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The Alteration of Aggressive Behavior in Mice Following Parenteral Administration of Sex Steroids and Synthetic Analogues

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THE ALTERATION OF AGGRESSIVE BEHAVIOR IN MICE FOLLOWING PARENTERAL ADMINISTRATION OF SEX STEROIDS AND SYNTHETIC ANALOGUES

by

Sister Elvera Abbatiello, O.P.

A dissertation submitted to the faculty of the Graduate School of Loyola University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

June, 1969

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--to one

who believes as I do --

Deus Providebit
LIFE

The author was born on September 17, 1932 in Jamaica, New York. She is the daughter of Antonio and Louise Abbatiello and is the fourth of eight children.

She received her primary and part of her secondary education in the public school system of New York City and completed her secondary education at Villa Maria Dominican High School, Watermill, Long Island.

In 1950 she was received into the Third Order of St. Dominic, Congregation of the Holy Cross at Amityville, N.Y. and was given the name of Sister Marie Antonita. She made her perpetual profession of vows in August, 1954.

From 1951 to 1953 she was a teacher in St. Joseph's Primary School, Long Island City, N.Y. In 1953 she was assigned to Mary Immaculate Hospital, N.Y. to begin studies at Fordham University, College of Pharmacy. She was graduated in 1958 and passed the licensure examination in Pharmacy in November of that year.

In September, 1959 she enrolled at St. John's University, Graduate School, majoring in Pharmacology. She received her M.S. degree from that University in 1962.

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It goes without saying that this work could not have been accomplished without the encouragement and assistance of my fellow graduate students at the Institute for the Study of Mind, Drugs and Behavior, nor would it have succeeded without the help of the technical staff employed there.

Finally, I would like to express my sincerest appreciation to the Sisters of St. Dominic whose confidence in me has enabled me to reach this goal.
FOREWORD

Little is known about the role of sex hormones on central nervous system development before birth. Most studies have been concerned with the prenatal development of endocrine functions. Hormones have been administered during pregnancy, but the study of their effects on the offspring have provided little information on CNS development.

The introductory chapters in this dissertation are concerned with the role of endogenous and exogenous gonadal hormones and their synthetic analogues on embryonic sexual differentiation, sexual behavior and aggressive behavior. It is intended that this background information be the framework from which this present investigation is formulated.

The series of experiments comprising this dissertation represent some preliminary attempts to elucidate the existence of critical periods of embryonic development of the central nervous system during which adult patterns of behavior are determined.
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CHAPTER I

A COMPARISON OF THE EFFECTS OF ENDOGENOUS AND EXOGENOUS SEX STEROIDS ON GONADAL AND GENITAL TRACT DEVELOPMENT
Genotypic sex is permanently established at fertilization and depends upon the sex chromosomes contributed by the parents. Although this aspect of the individual is determined by the chromosomes, many of the distinctive characters differentiating male from female are regulated mainly by appropriate gonadal hormones.

After an initial period of embryogenesis the different sexual characters appear during successive periods of development. The development of the gonad is closely linked with the development of the mesonephros and its associated ducts. Two systems of ducts appear in both sexes: the Wolffian or mesonephric ducts and the Müllerian or paramesonephric ducts.

Differentiation of the genital tract comprises the alternative development or retrogression of the sex ducts and the specialization of the common primordia. Thus, if the gonads are normal testes, the Wolffian ducts persist and develop into the male ductal system and seminal vesicles, while the Müllerian ducts regress. Conversely, if the animal possesses normal ovaries, the Müllerian ducts persist as the oviduct, uterus, cervix and anterior vagina, while the Wolffian ducts regress. The lower end of the reproductive tract is formed from the urogenital sinus, and the external genitalia develop from the genital tubercle.
At puberty, rapid appearance of the secondary sexual characters occurs. The role of gonadal hormones in this phase of development is well known.

Hormonal control of the development of two morphologically different types of individuals was suggested as early as 1903, (Bouin & Ancel, 1903), when it was suspected that the well developed testicular interstitial cells of the embryonic testes controlled masculine organogenesis.

In 1916 and 1917 the classical account of Lillie was published which presented an hormonal interpretation of freemartinism in births of twin cattle (Lillie, 1916; 1917). In this condition there is a fusion of the chorionic blood vessels of a male and female calf fetus resulting in feminization of the male twin which is born with a pseudohermaphroditic sexual system and is sterile.

Although the theory of Lillie is accepted with some reservations today, from his work a vast amount of research evolved on the hormonal control of sexual development.

The problem of sexual differentiation of the gonads has also been extensively investigated. Two distinct stages can be recognized: (1) an early phase which is independent of sex and which follows a virtually identical course in all individuals, and (2) a later phase, the period of sexual differentiation, which is chiefly hormonally conditioned. During the first phase, the primordial structures necessary
for the development of both sexes are formed and develop in a similar or identical fashion in both sexes up to a point when each embryo possesses morphologically, and for a certain time only, the capacity to develop anatomically into an individual of either sex. In this early indifferent phase of development the sex primordia show little reactivity to hormones. The onset of sexual differentiation of the accessory structures follows the appearance of sexual differentiation in the gonads and this is the phase of development when hormones are effective.

The basic pattern of the genital tract is established very early in development. During the relatively long period before the onset of adult sexual function the genital tract is highly susceptible to external influences which may produce alterations in structures or functions.

Witschi (1951), formulated a theory to explain many of the phenomena of apparent alterations in anatomical sex. He described the development of the embryonic gonad in two stages: (1) an initial proliferation of the peritoneal epithelium which forms the medulla of the gonad and is testicular, and (2) a second, superimposed proliferation which gives rise to the cortex and which is ovarian. It was suggested that the medulla secretes an inductive substance ("medullarin") which causes the involution of the Müllerian derivatives in the male embryo. The cortex secretes "corticin" which inhibits the "medullarin"
and causes the continued differentiation of the Mullerian ducts in the female. It has not been stated whether or not these inductors secreted by the cortex and medulla are true hormones.

Moore (1950), states that steroidal hormones play no part in early sexual differentiation. His reasons for supporting such a view are as follows:

1) Subcutaneous transplantation of kidney implants of undifferentiated embryonic reproductive tissues - gonads, ducts, urogenital empyric reproductive tissues - eliminated any evidence for a hormonal effect upon the female tissues, whereas the reverse would be expected on the basis of hormone theory. Androgens frequently stimulated the duct systems of developing embryos, gave little evidence of specific effects especially on the female tissues. Whereas the reverse would be expected on the basis of hormone theory, adrenal transplantation or kidney implants into newborn, juvenile or adult normal hosts failed to provide any evidence for a hormonal effect upon the female tissues at any stage of differentiation.

2) There is no proof that very immature embryonic mammalian gonads secreted any substance, other than androgenic steroidal hormones, at any other type of secretion.

Such evidence as follows:

Intrauterine castration experiments were performed on embryos at early stages of differentiation of sex hormones, and this is regarded as detectable amounts of sex hormones, and this is regarded as evidence of their involvement with the production of Mullerian ducts. It has not been stated whether or not these inductors secreted by the cortex and medulla are true hormones, and causes the continued differentiation of the Mullerian ducts.
rabbit fetuses (Jost, 1947a; 1953; 1954) at one day intervals between days 19 and 24 and examined on day 28 in order to analyze the part played by the gonads in genital tract differentiation. Removal of the testes on day 24 had little or no effect on subsequent masculine sexual differentiation: male ducts, prostate gland, and external genitalia continued their usual masculine differentiation and female ducts underwent the usual regression seen in normal males. However, if castrations were performed earlier, growth and differentiation of male structures were more and more atypical. Testes removal on day 21 resulted in some male duct regression and persistence of some portions of the female ducts, and when removal occurred on day 19, just prior to the appearance of prostate primordia the fetus on day 28 was found to be devoid of a prostate, male ducts had atrophied and there was complete retention of the female duct system. External genitalia were also of the female type. Thus, early testes excision resulted in a female type of fetus. Removal of the ovaries, on the other hand, exerted no appreciable modification or retardation of female differentiation.

Raynaud (1950) observed a feminine organogenesis of the urogenital sinus, genital tubercle, and nipple of male mice in which the gonads and the anterior part of the genital tract had been destroyed.

Using a method of exteriorization of the fetus, Wells,
(1950, 1954), castrated the male rat fetus at the 18th day (two days before term). This caused a reduction in the size of the accessory sexual glands - the seminal vesicle, coagulating gland, and dorsal and ventral prostate. These experiments of Wells also demonstrated (1) that the fetal adrenals do not appear to have a masculinizing effect on the sexual structures and (2) that hypophysectomy was also without effect on the differentiation of the sexual structures.

Price, (1957; 1958), studied the differentiation of the sexual ducts by a difficult technique of explanting and tissue culture. Reproductive tracts were isolated at early stages, cultured on a controlled medium and observed during the culture period.

The results of this investigation indicated in the male, that Wolffian duct maintenance and the development of the primordia of seminal vesicles and prostate glands depend upon the presence of fetal testes or an exogenous male hormone such as testosterone. Wells and Fralick (1951) and later Wells (1957) had established the fact that fetal rat testes secrete a hormone late in gestation and that this hormone stimulated the growth of accessory reproductive glands. However, Price did not identify the hormone which diffuses from fetal testes in the culture.

The female explants performed by Price demonstrated that the Müllerian ducts appeared to be independent of the ovary.
They were retained whether ovaries were present or not and testosterone pellets failed to inhibit them.

It is important to direct attention here to the female type of development which occurs in both sexes in the absence of the gonads. This suggests that there is a basic pattern, genetically, of the female type which requires the action of the testes to bring out the male components of differentiation. Embryonic testes secretions are required to induce a prostate anlage or to fix a masculine tendency on the embryonic tissues of the genital ducts and the external genitalia. Testes secretions are also required to inhibit female duct growth.

The responses of the gonads, of the accessory reproductive organs and of the brain to exogenous hormones depend on many factors: (1) the chemical nature of the hormones, (2) the length of the treatment, (3) the dosage employed and (4) the age of the treated animals. At the indifferent stage, for example, the embryo is susceptible to the actions of androgenic and estrogenic hormones which may swing the balance in favor of the male or female duct system.

