old females resulted in fertility in 16 out of 16 cases. Thus there is a period of sensitivity somewhere between birth and ten days of age.

This critical period was narrowed still further (Swanson and van der Werff ten Bosch, 1964) by giving various doses on various days. Small doses, five and ten μg, were more effective the earlier they were given. Five hundred μg induced persistent estrus and infertility whether given on day one, two or four. Thus, with small doses the timing is more critical. It appears that physiological masculinization does not take place, or has not been completed, before day four since removal of the testes on that day allows the occurrence of ovulatory cycles in ovaries implanted at a later age (Swanson and van der Werff ten Bosch,

1964). Thus the critical period appears to be between days four and six post-natal. It seems that by day four either the level of endogenous androgen has not yet increased sufficiently to cause an effect on the gonadotropic pattern, or that the brain is not yet sufficiently sensitive. Swanson and van der Werff ten Bosch (1964b) estimate that the amount of androgen secreted by the newborn male rat during the critical period is equivalent to five-50 μg.

Work has also been done (Swanson and van der Werff ten Bosch, 1964a) in which it was shown that the rats do not become anovulatory until some time after reaching sexual maturity (the time depending on the dose -- at five μg animals became anovulatory at 21 weeks). Doses of 50-100 μg resulted in modified estrous cycles with prolonged periods of vaginal cornification until the age of ten weeks, at which time most of the animals were anovulatory. The larger the dose, the sooner the anovulatory syndrome develops, with a sufficiently large dose blocking even the first ovulation.

The hypothalamus has been shown, as previously mentioned, to be the structure of the central nervous system which is affected by TP administration. The area within the hypothalamus which undergoes sexual differentiation has been localized (Gorski, 1965). This has been found to be the anterior hypothalamic-preoptic area (AH-POA). It is this region which regulates the cyclic discharge of LH necessary for ovulation. This

was refined to a still greater degree by the use of a small knife developed by Halasz which made it possible to sever certain neural connections of the hypothalamus to the median eminence region (MER). It was discovered that when dorsal, lateral or posterior connections to the MER were cut, 90% of the rats continued to ovulate cyclically. When the connections between the anterior hypothalamus and the MER were severed, however, (by a frontal cutting just behind the optic chiasma and extending 1.5 mm lateral), more than 90% of the rats became anovulatory. Gorski goes on to explain the theory of the cyclic and tonic centers of the hypothalamus. The tonic discharge of gonadotropic hormones (male type) is under the influence of the arcuate-ventromedial nuclei (Arc-VMN). The anterior hypothalamic regulation of FSH secretion is unaffected by androgen treatment (Gorski and Barraclough, 1962; Swanson and van der Werff ten Bosch, 1964b). From this then one may say that the hypothalamus exerts a dual control over gonadotropin secretion. The Arc-VMN regulates the tonic discharge of gonadotropins while the AH-POA is responsible for ovulation by eliciting LH release and for sexual behavior (Gorski, 1965). A working hypothesis is put forward by Gorski in saying that by administering androgen to the female, we duplicate what nature does in the male.

If ovulation can indeed be induced by hypothalamic stimulation in the androgen treated rat, then there must be sufficient LH present in the adenohypophysis to cause ovulation when

released. Here the effect of age becomes a factor. Studies on androgen-sterilized, persistent estrous rats at the age of 150-200 days demonstrated that the anterior lobe content of LH was 1/3 that found in the normal rat (Gorski and Barraclough, 1962). Other workers (Segal and Johnson, 1959), on the other hand, used rats 60 days old and found that adenohypophysial LH content was normal. Thus Gorski and Barraclough propose a difference in the storage ability of the anterior pituitary due to the longer exposure to the unusually high and constant estrogen titers present in the TP-sterilized female rat.

The mating behavior of the rat treated with various doses of TP has been investigated as well as the effects of mating on the constant vaginal estrus, ovulation and presence or absence of corpora lutea. Barraclough and Gorski (1962) report that females receiving 1.25 mg of TP were nonreceptive to males after caging with proven males for 30 days. These animals never ovulated. This same paper also reports that females receiving ten μg did not ovulate but did receive the male. In further work at the ten μg level, Ericsson and Baker (1966) report that not only will the female receive the male but will do so as many as seven times after caging with the male for ten consecutive nights. Of the animals which mated in this study, 44% conceived with 2/3 of this number going to term. Females which received 100 μg of TP were receptive to the male in six out of 13 cases after remaining with the male for six nights (Cornette, personal communication).

When this group was autopsied there was no evidence of ovulation ever having occurred and no corpora lutea were present. In another study at 100 μg (Segal and Johnson, 1959) 18 out of 60 females accepted males after 3 months of cohabitation. At this dose ovulation occurred neither before nor after mating. Of these 18 females which mated, all mated more than once, one as many as nine times. From these data concerning mating behavior at varying doses, it appears that these animals did not ovulate spontaneously nor did they ovulate as a result of mating stimuli except in the instance reported by Ericsson and Baker. In this latter case it appears that TP treatment resulted in an ovulatory pattern similar to that which occurs naturally in the rabbit and other induced ovulators.