The contrasts seen in the results produced by gonadal hormones in the male and female respectively are not primarily the result of the recipient's genetic sex. Broadly speaking, the organs which are present in both sexes, as for example, the Wolffian and Müllerian ducts as well as the external genitalia, react alike to the same hormones. The mammary
glands afford a good example. Their reactions to estrogen in both males and females have been investigated in detail by many workers and have been found to be identical in both sexes.

I. ANDROGENS:

In any consideration of the effects of androgenic compounds on the fetal organism it is important that the term "androgen" be defined. An androgen is usually regarded as a steroid which, in the mature animal, is capable of compensating for the effects of castration and which, in the immature animal, stimulates the development of male accessory sex organs and secondary sex characteristics. It may also be defined as a substance which, in the fetus, promotes masculinization of the primordial genital tract. To this group belong such compounds as androsterone, testosterone and androstenediol.

The appearance of the genitalia of the normal female rat at birth was described in detail by Greene et al (1939; 1949), together with the effects in the offspring when the mother was treated with androgens or estrogens (1937). On the basis of embryological facts the idea was suggested that testosterone when given to the pregnant rat might (a) produce an arrest of the development of the vagina in the female or (b) produce intersexuality in the female. These two possibilities were shown to be true.
The external genitalia of the affected animals varied. The offspring of mothers receiving the androgenic hormone late in pregnancy had a crescentic fold of skin that represented the vaginal orifice surrounding the caudal base of an organ that resembled a "hypospadiac" clitoris. With larger doses given earlier in pregnancy the organ resembled a penis, but was smaller than the penis of a litter brother.

The effects of androgenic hormones on the Müllerian ducts were variable. Suppression of regional parts of ducts sometimes occurred (Greene et al., 1940). In mice, the vaginal segment was frequently inhibited (Raynaud, 1942). Also in the mouse, as well as in the rabbit, failure of the posterior ends of the ducts to unite to form a vagina had been reported (Raynaud, 1942; Jost, 1947b).

If low doses of androgens were used only those parts of the genital tract responded which had a low threshold of sensitivity. The most sensitive parts were found to be the urogenital sinus and the external genitalia, which may, to some extent, explain why these parts respond almost selectively in cases of female pseudohermaphroditism.

Raynaud (1947) also described histological changes in the mammary gland of the masculine type following injection of testosterone into the pregnant mouse at the 15th day.

As previously mentioned, the degree of masculinization of the female fetus can be correlated with the dose and type
of androgen administered to the pregnant female and with the
stage of pregnancy when treatment was begun, (Greene & Burrill, 1938a & b; Moore, 1945; Jost, 1956). A maximal degree of
masculinization could be produced in the female rat fetus
with a total dose of 5mg of testosterone propionate if injec-
tions were begun before the 16th day of pregnancy. Ten times
this dose did not produce a greater degree of virilization.

The important principle of the limited period of sensiti-
vity of the genital ducts and urogenital sinus to androgens
was emphasized by Jost (1955) and convincingly demonstrated
in fetal castration experiments. The capacity to stimulate
development of the Wolffian duct, masculine derivatives of
the urogenital sinus, and a male type external genitalia in
the female fetus was limited to a sharply defined period of
fetal life. Once the future differentiation or regression of
these structures had been determined, it was no longer
possible to modify their development.

The more recent attempts by Suchowsky et al (1967) to
analyze the alterations produced in the secondary sexual organs
in female offspring of rats treated with androgenic steroids
during pregnancy extend the period of sensitivity. In this
research the test compound was administered beginning the 16th
day of pregnancy and continued for four consecutive days. On
the 21st day of pregnancy the animals were killed and the
fetuses removed from the uterus, cut at the umbilical level
and fixed for 8 to 10 days in Bouins solution.

Testosterone propionate caused inhibition of the development of the urethro-vaginal septum, of the cloacal and the urethral vaginae. It stimulated the development of the anterior, lateral and dorsal prostatic buds as well as the coagulating glands. Extreme malformations appeared at the 3mg dose level, i.e. male urethra, complete lack of cloacal and urethral vaginae and the appearance of prostate, seminal vesicles and deferential ducts.

Since variations might exist in the transplacental transmission of hormones injected into pregnant rats, Maqueo and Kincl (1965) attempted to inject androgens directly into the fetus during the last part of pregnancy. They sacrificed 100 previously injected fetuses on the 21st day of pregnancy. The external appearance of the female offspring was normal but autopsy demonstrated that the normal appearing ovaries were without corpora lutea and a small testis and a rudimentary prostate were present.

Androgenic hormones have not been shown to have a deleterious effect on the development of the genital tract of the male fetus. The masculinizing morphogenetic secretion of the fetal testes plays a primary role in the differentiation of the accessory sex structures (Jost, 1953). Although the effects of synthetic androgens on these structures are similar in many ways to that of the androgenic fetal testicular
secretion, there is an important difference - the fetal testis inhibits the development of the Müllerian ducts whereas the known steroid androgens do not (Jost, 1953; 1957).

II. ESTROGENS:

Hormones with chiefly "female" properties (alone or in combination with others) should, in theory, produce in ovariectomized rats typical female sexual changes such as estrus, changes of pregnancy or a return toward the normal condition. In castrated male rats these hormones should not be able to stimulate strongly the development of all male sexual organs.

Attempts to produce feminized male rats by the antenatal administration of estrogenic substances have not always been successful. Dosages that would conceivably cause feminization of the genetic male fetuses, when administered to pregnant rats, often caused resorptions of the pregnancies (Greene, 1938). However, Greene observed that if estradiol dipropionate was used, this compound was very slowly absorbed and consequently had a prolonged estrogenic effect.

In 1940 (Greene, 1940), it was found that prenatal treatment of rats with estradiol had a profound feminizing effect on the sexual development of the male embryo. It was simultaneously observed that administered estrogens produced modifications in the sexual development of the genetic female
embryo. The female offspring were paradoxically "masculinized" inasmuch as the Wolffian ducts persisted and differentiated to a limited extent and the typical female development of the urogenital sinus was partially inhibited.

In the modified newborn male of estrogen treated mothers the testes were retained in the position typical of female gonads. Both Wolffian duct derivatives and masculine development were inhibited to varying degrees. No prostates were present but the lower vagina (that part which arises from the urogenital sinus) was partially developed. Stimulation of the Müllerian duct was limited to the development of an upper vagina, the ostial portion of the oviduct, and occasionally, portions of the uterus.

In the newborn female offspring of estrogen treated mothers the uteri were enlarged because of distention and advanced differentiation (Greene, 1940). Development of normal female structures, the ovarian capsule and the lower vagina were inhibited while structures normally found only in the male were present, namely, the vasa deferentia and seminal vesicles. Thus, modification of the genital tract of female rat fetuses can be achieved by treatment of rats in late pregnancy with either androgens or estrogens.

Raynaud (1939) found the situation to be somewhat different in the mouse. With the dosages he used there was less inhibition of the Wolffian duct, less effect on the urogenital
sinus, and more effect on the Müllerian duct. He further reported some inhibition of the prostatic lobes in the mouse, but none of his animals showed a complete absence of prostates. He did not refer to the development of a vagina.

In the rat, any dose that causes abnormalities in the internal genitalia also causes changes in the external genitalia so that the newborn male externally resembles the female. In the mouse, however, the external genitalia apparently are not easily affected.

In 1964, a reduction in the anogenital distance in the male fetuses of rats treated with high doses of estradiol dipropionate was reported. Such evidence alone, however, is inconclusive for even doses of 10mg/day produced a modification of the phallus to the female type in 2 out of 14 fetuses (Moreau, 1964).

Although a dose of 100 mcg estradiol dipropionate caused considerable fetal resorption in mice and many of the young did not live to maturity, Jean and Delost (1964) did not observe any abnormalities in the male offspring except for a single case in which spermatogenesis was reduced. Later, using high doses of estradiol dipropionate on the 14th day of gestation in mice, these same authors (1964, 1965a) observed cryptorchidism in a high percentage of the young. A more detailed study of these fetuses by Jean (1965b) revealed that the 5000 mcg dose caused atrophy or reduced
development of several parts of the male genital tract (epididymus, prostate, penis, anogenital distance) and a persistence of certain female structures (uterus and tubes).

III. ANTIANDROGENS:

It has been shown that the administration of androgenically active steroid hormones during pregnancy produces varying degrees of masculinization of the female fetus. The suggestion, therefore, that substances with antiandrogenic properties were able to inhibit the effects of testosterone presented the possibility that masculinization of female fetuses caused by androgenic agents could also be inhibited by antiandrogenic compounds. Neumann and Kramer (1964) injected mice simultaneously from the 16th to the 19th day of pregnancy with testosterone propionate an androgenic compound and cyproterone acetate an antiandrogenic compound.

The masculinizing activity of testosterone propionate was diminished dose-dependently as a result of the antiandrogen administration. On the other hand, testosterone propionate did not influence the feminization of male fetuses caused by the antiandrogenic agent to any great extent.

The mechanism of action of antiandrogenic agents has been explained as a competition for the receptors of androgens at the target organs (Randall & Selitto, 1958; Lerner et al, 1960; Bridge & Scott, 1964).
Thus the administration of an antiandrogenically active steroid provided a new method for the inhibition of the effect of fetal testosterone. This technique permitted animals to be raised and studied for permanent changes in the genital organs and the mammary glands after transient inhibition of fetal testosterone (Neumann & Elger, 1965).

Administration of cyproterone acetate in doses of 3mg/mouse/day from day 12 of pregnancy to the day of autopsy (day 14, 15 or 18) revealed that mammary bud and teats in male mouse fetuses as well as those in female fetuses will develop under the influence of an androgen antagonist (Elger & Neumann, 1966). The continuity of the glandular process normally lost in male fetuses because of a destruction of the epidermal sector, is maintained by cyproterone acetate. Inhibition of the endogenous androgen, therefore, resulted in a female organogenesis of the mammary glands.