The objectives of this study then were to investigate the sexual receptivity and fertility of female rats receiving low doses of TP at the age of five days.

PROCEDURE

Two groups of rats were used in this study. Group I was composed of 120 female Spartan (Sprague-Dawley strain) rats which were injected s.c. with ten μg of testosterone propionate (TP) in 0.05 ml of peanut oil on day five of life (day of birth called day one). Group II was composed of 83 Upjohn (Sprague-Dawley strain) female rats. This group received 25 μg of TP s.c. in 0.1 ml of peanut oil on day five. Both groups were weaned at 21 days of age.

Group I was maintained at 72 degrees F ± 2 degrees with 14 hours of artificial light per day. They received Purina Laboratory Chow and water ad libitum. Daily vaginal smears were begun at the age of 92 days. The vaginal smears were taken by lavage using about 0.5 ml of tap water. The washings were placed on a glass slide and the types of vaginal epithelial cells were noted under a microscope and recorded.

Group II was housed three per cage with daily hours of light corresponding to outdoor cycles except for the last month and a half of the study, during which time they were maintained with 12 hours daily of artificial light. They received Wayne Lab Blox and water ad libitum. Vaginal smears were taken from 38 rats from this group when they were 61 days old and continued

daily for ten days. Smears were recorded on all animals beginning at the age of 90 days and continued throughout the study. These two groups of animals were used for the following experiments.

Group I. Forty females were placed one per cage with proven males for six consecutive nights beginning at 97 days of age (mating no. one). The males were removed each morning and replaced that evening. No effort was made to insure that the females were caged with a different male each night. Vaginal plugs and/or sperm in the vagina were taken as evidence that mating had occurred. The types of epithelial mucosa cells seen in the smears were recorded.

Over a period of about three weeks 105 females were exposed to males (mating no. two). On the day they were placed with the males, all females had predominantly cornified cells in their vaginas and all were treated with 15 I.U. of chorionic gonadotropin (Chorionic Gonadotropin, Upjohn). Twenty-three of the 105 females were used twice with at least two weeks between exposures. Thus 82 females were used once and 23 were used twice for a total of 128 exposures. All females were checked the following morning for evidence of mating. The percentage of females which bred was calculated and, again, the types of epithelial cells were noted and recorded.

To determine if mating would provide a sufficient stimulus to cause ovulation, eight animals were killed by cervical

dislocation the morning after mating and the oviducts flushed with about 0.5 ml of isotonic saline. The flush was then examined for ova. The oviduct was dissected from the ovary and placed on a glass slide under a dissecting microscope. If ova were seen in the ampulla, a slit was made at this point and the ova emerged. If no ampulla was seen, the oviduct was straightened and flushed by inserting a 30 gauge needle in the ovarian end.

To determine if chorionic gonadotropin (HCG) would induce ovulation, ten animals received 15 I.U. of HCG between 12:30 p.m. and 1:00 p.m. on the day they were placed with the males. They were killed the following morning regardless of whether mating had occurred and examined for evidence of ovulation.

Another group of animals received 15 I.U. of HCG, again between 12:30 p.m. and 1:00 p.m., and were placed with males that night. Fifteen animals which mated were killed three per day for five days to determine: a) if ovulation had occurred; b) the location of the ova in the oviduct; and c) if fertilization had taken place. The location of the ova was determined by pressing the oviduct between two glass slides and observing it under the dissecting microscope. In all the preceding cases where the animals were killed, the ovaries were removed. One ovary from each animal was placed in Bouin's fixative and serial sections were prepared. They were cut to a thickness of five microns and stained with hematoxylin and eosin. The second ovary

from each animal was placed in 2.5% sodium hydroxide solution and frozen to be later assayed for progesterone content by thin layer and gas chromatography. For the thin layer chromatography, the ovaries were extracted with ether and the residues were transferred with chloroform to glass plates spread with a 0.5 mm thickness of silica gel (Silica Gel G, Merck). Chromatography was accomplished in a saturated chamber with a cyclohexane-ethyl acetate (1:1) mixture. Gas-liquid chromatography was performed on an F and M Model 500 gas chromatograph equipped with a flame ionization detector and an automatic attenuator (Neill, et al., 1964).

Thirty-five animals ranging in age from 138-152 days, which mated (after receiving 15 I.U. HCG to insure ovulation) were divided into 3 groups. 1. Fifteen animals received no progesterone replacement therapy. 2. Ten animals received 2 mg of progesterone s.c. in sesame oil and 5% benzyl alcohol daily for 20 days starting on day two post-mating. 3. Ten animals received 4 mg of progesterone for 18 days starting on day two post-mating. Groups 2 and 3 were autopsied on days 22 and 20 post-mating, respectively.

Ten animals were primed with two mg of progesterone and placed with males two days later. They remained with the males for five consecutive nights. Starting the next morning, these animals were killed two per day for five days and examined for evidence of ovulation.

Group II. The work with this group followed the same basic pattern as with the Group I animals. For the study of mating behavior the animals were divided into four sub-groups. Mating no. 1. Forty females were placed one per cage with proven males for one night when they were 100 days old. Mating no. 2. Twenty-two females were placed one per cage with proven males for five consecutive nights. Mating no. 3. Nineteen females were placed one per cage with proven males for ten nights. Mating no. 4. Over a period of about two months 71 females were exposed to males. All females received 15 I.U. of HCG the day they were placed with males. Seventy of these females were used twice in the mating study and the other animal was used once. At least three weeks elapsed between exposures. Thus there was a total of 141 exposures of females to proven males in this sub-group. In all four of the preceding sub-groups females were checked each morning for evidence of mating and the types of epithelial cells in the smears were recorded.

The procedure employed with the rats from Group II in regard to ovulation data was identical to the method used with Group I with the exception that the location of the ova was not determined in Group II.

Again, three groups were set up to test the effect of progesterone replacement therapy on fertility. These animals ranged in age from 170-185 days and received HCG on the day they were placed with the males to insure ovulation. The three groups

were as follows: Group 1. Fifteen animals received no progesterone replacement therapy. Group 2. Ten animals received two mg of progesterone s.c. for 20 days starting on day two post-mating. Group 3. Ten animals received four mg of progesterone daily for 18 days starting on day two post-mating. The same batch of progesterone was used throughout this study.

RESULTS

Vaginal smear records were not taken on Group I before 92 days of age. However, daily records of the vaginal smears of Group II were taken for ten days beginning when the animals were 61 days old. Figure 1 illustrates that most of the rats in Group II were not in persistent vaginal estrus at this time. Rather, they exhibit somewhat modified cycles prior to going into constant estrus. The smear records of both Groups beginning at about 90 days of age show that most animals displayed prolonged periods of vaginal estrus. In both groups these extended periods of cornified cells were in many cases interrupted only after mating. They resumed a few days after mating except in the cases where progesterone replacement therapy was used. In this latter case the animals retained leukocytic smears until autopsy.

In considering the results of mating studies, Group I (ten μg) will be considered first. When 40 females 97 days old were exposed to males for six consecutive nights (mating no. 1) 16 mated (40%). Of the 16 which mated, one bred on three consecutive nights and three mated on the first and last nights of exposure. The remaining 12 mated once. In the case of the 128 exposures of females to males for one night (mating no. 2),

Figure 1. Vaginal Smears of Female Rats 61-70 Days Old After Receiving 25 μg of TP at 5 Days of Age

Rat Number	Days 61	62	63	64	65	66	67	68	69	70
1-1*	Ns**	S	S	S	Sw	S	Ns	S	N	S
1-2	W	Sn	N	N	Ns	S	Ns	Ns	Sn	Ns
1-3	Ns	NS	Ns	Ns	Sn	Sw	SN	S	N	S
2-1	Ws	Sw	W	Nw	N	Ns	S	Sw	SN	WN
2-2	S	S	Ns	Ns	N	N	Sn	S	S	Ns
2-3	Sw	W	N	Sn	S	Sn	S	S	S	Sn
4-1	S	Sn	N	Ns	Ns	Sn	S	SN	Wn	Sw
4-2	Sw	W	Nw	N	Ns	S	Ns	W	W	Nw
4-3	Sn	W	W	Ns	S	Sn	Sn	Sn	W	W
5-1	S	Sn	S	S	S	S	Sn	Ns	N	N
5-2	W	W	N	S	S	S	Ws	Wn	Ns	Ns
5-3	Sn	Ns	N	Wn	W	Sw	Sn	Ns	Wn	W
6-2	Sn	Ws	N	Ns	S	S	Nw	SW	W	N
6-3	Sn	Ns	Sn	Ns	Sn	S	Sn	SN	S	S
7-1	Ws	N	Ns	Sn	S	S	S	S	S	S
7-2	Sw	Ws	S	S	Sn	S	Sn	SW	W	N
7-3	S	Sn	Sw	N	Sn	Sn	SW	W	Ws	S
8-1	S	Sn	N	Sn	Nw	W	W	Ns	Sn	S
8-2	S	Sn	Wn	W	Ws	Ns	S	Sn	Sn	W
8-3	S	S	S	Sn	N	Ns	Ns	S	S	Sn
9-1	W	W	W	Ns	NW	Wn	W	Ns	Ns	W
9-2	Ws	N	S	Sn	Sn	Ns	NW	N	NS	S
9-3	S	S	SN	SN	S	S	S	S	Sn	S
10-1	S	Sw	W	W	N	Sn	S	Sw	Sw	S
10-2	Sw	Sw	Ws	Ns	Ns	Ns	Sw	W	N	Ns
10-3	S	S	Sn	Ns	N	Ns	Sw	S	S	Sn
11-1	Ns	Sn	S	S	Sn	N	Ns	Sn	S	S
11-2	S	Sn	S	S	S	S	Sn	Sn	Sn	S
11-3	Ns	Sn	S	S	Sn	Sn	Sw	Ns	Sw	Sn
12-1	N	Ns	Ws	W	N	S	S	S	S	Ns
12-2	Wn	N	Sw	S	S	Ns	SN	Ws	N	S
12-3	S	S	S	S	S	SN	Sn	S	S	Ns
13-1	N	N	Ns	NS	Ns	Ns	N	N	Nw	NS
13-2	Ns	W	W	Sn	S	N	Wn	Nw	N	NS
13-3	Sn	Ws	S	S	S	Ns	S	S	S	S
14-1	N	N	S	S	S	Sn	N	S	S	NS
14-2	Sn	Ns	Ws	W	W	W	Ns	S	S	S
14-3	N	S	S	S	S	SW	W	W	N	Sn