The treatment of pregnant rabbits from the 13th to the 24th day of pregnancy with cyproterone acetate was also investigated (Elger, Steinbeck & Neumann, 1967) and the genital organs of the litters were examined histologically. The results in this species were similar to those observed in male rats consisting of a dose-dependent destruction of the Wolffian ducts, inhibition of the prostate glands, and external genitalia which were indistinguishable from those of normal females.
Although differentiation of the male and female genital tracts was altered by the administration of cyproterone acetate, neither in male nor in female fetuses did the exclusion of androgens by an antiandrogen induce a deviation from the normal differentiation of the gonads (Neumann, Elger & vonBernsworrt-Wallrabe, 1967). The authors therefore stated that gonadal differentiation does not depend upon the influence of androgens.

IV. PROGESTINS:

The progestins are active principles of the corpora lutea of the ovary. They are chemical compounds whose biological actions are chiefly concerned with conception, gestation, parturition and perhaps, lactation. The molecular structure of progesterone closely resembles that of hormones formed by the testes and the adrenal cortex, and the resemblance extends to many of their biological actions. Some examples of progestins are: progesterone, 6 alpha methyl 17 alpha acetoxyprogesterone and norethynodrel.

Revesz (1960) subcutaneously injected progesterone, norethisterone and 6 alpha methyl 17 alpha acetoxyprogesterone into pregnant female rats from the 15th to the 20th day of pregnancy and showed that progesterone in daily doses up to 50 mg produced no abnormalities. The administration, however, of either norethisterone or 6 alpha methyl 17 alpha acetoxy-
progesterone at the daily dose of 1 mg produced masculinized females showing clitoral hypertrophy, increased anogenital distance, urethrovaginal fistulas and blind vaginas. Similar results were reported by Suchowsky and Junkmann (1961) when certain progestational steroids were injected into pregnant rats.

Schöler and deWachter (1961) elaborated on the technique of Revesz (1960) by inspecting the gonads and performing serial histological examinations of offspring treated during pregnancy with one of nine progestational compounds studied. In general, the finding of a large anogenital distance is characteristic for the male external genitalia of control animals. Masculinization of female fetuses may be characterized by a similar finding of a large anogenital distance. However, some female fetuses may normally show an anogenital distance similar to males although examination reveals no signs of masculinization.

No abnormalities were found in the genital tract of animals treated with progesterone. With all other compounds tested, however, obvious evidence of masculinization was found. Externally, those animals classified as pseudohermaphrodites at birth had a masculine appearance such as an enlarged anogenital distance and a scrotum-like perineum. Histologically, either a blind vagina, a urethro-vaginal fistula, or a penis-like clitoris was found, not only in the female pseudo-
hermaphrodites but also in some of the animals with a normal female anogenital distance.

Kincl and Dorfman (1962) studied the influence of various progestational steroids on the female rat fetus when the test compound was administered orally to the pregnant rat once daily from day 15 to day 20 of gestation. Their study confirmed the masculinizing effects of 6 alpha methyl 17 acetoxyprogesterone and norethynodrel. The authors suggested the possibility of metabolism of these progestational agents to more potent androgens in the mother, fetus or both. They further postulated that these agents may cause steroid hormone producing tissues to form strong androgens.

Many other similar reports of masculinization of female fetuses by progestational drugs have appeared recently (Duncan, Wyngarden, Cornette, 1967; Edgren, Jones & Peterson, 1967; Saunders, 1967).

Thus, in review, many facts have accumulated concerning the effects on the fetus of exogenous androgenic, estrogenic and progestational hormones. The effects of such hormones may be transient or persistent generally according to the stage at which they act. Hormones acting upon the fetus at late stages, once the endocrine glands or receptor organs are well differentiated generally induce transient changes. When estrogenic or androgenic hormones are administered to pregnant females or to fetuses during early stages, they cause permanent
intersexuality, as has been observed in the above discussed series of classical investigations. There is also a large amount of research literature on the effects of such administration to the neonatal animal.

As a result of the studies of Pfeiffer (1936) it was known that male sex hormone continues to play an important role in the differentiation of the sexual centers in the central nervous system of the neonatal rat.

Implantation of ovaries following castration of female rats during the first few postnatal days, showed a normal cycle accompanied by development of corpora lutea. However, after implantation of testicular tissue into newborn female rats, no corpora lutea were seen to develop in the implanted ovaries; the animals were sterile and remained in a state of continuous estrous. In the castrated newborn male rat, normal corpora lutea developed in the implanted ovaries indicating a cyclic functioning of the sexual centers. Pfeiffer concluded that the sex-specific function of the pituitary is not determined genetically. In male animals, it is determined secondarily by an action of the testicular androgens during one very appropriate phase of development.

During subsequent years these findings were confirmed by various investigators, although contrary to Pfeiffer's assumption, the target organ was not believed to be the pituitary but rather the hypothalamus (Barraclough & Gorski, 1962;

In female mice (Barraclough & Leathem, 1954), a single injection of androgen, administered at 5 days of age, induced infertility when the animals were mated at 90 days of age. In contrast, fertility was not influenced in mice receiving androgens at 20 days of age whereas mice treated at 10 days of age assumed an intermediate position.

In 1955, Barraclough observed the effect on the developing gonad of a single injection of 1 mg of testosterone propionate. Intact female mice of 5, 10 and 20 days of age were studied. Corpora lutea formation was prevented in 5 day old treated mice, but neither fertility nor ovarian development was influenced if mice received the androgen at 20 days of age.

A similar investigation was made using intact male mice of 5, 10 and 20 days of age (Barraclough & Leathem, 1959). Each animal received a single injection of testosterone propionate. A suppression of testicular growth was noted in mice treated at 5 or 10 days of age and was apparent throughout all autopsy periods whereas mice treated at 20 days of age failed to show any alteration in testes weight.

Whalen and Edwards (1966) observed that neonatally castrated male rats exhibit infrequent intromission and ejaculation responses in comparison with intact males. Examination of these animals prompted the suggestion that these infrequent responses reflected the reduced penile development
characteristic of neonatally castrated males.

The neonatal period during which androgen injection to the female rat can permanently alter ovarian function, apparently by an action on a hypothalamic system has often been referred to as the "critical period". More recently, however, Gorski (1968a) has considered this to be a nonspecific term and believed it more useful to consider the period during which gonadal steroids exert an organizing action on the central nervous system in terms of the competence of the developing cells of the hypothalamus to respond to androgen, i.e. the period of neuronal competence. As a consequence of his investigations he proposed that the competent period for 10 mcg of testosterone propionate can extend only through neonatal day 4.

The administration of a single dose of estradiol benzoate to rats under five days of age also causes alterations in the genital sphere (Heinrich et al, 1964). Injected rats in both species showed hypospadias. A high percentage of injected males had a malformation of the inguinal canal which was one of the causes of the cryptorchidia shown by these animals.

The data of Kincl et al (1965) suggest that in the newborn male animal only estrogens but not androgens are capable of inhibiting spermatogenesis. This effect was observed in guinea pigs, hamsters, rabbits and mice. However, it was considered unlikely that the steroids act directly on the
Treatment of female rats with estradiol dipropionate begun on the 15th, 20th, 30th and 40th days after birth caused no permanent impairment of reproductive functions. The females upon maturing displayed regular estrous cycles, mated, bore litters and lactated (Duncan, Wyngarden and Cornette, 1967). In contrast, when injections were started on the 1st, 5th and 10th days after birth none of the adult females either displayed cyclic manifestations of estrous or mated with normal males. The ovaries were small and contained no corpora lutea. Uteri were also small, showing severe pathological changes and responding poorly to estrogenic stimulation. Also, injection of large doses of estrogens into lactating rats over the first four or five days after parturition resulted in a permanent modification of the external genitalia of the female young.

When estrogens were administered to female rats four to five days after parturition subsequent testicular and accessory sex organ development was inhibited in the lactating male pups. There was also abnormal development of the rete tubules and intense epithelial cornification of the ejaculatory ducts (Weichert & Kerrigan, 1942). A single injection of 30 to 240 mcg of estradiol benzoate to a five day old male rats produced small testes, atrophied accessory sex tissues and later infertility (Harris & Levine, 1962; Kincl et al, 1963).

Following these observations various investigators
undertook an evaluation of the effects of progesterone on the sexual development of the neonatal rat. The work of Dorfman and Kincl (1963) implicated progesterone in the protection of the sensitive hypothalamic-gonadal system against the effects of increases in androgen or estrogen concentrations. In the absence of the "buffering" influence of progesterone, estrogens produced an action which resulted in permanent change and damage to the testes. Protection against the "early androgen" syndrome was also reported by Kincl (1965b) when small doses of progesterone were administered to newborn female rats 12 hours before testosterone propionate administration.

Various synthetic progestational compounds are androgenic. For comparative purposes Edgren et al (1967) employed the growth response of the ventral prostate of young, castrated rats treated for seven days. The androgenic effects of progesterone were trivial and appeared to be of little practical significance. Of the patently androgenic progestational compounds studied only one of the 18 halogenated steroids related to 19 nor-testosterone was found to be the most potent.

A more recent investigation by these same authors (Edgren et al, 1968) examined the effects of interactions of the progestational compound, norgestral, and estrogenic ethinyl estradiol, on the offspring of rats treated during lactation. During this period, mortality of the young, which
was largely the result of cannibalism was noted, but the authors believed the incidence of this was not drug related.

At autopsy at six weeks of age, the male offspring showed no significant variations from controls in the morphology of the testes, ventral prostate, seminal vesicles or levator ani and the females showed no effects on uterine, ovarian or adrenal weights. The data of these investigators would appear to indicate that neither norgestral nor ethinyl estradiol at 1, 5, and 25 fold multiples of the human dose have any biologically significant effects upon lactating female rats, the lactation process, or the offspring.

Thus, there is a wealth of data to suggest that hormonal factors participate actively in the differentiation of the genital tracts and that the testis itself is an important site of hormone elaboration. The effects of fetal gonadectomy upon sexual organogenesis have also been shown to be related to the stage of development at which the procedure is performed - after or before differentiation of the genital tracts.