*Litter number and rat number.

**S = cornified cells; W = leukocytes; N = nucleated epithelial cells. Lower case letters indicate that these cells were present in fewer numbers.

54 mated (42%). All females were in vaginal estrus when placed with the male. The smear records for these two mating groups show that of the 16 which mated in the first group, all displayed leukocytic cells in the vagina the following morning. Eight of these animals were killed the morning after mating to test for ovulation. The other ten which copulated maintained leukocytic cells in the vagina for an average of 11.8 days (range 3-21). This indicates that apparently these animals became pseudopregnant but that none became pregnant. Of the 54 which mated in the second group, 47 had leukocytes in the vagina the next morning, four displayed leukocytic cells two days after mating and three animals retained cornified cells despite copulation. Fourteen of these 54 animals were killed the morning after mating and thus no data is available on how long they retained leukocytic smears. Twenty of these animals were put on progesterone replacement therapy and retained leukocytes in their smears until they were autopsied. The remaining 13 animals which had vaginal leukocytes the morning after mating retained this type of smear for an average of 11 days. Again, mating apparently resulted in pseudopregnancy but no pregnancies occurred.

The mating behavior of Group II (25 μg) was as follows. Mating no. 1. Thirteen out of 40 females bred when placed with males for one night at the age of 100 days (32.6%). Mating no. 2. When 22 females were exposed to males for five nights, six bred (27.3%). Mating no. 3. Of the 19 females placed overnight

with males for ten consecutive nights, eight bred (42.1%). Four of these animals bred twice on successive nights. Mating no. 4. For the 141 exposures for one night a total of 46 rats mated (32.6%). In mating no. one, five of the animals which bred retained cornified cells in their vaginas; the other eight animals which mated had leukocytic smears for an average of 3.9 days. In mating no. 2, two of the six animals which bred retained cornified cells while the other four had leukocytes in their vaginas for an average of 6.7 days. In mating no. 3, two of the eight females which bred had only cornified cells in their smears. The remaining six retained leukocytic smears for an average of 7.3 days. In mating no. 4, the 44 animals which had leukocytic smears the morning after mating remained with this type of smear for an average of 10.4 days. These data are summarized in Table 1.

Animals from Groups I and II were assayed for ovulation the morning after mating to determine if copulation could induce ovulation. In Group I, no ova were found in the oviducts of seven of the eight animals tested. The eighth shed one ovum which appeared abnormally large. Histological examination of serial sections of the ovaries of these animals removed the following morning bore out these findings in that no ovulation points were seen nor was there any sign of luteinization beginning. In Group II ova were absent in all eight animals killed the morning after mating. Again, these results were

Table 1. Mating Percentage of TP-treated Female Rats with or without HCG Treatment, Number of Females with Leukocytic Smears the Following Morning and Length of Time these Animals Retained Leukocytic Smears

	No. of Females Exposed to Males	No. of Exposures*	No. of Females Mating	No. of Matings	No. of Females with Leukocytic Smears	Av. No. of Days with Leukocytic Smears
Group I.						
Mating no. 1.	40	240	16	21	16	11.8
Mating no. 2. (HCG)	128	128	54	54	47	11.0
Group II.						
Mating no. 1.	40	40	13	13	8	3.9
Mating no. 2.	22	110	6	6	4	6.7
Mating no. 3.	19	190	8	15	8	7.3
Mating no. 4. (HCG)	141	141	46	46	44	10.4

*Number of exposures is obtained by multiplying the number of animals times the number of nights with the males.

borne out by histological examination.