Endogenous estrogens and progestins play a vital role in control of the normal course of pregnancy, but balance of these two classes of hormones must be maintained for a relative excess or deficiency of either can be detrimental to the offspring. The development of the external genitalia of mammals is also influenced markedly by testicular secretions of the fetus and by excess androgens in the mother. In
laboratory animals an alteration in hormonal balance during specific periods of development can have permanent effects on the sexual structures as well as on the reproductive capacity of the animals.
CHAPTER II

ENDOGENOUS AND EXOGENOUS GONADAL HORMONES

AND

SEXUAL BEHAVIOR
A review of the research on the consequences of hormone action on behavior emphasizes the fact that the physiological mechanisms by which glandular secretions influence sexual behavior are not well understood or defined. Recently it has been suggested that hormones exert a dual influence on behavior: organizational or inductive during the period of development and activational or excitatory in the adult (Levine & Mullins, 1966).

In the immature (prenatal or neonatal) animal, hormones seem to affect future biological functioning permanently. They are responsible to some extent for the organization of control mechanisms in the central nervous system which will later regulate hormone activity in the adult. Specifically, the presence of these hormones seems to determine the sensitivity of a hormone regulating homeostatic feedback mechanism which later influences the character of sexual behavior (Marvin, 1968). The sex hormones of the embryo also affect ultimate sexual behavior by their control of morphogenesis of the genitalia. The possibility also exists that brain circuits or centers which are directly involved in perceptual or behavioral routines might be similarly affected by the sex hormones.

The term sexual behavior refers to a complex series of responses directly associated with genital stimulation and copulation whether homosexual or heterosexual. For each species
there is a generally recognizable sequence of postural configurations which, taken together, constitutes a sexual behavior pattern.

In the adult female rat, for example, the secretions of estrogen and progesterone, which accompany the cyclic process of ovulation and luteinization, act upon the brain so that the animal is sexually receptive only at the time of ovulation. The primary indication of receptivity is the lordosis response. This consists of the arching of the back with elevations of the head and hind quarters so that the vagina is presented to the male. Lordosis is basically reflexive, is almost never seen during other phases of the sexual cycle, and has rarely been observed in normal male rats.

The sexual behavior of the male, however, is more complex and consists of a sequential pattern of responses. During the first stage of this sequence the male mounts the female from the rear and clasps her sides with his front paws. In the second stage the male palpitates the sides of the female with his front paws and at the same time makes rapid pelvic thrusts. Stage three of this pattern is associated with the penetration of the vagina by the penis and involves rapid pelvic thrusts of stage two terminated by a deep thrust and a very quick and forceful dismount which usually carries the male several inches away from the female. The fourth and final stage is the ejaculation which consists of a mount about three times longer.
than normal, several deep pelvic thrusts, and then a slow dismount and a dramatic raising of the front paws (Levine & Mullins, 1967).

In the rat, because of the extreme immaturity at birth, it is possible to inject hormones into the newborn young instead of the pregnant mother to see the effects on the animal. Barraclough and Gorski (1962) found that testosterone propionate administered to the female rat at five days of age permanently impaired the regulation of the sexual cycle postpuberally and rendered the rats sterile, in a state of anovulatory persistent estrous, constantly sexually unreceptive to the male. Mating behavior could not be restored by replacement therapy with estrogen and progesterone, as is the ordinary case of the ovariectomized rat. When a smaller dose of testosterone (10 mcg) was used at five days of age, the results were similar but these rats subsequently accepted the male at bizarre intervals such as nine days consecutively.

The relationship between neonatal hormone injection and subsequent adult sexual behavior is sometimes paradoxical. On the investigation of Whalen and Nadler (1963), which was designed to study the effects of homotypical hormones administered during infancy in a single injection, the administration of 200 mcg of estradiol benzoate subcutaneously to four-day-old female rats resulted in reduced mating in response to estrogen and progesterone replacement in adulthood. When mounted by males,
the estrogen treated females did not show lordosis; on forty percent of the mounts the females responded by kicking the male. Thus estrogen treatment at the appropriate dose level during the neonatal period acts, as does testosterone, to reduce in females the sexual responsiveness which is normally induced by estrogen and progesterone.

These data conflict with the findings of Harris and Levine (1965) that neonatal female rats treated with 50 mcg of estradiol benzoate showed sexual receptivity when given estrogen and progesterone replacement. The one obvious difference in these studies is between the doses of estradiol administered to the neonate. It has been shown that the dose of hormone given to the neonatal female is critical with regard to sexual behavior (Barraclough & Gorski, 1962).

Female rats, in another study by Levine and Mullins (1964) were given 100 mcg of estradiol benzoate as neonates. They were, likewise, unreceptive in adulthood both intact and when castrated and given estrogen and progesterone replacement. Although no sexual receptivity was found among these neonatally estrogenized females, some of them did show male sexual behavior when castrated and administered testosterone. Not only were all the components of the ejaculation pattern exhibited, but also the post-ejaculation latency before the next mount!

The above studies suggest the hypothesis that, as in the
case of somatic tissue development, neural regions pass through an undifferentiated stage during which they possess bisexual potentiality behaviorally. Whether the hypothetical neural tissue develops into a female or male behavior integrating unit is determined primarily by the presence of an androgen which, either elaborated by the embryonic testes or injected, "organizes" or induces the undifferentiated sexual-neural areas to develop into a center regulating male behavior. In the absence of such a substance the intrinsic tendency in mammals is for the relevant neural tissue to be organized into female behavior controlling patterns (Phoenix et al, 1959; Young, Goy and Phoenix, 1964; Harris, 1964; Levine and Mullins, 1964).

This hypothesis was supported by the investigations of Gerall (1966) using guinea pigs and Gerall and Ward (1966) using albino rats.

In the guinea pigs, exogenous gonadal hormones (in this study, testosterone propionate), administered from the 10th day of pregnancy until parturition affected the adult mating behavior of the pseudohermaphroditic female offspring.

When albino rats were used for an investigation of the modifiability of both female and male behavior patterns in genetic female rats prenatally injected with testosterone propionate, the following were varied: (a) dosage level, (b) time of injection and (c) duration of injection series. Given during days 16 - 20 of gestation, 1 - 2 mg of
testosterone propionate appeared to be minimal for producing somatic masculinization, and 2 mg of testosterone propionate appeared to produce the highest degree of masculine behavior. After receiving daily injections of testosterone propionate during adulthood, the androgenized females began mounting sooner and attained higher rates of male behavior than normal females; in fact, the mean rate of mounting of the experimental animals was not significantly different from that of normal males tested at the same time.

Since male responses appeared in this study when the clitoris of the treated animals assumed prominent penile characteristics in response to testosterone propionate, it was proposed that if the female, in fact, possesses a potentially active male neural center, its activation may occur because of a feedback from peripheral centers. Activation may not then result from sensitization attributable to androgen present during development.

In a later study Gerall (1967) injected 0.25 mg estradiol benzoate and 1.25 mg testosterone propionate into five day old intact and spayed albino female rats. This treatment either eliminated or shortened the period of estral activity. Also, none of the steroid-treated females exhibited normal receptivity to vigorous males after hormone replacement therapy.

The most recent study of Mullins and Levine (1968) places some doubt on the adequacy of "mounting behavior" as a parameter
for determining masculinization. They observed that untreated females exhibit some mounting behavior both during periods of estrous and when injected with testosterone in adulthood. Whalen and Edwards (1967) suggested that mounting may be an indicator of sexual arousal which can be exhibited under the appropriate conditions by either genetic sex.

In all the above cases treatment was through the systemic route. Therefore, Nadler (1968) presented a study to demonstrate the central organizing action of androgen by directly implanting the hormone in the brain of the neonatal female rat. His results revealed that intracranial implantation of testosterone propionate in neonatal female rats induced advancement of vaginal opening. Implants containing as high as 12.5 mcg of testosterone propionate inhibited both ovulation and female sexual behavior while those containing small amounts of testosterone propionate (1.25 mcg and 1.67 mcg) inhibited ovulation but did not inhibit female sexual behavior. Although augmentation of male sexual behavior did occur it would be unwarranted to account for this alteration in behavior in terms of an action of androgen on the brain since apparently there was sufficient testosterone propionate in the systemic circulation with the 12.5 mcg implant to produce clitoral hypertrophy. The author also failed to localize an area of influence in the brain and therefore, suggested the need for further research on the mode of action of neonatal testosterone propionate in
augmenting male sexual behavior in the female rat.

As a consequence of the indirect evidence implicating hypothalamic mechanisms in the mediation of alterations in sexual behavior, a study was undertaken (Michael, 1966) to obtain more direct evidence for the existence of an estrogen receptor system in the brain of the neonatal rat, which, if present, might be regarded as capable of mediating these effects.

Female rats under three days of age were injected subcutaneously either with 0.02 ml ethyl oleate (control) or with a similar volume of oil containing 0.05 mcg H$^3$ hexestrol. The neonates were killed by decapitation four hours after injection. Sections through the whole head were cut at five microns in the cryostat and autoradiographs prepared.

Grain counts were made of the lateral hypothalamic area, medial forebrain bundle, dorso medial nucleus of the hypothalamus, the area of the tuber cinereum, the antero-ventral and antero-medial nuclei of the thalamus and various areas of the neocortex.

Grain counts were significantly above background only in the nucleus arcuatus and in the basal part of the ventromedial nucleus of the hypothalamus. These data may indicate, therefore, the existence of a receptor or storage system in the neonatal hypothalamus possessing an affinity for hormones.

There is considerable evidence to implicate progesterone in the mating behavior of mammals (Young, 1961). The hormone
seems to work in a synergistic way with estrogen and effects of progesterone upon copulatory behavior have been found to require previous estrogen "priming" of the ovariectomized female.

Experiments by Goy, Phoenix and Young (1966) suggested that progesterone was able to suppress sexual responsiveness of female guinea pigs. They demonstrated that in intact females the presence of a functional corpus luteum was correlated with a decreased effectiveness of exogenous estrogen and progesterone in inducing estrous behavior.

Zucker (1966) undertook a study to evaluate further the role of progesterone in the suppression of female sexual behavior in the guinea pig and to define more precisely the conditions under which progesterone exerts facilitatory and inhibiting effects on female sexual responsiveness. This author concluded that progesterone may depress sexual responsiveness by preventing estrogen from acting at hypothalamic and limbic sites. Indirect evidence was provided that the inhibitory effect of progesterone was not due to a progesterone-mediated decrease in the systemic availability of estrogen but might be due to a blockade of estrogen action. In their proposed mechanism, progesterone was visualized as functioning as a competitive inhibitor of estrogen on suitable neurons.

On the other hand, once the appropriate neurons have been properly conditioned by estrogen, progesterone cannot exert its
inhibitory effect, but facilitates the expression of sexual behavior. This was demonstrated by the fact that progesterone could induce estrous behavior only following a conditioning period with estrogen of approximately twenty-four hours.

The most recent studies of Zucker (1968) on the biphasic effects of progesterone on sexual receptivity in the female guinea pig showed that sexual refractoriness at the late luteal phase of the cycle reflected hormonal influences on the brain and not activation of an intrinsic neural state of the brain.

Having reported that progesterone exerts both a facilitatory and inhibitory effect on sexual receptivity of the spayed female guinea pig, Zucker (1967) then evaluated the role of progesterone in the control of sexual behavior of the female rat.

Following the termination of heat, spayed rats could be made sexually receptive a second time by treatment with progesterone. This was in sharp contrast to results obtained with the guinea pig under similar conditions. Female guinea pigs were almost completely refractory to progesterone shortly after the termination of heat, thus a second period of receptivity could not be induced. This would indicate that the role of progesterone is probably different in the regulation of estrous behavior for the two species. In the rat progesterone does not seem to have a biphasic action, i.e. it acts synergistically with estrogen to facilitate receptivity but does not induce refractoriness following estrous.
Unlike the female, whose display of sexual behavior is generally cyclic, the male of most species of mammals characteristically shows a constant readiness to engage in sexual behavior. Beach (1941; 1945) has reported one case of a male rat with normal testicular function that displayed female behavior but such display by males is indeed infrequent.

Considerable evidence now exists supporting the theory that in the genetic male some androgenic substance secreted by the developing testes acts on neural tissues during the period of psychosexual differentiation to organize the male pattern of behavior. In the absence of this testicular support, development of male behavioral patterns is deficient and female patterns are developed or retained instead.

One of the accepted generalizations from studies on sexual behavior is that the behavior of animals high on the phylogenetic scale is less dependent on gonadal hormones than is the sexual behavior of animals with a lower phylogenetic status. For example, the sexual behavior of experienced male cats and dogs (Rosenblatt & Aronson, 1958; Beach, 1952) persists much longer after castration than does the behavior of experienced castrated rats and guinea pigs (Beach, 1944; Beach & Pauker, 1949; Grunt & Young, 1952).

The decline of sex behavior following castration and the qualitative changes in behavior resulting therefrom were studied in adult male rats (Davidson, 1966). The findings were
summarized by the statement that, while the efficiency of the "copulation mechanism" appeared to be enhanced for some time after castration, measures related to sexual arousal showed immediate and continuing deterioration. Thus, latency to first intromission after commencement of the test, as well as following a prior ejaculation, were both increased in the first postoperative week, and showed greater increases in successive weeks.

Castration produces regressive changes simultaneously in mating behavior, in the glans penis, and in the vesicles. Furthermore, the restorative effects of exogenous testosterone are exerted equally upon behavior and the morphological target organs. It is, therefore, difficult to determine whether the effects of endogenous or exogenous androgen upon behavior are due to influence of the hormone on the central nervous system, to the stimulation of more peripheral tissues involved in the reception and discharge of stimuli, or to a combination of both effects (Beach & Westbrook, 1968).

Grady and Phoenix (1963) devised an experiment in which male rats were deprived of androgen by castration instead of being given estrogen neonatally. Males that had been castrated prior to the tenth day of age were compared with those castrated later. The comparisons were begun at 120 days of age, at which time the animals were given estradiol plus progesterone
replacement treatment. They were then tested for feminine behavior in response to mounting by intact males. Castration prior to day ten resulted in significantly more feminine behavior.

Levine and Mullins (1964) also performed the experiment of neonatally injecting male rats with 100 mcg of estradiol benzoate. In adulthood these rats were mating-tested before and after castration and again after seven days of testosterone replacement therapy. Their performance paralleled that of the oil injected control animals fairly well, except for a lesser intromission and ejaculation rate which could be attributed to poor morphological development of the accessory sex organs following neonatal estrogen. The mounting activity of the experimental animal was, however, bizarre. They tried to mount from the head, the side or high up on the back of the receptive female.

Male rats injected with estrogen on the fourth day of postnatal life showed reduced male sexual behavior as adults (Whalen, 1964). Such estrogen treated males also displayed some female behavior in adulthood. It was suggested that these animals might have been feminized, perhaps by chemical castration attributable to the injected estrogen. However, Feder and Whalen (1965) in their experiments showed conflicting results, namely that estrogen injected into newborn male rats did not induce female behavior, in fact it appeared to suppress
its display. These authors concluded that it is the absence of androgen during the period of neural differentiation rather than the presence of estrogen which induces female sexual behavior.

The data of Whalen and Edwards (1967) showed that male rats stimulated by endogenous androgen (sham operated only) or exogenous androgen (castration + testosterone) in infancy, did not mount more frequently than males not stimulated by gonadal hormones after birth (castration only). Furthermore, exogenous estrogen treatment in infancy neither facilitated nor inhibited adult mounting behavior in males. However, the sham operated males and those which were castrated then treated with testosterone in infancy exhibited the intromission pattern more frequently than animals who were castrated in infancy and were not androgen stimulated. On the other hand, neonatal estrogen treatment did not facilitate or inhibit the display of intromission behavior in adulthood.

Male rats castrated 16 hours after birth and then either untreated or injected with oil at 96 hours were not different from untreated female rats in their ability to display both lordosis and mounting behavior when given the appropriate injections of estrogenic or androgenic hormones in adulthood (Mullins & Levine, 1968).

Neumann, Elger and Kramer (1966) were able to induce the formation of a vagina in male rats by treating gravid rats
during the second half of pregnancy and newborn animals during
the first three postnatal weeks with an antiandrogen,
cyproterone acetate. Subsequent castration and implantation
of ovaries allowed for a determination of cyclic functions in
these animals using, among other means, the vaginal smear
technique. In other words, they determined whether the anti­
androgen had prevented the differentiation of the sexual center
to the male type. In addition, they investigated the sexual
behavior of these animals in order to determine whether the
differentiation of centers which determine the specific sexual
behavior was influenced.

Since the antiandrogen used by these investigators inhibits
the androgenic effect competitively, castration can only be
partially achieved. Most of the feminized animals in this
study showed a tendency toward permanent estrous. In those
animals with relatively well pronounced cyclic functions the
estrous phase was prolonged.

Male rats affected by this antiandrogenically active steroid
during the critical phase of receptivity were believed to
possess an inhibited pituitary-diencephalic system. The effect
was similar to that occurring as a result of removal of the
gonads.

The administration of antiandrogen prevented the effect
of androgen necessary for the differentiation of the mating
center. Consequently at a later stage the female mating
center is dominant in these animals, and in the presence of female sex hormones (in this case, estrogens and progesterone of the implanted ovary) female sexual behavior became evident.

It was also of particular interest to these authors (Neumann, Elger & von Berswordt-Wallrabe, 1967) from a clinical viewpoint, to explore the possibility whether male sex drive could be inhibited by antiandrogens. They measured this by counting the mountings with complete copulatory patterns. For two short periods of time females in estrous were caged with untreated control males and males that were under the influence of antiandrogen. Compared with the treated males, libido was decreased by 70 percent within 14 days under cyproterone acetate.

It is therefore believed that gonadal androgen has two central roles in influencing sexual behavior of male animals. One concerns the influence of androgen in the prenatal or early postnatal organization of neural tissue that mediates sexual behavior; the other concerns the postpubertal activation of previously organized neural tissue resulting in the onset of mature patterns of sexual behavior.

Hart (1968) attempted to locate the part of the central nervous system that is apparently organized by androgens in the neonate and irreversibly altered by neonatal castration. Male rats were castrated four days after birth and given exogenous testosterone in adulthood. When tested for sexual
reflexes after spinal transection these animals displayed impairment of genital responses. Similarly, treated animals castrated on the twelfth day exhibited a complete mating sequence and had normal sexual reflexes. The author concluded that neonatal testicular androgen appeared to have an organizational influence at the spinal level on neural tissue mediating sexual reflexes.

The adult rodent is characteristically unisexual. That is to say, both male and female patterns of behavior are not displayed with equal ease or equal frequency by the same individual. Customarily, one pattern predominates and the potentiality for heterotypical behavior is limited.

The predominant pattern of behavior usually corresponds to the gonadal, genotypic and morphological sex of the individual. Each of these factors contributes to unisexual orientation in a unique way. The gonad contributes to unisexuality by the type of hormone secreted, genetical factors, by regulating the thresholds for male and female responses and morphology by imposing limitations on heterotypical responses according to the structure of the external genitalia. These factors never operate with complete independence in the normal individual. From the beginning the sex genotype influences the formation of the gonad and its subsequent pattern of secretory activity determines the morphogenesis of the external genitalia. In the adult their joint action determines that intromission and
ejaculation for the genetic male and permitting intromission for the genetic female are sex specific behaviors. Other components of the sexual repertoire are not limited to the same extent and may be displayed by both sexes (Goy, 1964).

At present any statement as to the exact nature of androgen action upon the central nervous system cannot be made with complete certainty. The neurological sites of hormone action remain to be located. The pathways of stimuli for the many responses given are still to be defined and the mechanisms of hormonal action in organizing these circuits in the central nervous system must be clarified.
CHAPTER III

SEX STEROIDS AND AGGRESSIVE BEHAVIOR
Intraspecific aggression, threatening or fighting, plays a major role in the organization of societies and populations. It is difficult to define "aggressiveness" accurately to cover all possible manifestations. By limiting the question to experimental psychology, Valzelli (1967) has stated that the term "aggressiveness" should be used to indicate a particular behavior, directed toward removing or overcoming whatever is menacing the physical or psychological integrity of the living organism.