Ten animals from both Groups (also used in the mating study) received 15 I.U. of HCG the day they were placed with males to determine if this would induce ovulation. All 20 of these animals were found to have ovulated when flushed the following morning regardless of whether mating had occurred. In all cases the ova were located in the ampulla.

Fifteen additional rats of Group I (again, also used in the mating study) received 15 I.U. of HCG and were killed three per day for five days after treatment. All fifteen rats showed evidence of ovulation. The locations of ova were noted. A summary of the ovulation results is presented in Table 2. The number of ova ovulated was not recorded but it ranged from three to eight per ovary in each rat. The location of ova in the oviduct on the five succeeding days after HCG treatment is given in Table 3.

On day 3 the ova were in the two-cell stage and the polar body was clearly visible. An effort was made to place one of these cleaved ova on a glass slide under a microscope to examine it for evidence of fertilization. The procedure failed. Although no ova were found on days 4 and 5, it was evident from gross inspection of the ovaries and from the examination of histological sections that ovulation had occurred.

Ten rats which received ten μg of TP were killed after progesterone priming and flushed and examined for evidence of

Table 2. Incidence of Ovulation as a Result of Mating, HCG Treatment or Progesterone Administration

	No. of Animals Flushed	No. of Animals which Ovulated
As a Result of Mating		
Group I.	8	1*
Group II.	8	0
15 I.U. HCG		
Group I.	25	25
Group II	10	10
2 mg Progesterone		
Group I.	10	2*

*Abnormally large ova.

Table 3. Location of Ova in the Oviduct of Female Rats Treated with 15 I.U. HCG

	Location
Day 1.	Ampulla
Day 2.	Posterior 1/3
Day 3.	Uterine-Oviducal Junction
Day 4.	No Ova Found
Day 5.	No Ova Found

ovulation. They had been placed with males two days after receiving two mg of progesterone and remained with them for five days. The progesterone put the rats in a state of vaginal diestrus for at least two days followed in three cases by one day of a proestrus vaginal smear. Of the ten, only two ovulated -- one six days after progesterone administration and the other seven days after this treatment. The one which ovulated after six days had mated the previous night while the other animal had not mated on any of the four previous nights. In both cases only two ova were shed and both appeared abnormally large.

The progesterone analysis was carried out in the laboratories of The Upjohn Company and the results are summarized in Table 4. The results are given in micrograms but are not as meaningful as they might be because control values are not available and because the weights of the ovaries were not determined when they were removed.

Of the 70 rats used in the fertility study, none were pregnant. All animals receiving progesterone had corpora lutea at autopsy but none were pregnant. The females from Group I in this fertility study had leukocytic smears for an average of 11.3 days (range 8-14). The females from Group II had leukocytes in their vaginas for an average of 12.6 days (range 7-15). The results of this section are summarized in Table 5.

Table 4. Progesterone and 20α-hydroxypregn-4-en-3-one Content of Ovaries Removed from TP-treated Females on Days 1-5 after 15 I.U. of HCG

	Progesterone	20α
Day 1.	8.46 μg/3 ovaries	0.58 μg/3 ovaries
Day 2.	-----*	1.54 " "
Day 3.	0.18 " "	0.25 " "
Day 4.	0.29 " "	0.25 " "
Day 5.	0.95 " "	0.17 " "

*Not detectable.

Table 5. Incidence of Pregnancy, Length of Time with Leukocytic Smears, Number of Animals with Corpora Lutea in Groups Receiving no Progesterone, 2 mg Progesterone/day and 4 mg Progesterone/day.

	Treatment	No. of Animals Used	No. of Animals Pregnant	Av. No. of Days with Leukocytes	No. of Animals with Corpora
Group I.					
	None	15	0	11.3	-----*
	2 mg prog.	10	0	20	10
	4 mg prog.	10	0	18	9
Group II.					
	None	15	0	12.6	14
	2 mg prog.	10	0	20	10
	4 mg prog.	10	0	18	10

*Not autopsied.

There are two interesting results not yet mentioned. One animal receiving ten μg of TP was the only animal to get pregnant and go to term and she had received neither HCG nor progesterone. One animal from Group II was autopsied at 26 days post-mating and was not pregnant but had numerous corpora lutea. It is doubtful that this animal had ovulated during this interval as she had only leukocytes in her smears for the entire time.

DISCUSSION

The administration of testosterone propionate (TP) to five-day old female rats approximates what nature accomplishes in the male (Gorski, 1965). The presence of androgens (as well as a number of other compounds) at the time of sexual differentiation of the hypothalamus results in the tonic, male pattern of gonadotropin release. This differentiation can be considered to be a normal step in the development of the animal, but one which occurs after parturition rather than during gestation. This is demonstrated by the fact that when 20-100 μg of TP are given s.c. to fetuses one to four days before birth, masculinization of the external genitalia occurs as well as the "androgen syndrome" (Swanson and van der Werff ten Bosch, 1965). Thus the presence of androgen affects the process of sexual differentiation and any physical sexual development which occurs after its administration. This includes the very early stages of development in which either the Mullerian or Wolffian duct systems develop depending upon the presence or absence of male hormones.