Aggressive behavior occurs when particular circuits in the brain are active in the presence of specific external stimuli (Moyer, 1968a). The types of external stimuli which evoke overt attack are determined in part by heredity, in part by the reinforcement history of the individual, and in part by the particular neural circuits which are sensitized. The ease with which the hostility circuits may be activated will depend on the degree to which they have been sensitized.

How to evaluate or induce aggressive behavior in laboratory animals has been studied considerably. The earliest papers dealing with this problem were published between 1930 and 1938 (Anderson, 1938; Hall, 1934; 1936; Higginson, 1930; Stone, 1932), but only in the past decade has experimental aggressiveness been more carefully investigated and used as a scientific tool.
In 1944, Collias presented a survey of (1) the role of aggressive behavior in the ecological pattern of living systems, (2) the associated psychological, physiological and genetic mechanisms and (3) the evolutionary changes in aggressive behavior patterns. He arrived at three principal conclusions: first, a great deal of fighting in vertebrates is connected with the phenomenon of territoriality; second, fighting in animal groups tends to become organized into social dominance hierarchies which have the net effect of greatly reducing the amount of fighting and mortality; and third, one of the major physiological factors connected with fighting in vertebrates is the male sex hormone.

According to Scott (1958), fighting is so widespread a habit in animals that it cannot be considered as an accident or abnormal aspect of behavior but rather as a useful and necessary part of their life. Adult male mice which have never seen each other usually react during their first meeting by mutual investigation and grooming. The tactile stimulation of one mouse roughly grooming another is often adequate to start a fight. Male mice never fight over females, but this happens frequently in other species.

In 1962 (Scott, 1962), it was suggested that fighting is strongly affected by learning. Success in fighting is a powerful reinforcing agent. Successfully experienced animals are much more highly motivated to fight than inexperienced ones.
Animals also form strong habits of not fighting, either as a result of lack of success or more importantly, by simply not fighting.

Particular attention must be paid also to territoriality by which animals establish living space and concomitantly the level of interchanges with other animals. A well organized system of territories acts as a control on aggressive activity, since all violations of habitual territory will induce aggressive reactions (Fredericson, 1950; King, 1957; Scott, 1958). Again the aggressive response tends to remove the cause of the stimulus. It produces two effects; stopping the intruder from whatever it is doing, and driving away the intruder, leaving the winner in possession of the territory (Valzelli, 1967).

The classes of intraspecies aggression are diverse, possible examples classified by stimulus are: predatory, inter-male, fear-induced, irritable, territorial defense and maternal (Moyer, 1968a).

Predatory aggression is a type of aggressive behavior evoked by the presence of a natural object of prey.

In inter-male aggression the most potent releaser of the aggressive response is the presence of a male to which the attacker has not become habituated.

Fear-induced aggression, in its purest form, is always preceded by attempts to escape, thus, one of the components of the stimulus situation eliciting fear-induced aggression is
a degree of confinement of the defensive animal from which there is no escape.

The stimulus situation which evokes an irritable aggressive response is the presence of any attackable organism or object. The range of stimuli which will elicit irritable aggression is extremely broad, and may involve inanimate as well as animate objects.

In the case of territorial defense an area is involved in which the animal has established itself. Some species will attack an intruder of any type.

In most mammalian species, maternal aggression is the particular province of the female. The stimulus situation involves the proximity of some threatening agent to the young of that particular female.

A particular instance of aggression may appear to involve more than one of these classes. Possibly there may be different neural and endocrine bases for each class of aggression not discernable in a particular experimental situation.

The aggressive behavior of wild mice is essentially the same as that described for laboratory strains (Scott, 1966). There are characteristic patterns of behavior such as the defensive posture assumed by a beaten mouse. The animals rear up and sit quietly with forepaws rigidly extended toward the attacker who charges and attacks with his teeth, sometimes
rearing in a hunched posture before striking but never assuming the same posture as the defensive animal. Tail-rattling is commonly heard and seen in mice which are hesitating before an attack (Clark & Schein, 1966). This behavior suggests a signal conveying a warning or threat similar to growling in carnivores. It occurs in any irritated or excited mouse.

Aggressiveness in rats and mice can be increased by prolonged isolation. This aggressive behavior is complex and probably involves inter-male aggression. The aggressiveness increases proportionally to the period of isolation, thus after 6 days 0 percent of the animals will fight, after 10 days 42 percent and after 15 days 67 percent; a maximum is reached three weeks after the beginning of isolation (Suchowsky, 1968). This response is strain related.

The principal kinds of tests that have been successfully used to measure aggression are presented below (Scott, 1966):

1. TEST FOR THE ESTABLISHMENT OF DOMINANCE (fighting test):

In most of the early experiments with mice the animals were brought together in single pair contacts and allowed to fight in a round-robin fashion for half-hour periods eventually establishing dominance. The results were based on the winning fights. Various other measures can be obtained from the same situation; latency to first attack, total number of attacks, and number of attacks until a decision is reached as to which animal is dominant.
2. LATENCY METHOD:

The dominance method may result in more or less serious bite wounds and loss of valuable mice. In the method developed by Fredericson (1951), the mice are separated immediately after the first attack, no dominance is developed, and the only measure is latency. The mice are housed in adjacent pens for three days, in order to establish familiarity. At the time of testing a barrier between the pens is raised and behavior observed for a period of five or ten minutes or until fighting occurs. The test is repeated for a total of ten successive days by which time a stable response is usually established.

3. DANGLING METHOD (forced fighting):

In this method a mouse is dangled by the tail against another so that only one standardized stimulus is delivered at a time. The experimenter uses four stimulus animals five times each, for a total of 20 stimulations, and records the behavior patterns elicited for each stimulus.

A still more precise way of controlling stimulation is to introduce mechanical rather than social stimulation. A moving bottle brush, for example, will elicit attacks in highly aggressive mice (Lagerspetz & Mettälä, 1965).

4. FOOTSHOCK METHOD:

This method has been standardized for rats by Hutchinson, Ulrich and Azrin (1965) and has been used with some success in mice (Tedeschi et al, 1959). Two rats are placed on a grid
in a chamber approximately 9 x 12 inches square in which they are forced into close contact. In these conditions male rats will reliably assume the "boxing" posture when given shocks of two milliamperes intensity and will sometimes bite and continue to fight.

5. COMPETITIVE METHODS:

These involve object-directed behavior rather than behavior directed toward another animal. Consequently the behavior includes much more than aggressive behavior and is complex from a psychological point of view.

It is well known that evident differences in aggressive behavior are linked to the sex of the animal. In mice, fights between females are very unusual (Fredericson, 1952) and, if the fact is considered that males, in most cases, are the dominant sex, and that castration renders males placid and tractable, it is easy to accept the assumption of the importance of the androgen hormones for aggressiveness.

Androgens appear to be critical to the manifestation of aggressive behavior in males. Inter-male aggression does not appear in either mice (Fredericson, 1950) or rats (Seward, 1945) until after sexual maturity. In domestic mice, the young males will start to fight each other at about 32 days of age whereas females in most strains rarely fight at all.

There have been reports of increases in irritability in females given androgens, but these are difficult to evaluate
because they have, in general, been unsystematic observations made in connection with studies done for other purposes (Huffman, 1941; Ball, 1940).

Sex affects aggressive behavior in two ways. One of these is anatomical. The larger male has a physical advantage over females in any conflicts which may arise between them. However, this does not account for the fact that males fight each other more readily than do females.

Only one study was directly related to the importance of male hormone in stimulating aggressive behavior. Ulrich (1938) found that castration diminished the fighting reactions of some, but not all male mice.

Prepuberally castrated mice do not develop inter-male aggression. Beeman (1947) castrated male mice at twenty-five days of age and allowed them to recover from the operation. When given the opportunity to fight, they behaved like females and remained peaceful, regardless of their age at isolation if a period of 25 or more days had elapsed between castration and initial encounters. If the same animals were given implanted doses of androgenic hormone they started fighting immediately and usually stopped when the hormone was taken away.

The results of this investigation on castration differed from that of Ulrich, previously mentioned, who found that castration did not inhibit fighting in all mice. This discrepancy may be due to the fact that the experimental
conditions of the study of Beeman were not comparable with those of Ulrich since the mice in Beeman's study were isolated except during encounters, and thus could not form true social organization such as Ulrich described for groups of caged mice.

However, Ginsburg and Allee (1942) indicated that mice kept in isolation are much more prone to exhibit aggressive activity when brought together for staged encounters than are mice which are constantly caged together. It might be expected, therefore, that the mice in the study of Beeman would exhibit more aggression than those used by Ulrich.

By subcutaneous injection of 0.5 mg testosterone propionate in 0.25 ml of sesame oil, Levy and King (1953) were able to cause fighting between mice as early as 18 days of age in the experimental group whereas none of the control group fought before 34 days of age. The authors suggested that since the psychomotor development of these mice was apparently mature enough to display fighting, it was possible that testosterone propionate lowered the stimulus thresholds necessary for eliciting the fighting response.

Accepting the validity of the relationship between the androgens and aggressiveness left many unanswered questions. Two of the most obvious were: (1) What is the nature of the androgen-aggressiveness relationship? Is it all-or-nothing above or below a critical restorative dose level? Or is there a dose-response function? These last questions could be
answered by observing fighting in castrates given restorative doses of many different strengths. (2) What is the active principle in the androgens that maintains or produces fighting behavior in castrates? This could be investigated by comparing the effectiveness of the same strength doses of closely related androgens.

In an attempt to answer the second question, Bevan et al. (1957) compared the fighting behavior of mice after castration and after restorative doses of testosterone, androsterone, and dehydroisoandrosterone. The results obtained were totally unexpected and merely served to enhance the complexity of the situation.

When given doses of 150 mcg (uniformly inadequate for physiological maintenance) the hormones facilitated both frequency and vigor of aggressive encounter. In contrast, when given 300 - 600 mcg doses (physiologically adequate for testosterone but not for the other two compounds) they appeared to have no effect on frequency of encounter and actually to suppress vigor of aggressiveness in castrates.

In a later study (Bevan, Bevan & Williams, 1958), a comparison was made of the effects on fighting of testosterone therapy using different strengths. Male mice were isolated and operated on between 35 and 40 days of age. Oral doses of methyltestosterone were given daily in strengths of 400, 600 and 800 mcg. None of the operated groups approached the level
of aggressiveness displayed by the unoperated, untreated controls. Nor did the treated animals differ reliably in level from untreated castrates.

The dosage levels used in the 1957 study as well as in this study appear to have resulted in refractory effects. Furthermore, the results may be indicative of the fact that aggressiveness is more than simply a matter of androgen level as demonstrated by the finding of Tollman and King (1956) that testosterone had no effect upon the fighting of gonadectomized females. These investigators proposed that the nervous system of the two sexes responds differently to testosterone.

The relative influence of both androgen status and pre-test fighting experience upon competitive aggression was studied with seventy-two C57Bl/10 male mice kept isolated in individual living cages except for testing. Twenty-four animals were castrated at 35 - 40 days of age. Half of these were placed on replacement therapy of testosterone propionate for the remainder of the experiment. After several weeks of isolation all animals were trained to escape shock by jumping to a small platform at the center of the floor. One-third were then forced to compete for the platform with an aggressive trainer, one-third with a submissive trainer and the final third received no competitive experience. After training, an additional twenty-four animals were castrated. Twelve of them received replacement therapy. Two weeks after training each
mouse was placed in a round-robin test sequence of 17 pairings representing each combination of pre-test experience and androgen status (Bevin, Daves & Levy, 1960).

Analysis of the data from 1224 bouts yielded the following results:

(1) Time of castration relative to training had no effect on fighting in the competitive test situation.

(2) Winning during the training series was associated with the most intense aggression in the test situation. Meanwhile, no reliable differences were demonstrated between losers and animals allowed no pre-test competitive experience.

(3) Androgen status was shown to have some relationship to aggression, but this appeared to be evidenced through its effect upon body weight.

(4) Of the two variables tested, androgen status and pre-test experience, pre-test experience appeared to have been more influential.

Levine and Mullins (1965) conducted an experiment to study the effects of testosterone propionate injections to newborn female rats and castration of newborn male rats on pain-induced aggressive behavior (Ulrich & Azrin, 1962). Results indicated that neonatal castration of males produced a level of aggressive behavior in response to 2.5 milliamperes of electric shock that resembled that seen in normal intact females. However, neonatal testosterone propionate injections to females
suppressed aggressive behavior below the normal female level. In this instance, therefore, the newborn male, deprived of gonadal hormones in infancy, is feminized and the newborn female given testosterone propionate is hyperfeminized.

Following isolation of intact male mice for 21 days, Suchowsky (1967) showed that testosterone propionate (1 mg s.c. daily) only slightly increased aggressiveness. If adult males were castrated simultaneously with isolation no aggressive behavior developed. But if castrated, isolated males were simultaneously treated with steroidal hormones their behavior changed and they were aggressive. Testosterone propionate was shown to reinstate and maintain fighting behavior for as much as 15 days after suspension of treatment.

Bronson and Desjardins most recently (1968) performed a series of experiments in mice with the intention of extending the concept of androgen-dependent organization of adult characteristics to include aggressiveness. Single injections of 1 mg of testosterone propionate at 3 days of age increased the incidence of fighting among females tested during adulthood. Such females, in addition, usually attacked, wounded, and in one case, killed males paired with them. A second experiment showed androgen to be most effective in increasing aggressiveness in adult females when given on the day of birth while the effect was gone by 24 days of age.

A third experiment in which males were castrated at birth
and then given androgen as neonates and/or again as adults led these authors to the hypothesis that maximum aggressiveness in the adult male is dependent upon androgen being present during at least two developmental periods in the neonate. The effect of androgen during one period would be to elevate aggressiveness independently of androgen circulating in the adult while the effect in the second period would be to sensitize the brain to be more responsive to androgen circulating in the adult.

Further studies on male rats (Conner & Levine, 1968) demonstrated that castrates fought less in adulthood than sham operated controls, provided the animals were castrated at or before weanling age. The age of the male rat at the time of castration was found to be an important parameter of adult fighting behavior. Males castrated neonatally (within the first 96 hours) fought significantly less as adults than animals castrated at weanling age.

Hypophysectomy was observed to interfere with the development of fighting behavior (Sigg, Day & Colombo, 1966). Furthermore, 21 of 30 mice, aggressive before the operation, lost this behavior after hypophysectomy. Repeated injection of ACTH, TSH or LH did not induce aggressiveness in these mice. However, five of ten testosterone treated hypophysectomized mice became aggressive within 21 days with no further change up to 28 days. At that time the treatment was terminated and
all animals failed to exhibit aggressiveness by the 35th day.

It has been shown, therefore, that testicular hormone affects the fighting behavior of male mammals. Androgen has also been found to stimulate aggressiveness in the females of some mammalian species. In contrast to androgen, however, estrogen has been regarded as an inhibitor of aggressive behavior although this may not be true in all cases.

Clark and Birch (1945; 1946) thought they observed some increase in aggressiveness between a pair of castrate female chimpanzees by injecting estrogenic hormone, but no actual fighting resulted from either this or the androgenic hormone which they also tried.

Not until 1956 did Kislak and Birch make a similar suggestion that estrogen produced an increase in aggressiveness. This was indicated by two lines of evidence: First, spayed female hamsters became slightly more aggressive when given several injections of estrogen. Second, untreated spayed females were somewhat less aggressive than intact animals in the non-estrous condition. The authors believed that this difference in aggressiveness was due to the presence of estrogen in the unoperated females.

The studies of Gustafson and Winokur (1960) attempted to investigate the effect of sexual experience and of estrogen upon aggressive behavior in one inbred strain of mice. In their study the males were separated from each other at the age of
thirty days and from then on lived alone without contact with other mice. During the testing period the males were placed, for a set period of time prior to fighting, with females who were definitely in estrous. It was obvious from the results obtained that neither sexual satiation, nor female hormone administration (200 I.U. Theelin 15 hours prior to fighting), had any effect on aggressive behavior as demonstrated by the continued fighting of the mice. However, since only one strain of mice were used, the authors admitted the possibility that genetic factors might be partially involved in determining aggressivity.

Since there were no papers available regarding the effects on aggressive behavior of synthetic analogues of steroid hormones, Suchowsky (1967) programmed his work to study the influence of several classical sex hormones and of some synthetic derivatives. The results showed that progesterone and 6 alpha methyl 17 alpha acetoxyprogesterone (MAP) in doses of 1 mg s.c. daily, decreased aggression while estradiol at a dose of 1 mcg s.c. daily abolished combativeness in intact, isolated male mice. Suspension of treatment led to a progressive reestablishment of fighting behavior within the following 15 days. If castrated isolated male animals were treated simultaneously with progesterone a moderate appearance of aggressiveness was induced, whereas MAP did not promote fighting behavior at all.
Further investigations (Suchowsky, 1968) involved the administration of a combination of testosterone propionate and estradiol to castrated, isolated mice. The manifestation of aggressive behavior was negligible but did appear after the suspension of treatment. It was concluded that an androgenic effect may be masked or inhibited by an estrogenic side effect or by estrogens themselves. The suspension of treatment led to the manifestation of the longer lasting androgenic properties. The strongest inhibitors of aggression in these studies were represented by estrogens and followed by pregnane derivatives which seemed to produce an effect which was longer lasting.

Bronson and Desjardins (1968) observed that neonatal injection of estradiol increased aggressiveness in females after maturity though to a lesser extent than did testosterone. Although Feder (1967) had reported that masculine or aggressive responses interfered with normal female sexual behavior when rats were treated with estrogen in infancy, it was not as severe as that shown in this study where the females usually attacked and sometimes wounded males.

Little is known about the endocrine basis of predatory aggression. Castration and subsequent adrenalectomy does not reduce the tendency for killer rats to kill mice and administration of testosterone to non-killers does not increase the tendency to kill (Karli, 1958).

Essentially nothing is known about the endocrine basis
of fear-induced aggression. It has been suggested (Moyer, 1968) that certain endocrine balances might sensitize the brain areas which control this type of response to external stimulation.

There is some evidence that both the endocrine and the nervous system are involved in the control of irritable aggression but little is known about the interactions between the two. In one study it was shown that castration controlled both the hypersexuality and the aggressive behavior of amygdalectomized cats (Schreiner & Kling, 1953). These same experimenters reported a drastic increase in irritable aggression in two docile, spayed, but otherwise normal cats after the administration of daily doses of diethylstilbestrol. However, this finding has never been replicated.

Although gonadal hormones may be involved in the response of territorial aggression little if any work has been done on mammals to substantiate this. It is also possible, that in maternal aggression the hormonal changes which accompany delivery and lactation serve merely to increase the generalized irritability of the female.

Of all classes of aggression, however, inter-male aggression is particularly dependent upon the male hormone for its development and can, therefore, be characterized as a relatively unique class of aggression.
STATEMENT OF THE PROBLEM
Endogenous or exogenous sex hormones of the fetus have been shown to affect ultimate social and sexual behavior in mice by controlling morphogenesis of the genitalia, and, experimentally, in some species at least, directly by their neural organizer effect. Most of the available data, however, lend support to the theory that the critical time for either of these effects is the paranatal period of development.

In no instance has the effect of a single dose of a sex hormone on the developing fetus been studied in an attempt to ascertain whether or not one can alter, during early fetal life, specific circuits in the brain which are related immediately to a secondary sex-related behavioral characteristic such as aggression, without at the same time, producing gross genital abnormalities.