The administered androgen appears to affect the anterior hypothalamic-preoptic area (AH-POA). This is the region which has been found to regulate both ovulation and sexual behavior (Gorski, 1965). The area of the hypothalamus which regulates the

tonic discharge of gonadotrophin, the arcuate-ventromedial region (Arc-VMH), does not seem to be affected by the neonatal administration of TP. Evidence that it is indeed the hypothalamus and not the pituitary gland which is affected is presented in the Review of the Literature section of this paper.

The time at which persistent vaginal estrus is manifested depends on the dose given (assuming that all injections are given on day five of life). In rats receiving 25 μg, the vaginal smears at 61-70 days of age indicate that the animals are undergoing modified estrous cycles. By day 92 most of the females receiving both ten and 25 μg had established a more constant estrous pattern. This was characterized by prolonged periods of vaginal cornification extending for as long as 53 days. This vaginal estrus was often interrupted by mating which resulted in a smear composed of predominantly leukocytes. This would indicate that perhaps the act of mating triggered a neural stimulus sufficient to alter this constant pattern. More will be said of this later.

The mating behavior of these androgen sterilized rats provides an interesting extension of the vaginal cytology. The difference between vaginal estrus and behavioral estrus became quite pronounced in the group of rats receiving ten μg of TP. About 96% of these animals were in vaginal estrus at the time they were exposed to males and yet only 41% mated. A similar trend is also apparent in the groups receiving 25 μg. Here

again the concept of the cyclic and tonic centers of the hypothalamus arises. The administration of TP affects the cyclic center which is responsible for sexual behavior. However, sexual behavior is also affected by estrogen. Since FSH secretion is mediated by the tonic center which is unaffected by TP, the estrogen output of the polyfollicular ovary must also be a factor in the mating behavior of these females. These estrogen titers, however, are probably secondary in importance to the hypothalamus in the regulation of their sexual behavior.

When larger doses of TP are given (1.25 mg) mating is completely abolished (Barraclough and Gorski, 1962). Surprisingly, however, rats in which constant estrus has been induced by illumination will receive the male, conceive and lactate normally while retaining cornified cells in the vagina throughout this entire period (Maekawa, 1959). Barraclough concludes from this that the hypothalamus of the spontaneous or light-induced persistent-estrus rat is less refractory to extrinsic stimuli than is the hypothalamus of the androgen sterilized rat.

There was a small difference in mating percentages between the two dosage groups. In Group I an average of 41% of the rats copulated while 33.5% of Group II mated. This difference could be due to the fact that different strains of females were used. A more likely possibility, however, is that it was due to environmental conditions. The housing conditions of Group II were such that, for the early part of the study, the animals may

have been disturbed at night. This would have had a detrimental effect on their mating performance. A dose response is probably unlikely at the doses used in this study since previously mentioned studies at the 100 μg level (Cornette, 1965; Segal and Johnson, 1959) indicate that the percentages of females receptive to the males are 46.2% and 30.0% respectively. It may be that receptivity patterns of rats receiving lower doses of TP (up to 100 μg) are altered but to approximately the same extent while doses in the mg range are required to abolish mating behavior completely.

Barraclough and Gorski (1962) report that TP treated females are anovulatory at both small (ten μg) and large(1.25 mg) doses. Ovulation did not result consequent to mating. These results were contradicted by Ericsson and Baker (1966), who reported that some females which received ten μg of TP ovulated consequent to mating. In view of this contradiction, the incidence of ovulation was tested in the animals of both dosage groups in this study. Ovulation occurred in only one out of 16 animals flushed the morning after mating. The one animal which did ovulate as a result of mating shed only one abnormally large ovum.

It is difficult to explain why the Group I results differ from Ericsson and Baker (1966) since the same strain of rat was used, the same TP preparation and dose was used and the animals were housed under the same conditions. At least 44% of the

animals in the Ericsson and Baker study ovulated consequent to mating while in the current study, only two animals ovulated after mating. Furthermore, one of the two animals which did ovulate shed only one abnormal ovum. It may be mentioned, however, that while these disagree with Ericsson and Baker, they are in agreement with Barraclough and Gorski (1962). It is possible that ten μg is a "borderline" dose in that animals may or may not ovulate as a consequence of mating. Apparently in the present study the act of mating induced a neural stimulus which resulted in the release of factors from the hypothalamus which caused a change in the vaginal mucosa but did not cause ovulation. Ericsson and Baker, on the other hand, reported the possibility of a neural stimulus which not only caused a change in the vaginal mucosa but also induced ovulation. It may be that the strength of the stimulus is a factor here dependent perhaps upon multiple matings or activity during mating.

In this regard the question of LH content of the pituitary becomes important. Gorski and Barraclough (1962) reported that the LH content of pituitary glands removed from TP treated rats was one-third that of the normal proestrous pituitary. Segal and Johnson (1959), on the other hand, reported that adenohypophysial LH in TP rats was higher than in normal females. Gorski and Barraclough (1962) also found that rats treated with ten μg of TP would ovulate upon electrical stimulation of the hypothalamus; and that rats pretreated with 1.25 mg of TP would

also ovulate upon electrical stimulation if primed with two mg of progesterone two days prior to stimulation. From these data it appears that the hypophysis of the ten μg TP rat has sufficient LH present to induce ovulation if a stimulus is sent to release it, while the 1.25 mg TP rat requires progesterone priming to permit storage of LH before there is a sufficient amount present to cause ovulation. The assumed constant estrogen secretion by the polyfollicular ovary in TP treated rats apparently prevents proper storage of LH by the negative feedback mechanism described by Flerko and Szentagothai (1957). Thus the inability of the animals in the present study and in the study of Barraclough and Gorski (1962) to ovulate as a result of mating was perhaps due to the inability of the pituitary gland to store LH in sufficient quantities to cause ovulation when released or to an insufficient stimulus (mating) to the hypothalamus to signal the release of LH.

The fact that all animals tested ovulated after treatment with 15 I.U. of chorionic gonadotropin (HCG) further demonstrates that FSH was being released. The ovaries had numerous mature follicles which were capable of rupturing upon treatment with HCG. These mature follicles were evident also in serial sections of ovaries taken from HCG-treated rats. Follicles apparently grow and regress in the TP-treated female and thus there is always a number of mature follicles present which accounts for the presumed high estrogen titer.

In another attempt to get animals to ovulate in response to mating, progesterone priming was employed. The reasoning behind this was based on several assumptions. If females treated with doses of TP as high as 1.25 mg could ovulate after progesterone priming and electrical stimuli, wouldn't the priming of ten μg TP rats with progesterone enhance the possibility for ovulation after mating? The progesterone places the animal in two days of diestrus followed by one day of proestrus and thus permits storage of LH by the pituitary. As indicated by the results, only one animal ovulated after mating and progesterone priming and the two ova which were shed were abnormally large. Thus it appears that TP treated rats are anovulatory because the stimulus provided by mating is insufficient to cause the release of LH even when adequate amounts of it are present in the pituitary. Another assumption from the work of Gorski and Barraclough (1962), but one which was not substantiated here, is that TP treatment does not affect the production of LH but does result in an inability to store it and an inability to release it.

The location of the ova in the oviduct on days succeeding HCG treatment could provide a clue to the reasons behind the infertility of these animals. In the morning of the fourth day after HCG treatment no ova were found in the oviduct. Normally in the rat, the blastocyst reaches the uterus on the fourth day. The animals in this study were examined at about

9:00 a.m. and since no ova were found at this time, it is possible that transport of the blastocyst was too rapid to permit implantation in the uterus.

In considering the results of the progesterone determination, it should be borne in mind that no values are available for control animals at corresponding times in their cycles. Thus the results are of value in a comparative sense mainly of one day to the next. The results obtained are difficult to interpret. The progesterone content was 8.46 γ on day 1 and dropped to 0.18 γ by day 3, then rose to 0.95 γ by day 5. The 20-α-hydroxypregn-4-en-3-one content decreased daily from day 2 through day 5. Examination of histological sections of ovaries removed from the same animals used in the progesterone determinations revealed numerous corpora lutea which appeared normal. However, the corpora lutea may not have been functionally normal, since one would have expected the progesterone content to increase from day 1 through day 5 rather than decrease or remain constant. A brief consideration of the neurohumoral mechanisms involved in luteotrophic hormone (LTH) release may be of interest here. LTH does not depend on hypothalamic neurohumoral stimulation and, in fact, these neurohumors may inhibit LTH secretion (Bogdanove, 1964). It is possible that TP affects the region of the hypothalamus which is responsible for these neurohumors. If this is the case then the LTH secretion should be greater than normal and the observed infertility would not be

due to LTH mechanisms. In addition, if the LTH release is normal or greater than normal, there must be another explanation as to why the corpora lutea are non-functional if indeed they are.

An explanation of why none of the 70 animals in the fertility section of this study were pregnant is quite complicated and involves a number of possibilities. Infertility of the males which bred with the treated females can be discounted since all males were proven previously. The ova appeared normal in those animals killed and flushed after receiving HCG. Another basic possibility is that fertilization did not take place. As a working hypothesis it may be assumed that conception did occur in animals receiving HCG prior to mating. Since the males were fertile and the ova appeared normal, this latter assumption seems justified. The administration of HCG to induce ovulation complicates the problem further as will be mentioned later. In other studies of TP treated rats, the animals were infertile because they were anovulatory. In the present case ovulation did occur (after HCG) so the problem must lie elsewhere.

Animals which received HCG, mated and received no progesterone therapy apparently became pseudopregnant. The ten μg TP group had primarily leukocytes in their smears for an average of 11.3 days and those animals in the 25 μg group had leukocytic smears for an average of 12.6 days.

In the 40 animals which received daily injections of progesterone there were no pregnancies. Thus whatever the cause of infertility, it apparently is not due solely to a progesterone deficiency. It is perhaps unwise, however, to consider progesterone without giving some consideration to estrogen, since these two hormones no doubt interact with each other. Thus the question of estrogen output of TP rats arises. Are estrogen titers in the androgenized rat higher, lower or equal to that of the normal rat? This could be crucial in considering possibilities for infertility in these animals.

Barraclough and Gorski (1961) believe that the circulating estrogen is higher in the TP-sterilized rat than in normal animals. They base this mainly on two points: 1) androgenized animals display persistent cornified vaginal mucosa and, 2) there is hypertrophy of ovarian interstitial tissue. Both of these responses are caused by estrogen. If this is the case, and estrogen titers are high, then the uterine and oviducal motility may have been enhanced to such a degree that implantation of the blastocyst was impossible. Again, if the estrogen levels are high it would (as previously mentioned) inhibit the storage and ovulatory release of LH. LH is eight times more sensitive to estrogen inhibition than is FSH (Bogdanove, 1964), thus the estrogen level could be such that it is high enough to inhibit LH release and yet too low to inhibit FSH secretion. In addition, estrogen is luteotrophic in the rat (Nalbandov, 1964).

It can stimulate the corpus luteum directly and it can stimulate prolactin secretion. Thus if estrogen titers are high, LTH elaboration would be at least normal.

The opposite situation in which estrogen levels are low also presents several possibilities. Johnson and Witschi (1963) found that in 23-day old rats which were sterilized with TP on day 5, the estrogen production is less than normal and in 35-day old rats the estrogen output is barely detectable. A certain amount of estrogen is necessary for implantation of the blastocyst in the uterus. If the estrogen titer is below that needed for nidation, the blastocyst will be passed out and lost. Cochrane and Meyer (1957) found that 4 mg of progesterone per day s.c. will delay nidation until one μg of estrone is given. The embryo will remain in a resting, viable state until the estrone is given. If progesterone will delay nidation, the progesterone replacement therapy employed in this study may have had an adverse effect on fertility rather than enhancing it.

Another point which may be of interest is the possibility of hormonal interactions between estrogen and progesterone and among the estrogens themselves. Estradiol-17β has been shown to be five times as effective as estrone in promoting uterine growth and 20 times as effective as estriol (Hisaw, et al., 1954). In mice estriol competes actively at a physiologic site essential in the development of the usual estradiol stimulation of the uterus (Weeks and Segal, 1957). This was seen not

only by organ weights but also by diminished activity of particular enzyme systems. Johnson and Witschi (1963) have found that in young (35-day old) mice, the ovaries secrete a different estrogen or combinations of estrogen than normal while under the influence of HCG and small amounts of endogenous FSH. Since the rats in this study received HCG to induce ovulation, it may be possible that these combinations of estrogens which were released, competed for the active site in the uterus for proliferation and thus prevented the growth of the uterus. The uterus then could be incapable of maintaining the embryo if it was implanted.

Estriol has not been found to be a normal steroid in rats (Hisaw, et al., 1954). As mentioned previously, estriol inhibits the action of estradiol and estrone; also estriol causes the release of adrenal cortical steroids (via ACTH?) which also inhibit uterine stimulation by estradiol. Thus if estriol is secreted by the rat under the influence of HCG, it could inhibit uterine growth not only directly but also indirectly by causing circulatory levels of the adrenal-cortical steroids to rise.

A final possibility is an adverse interaction between estrogen and progesterone (endogenous and exogenous). There may be an upset of the endocrine balance between these two steroids resulting in an hormonal environment which is incapable of maintaining a blastocyst if it is implanted.

It is the investigator's responsibility to make an attempt to explain his data. Unfortunately, a good deal of the data in this study was negative. Due to the limited nature of these results I have been unable to answer all the questions which arose. Thus I was required to postulate on certain points -- a number of possible reasons for the observed sterility have been presented. It seems that the problem may lie at the ovarian level. Some questions which may be pertinent here are what is the titer of circulating estrogen(s); how is the already altered hormone balance affected by the administration of HCG; what is the state of the corpora lutea -- are they functional; what does the administration of exogenous progesterone accomplish? It is very possible that the problem causing infertility is one which arises after successful fertilization and is due to an imbalance of ovarian hormones. Ultimately the explanation of the TP-sterilized rat will most likely encompass most or all of the endocrine system due to the complex feedback mechanisms and interactions involved in maintaining an homeostatic hormonal environment.

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