The present investigation was, therefore, undertaken for a threefold purpose: first, to study the effects of sex steroids and their analogues on the adult behavior of the offspring when any one of these compounds was administered in a single dose to the mother at randomly chosen stages of gestation; second, to investigate the possibility of obtaining, through this method, a normally organized genital tract in the presence of deviated development of the brain, and third, to observe the acute effect of a similar dose of each of these compounds on the behavior of normal adult mice.
CHAPTER IV

MATERIALS AND METHODS
MATERNAL CARE AND TREATMENT:

The animals used in this study were Swiss-Webster female mice obtained from Camm Laboratories in New Jersey and Pel Freeze Laboratories in Minneapolis. Pregnancy determinations were performed prior to shipping. Copulation was ascertained by the presence of a vaginal plug following mating or by finding sperm in the vaginal washings. This was designated day one of pregnancy.

The mice arrived at this laboratory on day three of pregnancy at which time they were divided into groups of six and housed individually. Each group of six cages was labelled according to the day of gestation when each mouse in that group would receive a single subcutaneous injection of the steroid under investigation.

The days, randomly chosen for injections, were days 7, 10, 12, 15 and 17 of gestation. All injections were administered subcutaneously into the right hind leg with a 26 gauge needle. The vehicle for the steroids being tested was distilled water with propylene glycol as a suspending agent. This vehicle had been previously tested and found to be inert, having no toxic effects upon the mother or fetus in terms of fetal resorptions, retarded growth of the fetus or behavioral effects on either mother or fetus.
COMPOUNDS STUDIED IN THIS INVESTIGATION:

NORETHYNODREL 0.1 mg/kg (progestational)

MESTRANOL 0.0015 mg/kg (estrogenic)

TESTOSTERONE 0.5 mg/animal (androgenic)

CYPROTERONE ACETATE 0.5 mg/animal (antiandrogenic)
The doses of norethynodrel and mestranol are equivalent to those in the trademarked oral formulation "Enovid" and were found to produce the greatest number of viable offspring in mice when administered during pregnancy. The dose selected for testosterone is compatible with that used in earlier investigations by other authors and is within the physiological range. The dose of cyproterone acetate, however, was arbitrarily chosen due to the limited research with this compound on mice.

Following steroid treatment of each pregnant mouse on the designated day, the course of pregnancy was permitted to continue undisturbed until the animals came to term. This precaution was necessary since handling of pregnant mice has been shown to affect the behavior of the offspring (Adler & Conklin, 1963; Weboff, Anderson & Haggett, 1968).

The offspring were delivered normally by the mothers who cared for them until weaning at 21 days of age. At this time male and female offspring were separated.

**ISOLATION AND TESTING OF OFFSPRING:**

At 30 days of age male and female mice weighing 20 to 25 grams were divided into groups of ten according to the day of prenatal treatment of the mothers. The mice were then isolated individually in opaque metal cages measuring 9 x 6 x 4 inches (Fig. 1). All visual and social contact was
restricted. Food and water was available at all times.

The period of isolation was maintained for 21 days during which time handling of the mice was restricted to once a week when the cage was cleaned.

At the termination of the isolation period the procedure of round-robin encounters were staged (Ginsburg & Allee, 1942; Beeman & Allee, 1945) in which each mouse met every other mouse in its experimental group. The animals which were brought together in round-robin encounters were termed members of the same group on the basis of prenatal treatment of the mothers.

The encounters were staged in a neutral cage to which neither mouse was accustomed and which was divided into two equal sections by a partition that could be readily raised.

During the encounter all room lights were turned off and a lamp with a 60 watt bulb was placed over the cage. All noise and movement in the room was restricted.

Encounters were begun by raising the partition between the two mice. The latency to fight was timed with a stopwatch and was calculated as that time from the raising of the partition to the first occurrence of vigorous biting and fighting.

The mice were separated immediately so that neither dominance could be established nor serious wounds inflicted upon either of the mice. The maximum time of exposure was
ten minutes. If no fighting occurred within this period of time the latency was recorded as 600 seconds.

GROSS ANATOMICAL EXAMINATION:

Upon completion of aggression studies all treated and untreated offspring were subjected to gross anatomical examination.

Anogenital distance of each offspring was measured and recorded prior to dissection of the pelvis and abdomen. This is the usual criterion of apparent sex in the newborn (Johnstone & Franklin, 1964).

Since this parameter alone is not entirely adequate for determination of sex, other observations were necessary. Each animal was checked for the presence or absence of a vaginal orifice (Fig. 2). They were then necropsied and gross examination of internal anatomy was made with particular attention to the gonads and accessory sex organs (Fig. 3).

ISOLATION AND TREATMENT OF NORMAL ADULT MALE MICE:

In order to compare the effect on the offspring of sex steroid administration during pregnancy with the effect of sex steroid administration to normal male mice in adulthood, it was necessary to isolate the adult mice for 21 days.

Male mice, which were fifty days of age and weighed 25 to 30 grams were isolated in metal cages similar to those
described above. Handling of the mice was limited and food and water were always available.

These mice were used as their own controls and were, therefore, subjected to staged encounters on four consecutive days according to the following schedule:

- **day 1** - no treatment
- **day 2** - one hour following sham treatment with the vehicle alone
- **day 3** - one hour after steroid treatment
- **day 4** - no treatment

Fighting latency was calculated as that time from the raising of the partition to the first occurrence of vigorous biting and fighting. The mice were then separated immediately. The maximum time of exposure was ten minutes. If no fighting occurred within this period of time the latency was recorded as 600 seconds.

Adult female mice were not used in this part of the study since there is little evidence that adult females will fight even under conditions of isolation. Furthermore, adult male mice were used for the purpose of comparing developmental effects in the offspring of treated mothers with effects on normal animals whose development was essentially completed and not drug affected.
STATISTICAL METHODS:

The Student "t" test was used to determine the level of significance of the data obtained during this investigation (Hill, Bradford, 1961). The following formulae were employed throughout this dissertation for statistical evaluation of the data presented:

\[
\text{variance } = s^2 = \frac{\sum y^2 - (\bar{y})^2}{n-1} \\
\text{standard deviation } = s \\
\text{standard error } = \frac{s}{\sqrt{n}} \\
\text{unpaired "t" test } = t = \frac{\bar{y}_1 - \bar{y}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}
\]

PREPARATION OF DRUG SOLUTIONS:

Each compound was dissolved in 10 mls of distilled water with the aid of 0.1 ml of propylene glycol as a suspending agent, the final solution being a clear microcrystalline suspension.

DURATION OF DAILY AGGRESSION STUDIES:

Depending upon the aggressivity of the mice under observation the duration of round-robin encounters for an entire experimental group ranged from four to six hours. Each group was run consecutively depending upon the day of mother treatment with control groups being observed first.
FIGURE 2

Feminized male rat, note the presence of a vagina. (From Neumann, F. and Elger, W. 1965)
FIGURE 3
(Top) Drawing of internal anatomy of normal female. Note ovaries, oviduct, uterus and small phallus. (Center) Internal anatomy of normal male. Note testes and large phallus. (Bottom) Internal anatomy of virilized female, ovaries, oviduct, blind uterus, large, phallus. (From: Johnstone, E.E. & Franklin, R.R., 1964)
CHAPTER V

EXPERIMENTAL DATA FOR OFFSPRING OF TREATED MOTHERS
NORETHYNODREL + MESTRANOL (ENOVID) - 0.1 mg/kg

1. Aggressive behavior in female offspring:

The effect on female mice offspring of treatment of pregnant mothers with a single injection of norethynodrel plus mestranol on one specific day of pregnancy is shown in Figure 4. The ordinate represents the day of mother treatment following conception and the abscissa represents the average latency of the offspring to fight for a total of ninety trials per group of ten mice.

During the observation period of ten minutes for each staged encounter, neither the offspring of control mothers nor the offspring of treated mothers showed any tendency for aggressive behavior regardless of the day of mother treatment.

The mice were mutually compatible and manifested, for the greater part of the observation period, either exploratory or contactural behavior patterns. Attempts to elicit aggressive behavior by means of tail pinching or pencil-probing were futile.

There were no indications of bizarre sexual behavior during the encounter periods and in general all treated offspring displayed behavior patterns typical of normal, unisolated female mice.
AGGRESSIVE BEHAVIOR IN FEMALE MICE OFFSPRING OF MOTHERS TREATED DURING PREGNANCY

**Figure 4**

- Control Group
- Offspring of injected mothers (Norethynodrel & Mestranol: 0.1 mg/kg, 0.0015 mg/kg)

*Time (sec.)*

<table>
<thead>
<tr>
<th>Control</th>
<th>7</th>
<th>10</th>
<th>12</th>
<th>15</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGHTING LATENCY</td>
<td>(AVERAGE OF 90 TRIALS/ Group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2. Aggressive behavior in male offspring:

Figure 5 represents the effects of norethynodrel plus mestranol on the isolation-induced aggression of male mice offspring of mothers treated during pregnancy. The ordinate represents the day of mother treatment following conception and the abscissa represents the latency to fight for a total of ninety trials per group of ten mice.

A demonstrable alteration in the aggressive aspect of social behavior is evident in certain of the male offspring of treated mothers. The male offspring of control mothers showed an average latency to fight of 83 seconds which was not significantly (p > 0.5) altered in the male offspring of mothers treated on days 7 (103 seconds), 12 (80 seconds) or 15 (95 seconds) of pregnancy.

On the other hand, the male offspring of mothers treated on day 10 of pregnancy showed an almost five-fold increase in the average latency to fight with a latency of 390 seconds and were, therefore, significantly (p < 0.001) less aggressive than the control male offspring.

Of equal importance in this experiment was the fact that the offspring of mothers treated on day 17 of pregnancy showed a four-fold decrease in the average latency to fight with a latency of 20 seconds. They were, therefore, significantly (p < 0.001) more aggressive than control male offspring.
AGGRESSIVE BEHAVIOR IN G MICE OFFSPRING OF MOTHERS TREATED DURING PREGNANCY

(Fig. 5)

Range of values in seconds: Control (75-92); Day 7 (85-125); Day 10 (358-453); Day 12 (76-99); Day 15 (83-120); Day 17 (5-35).

FIGURE 5
However, as can be seen in Table 1, subsequent experimentation failed to reproduce the initial increased aggression seen in day 17 offspring, whereas the decrease in aggression observed in day 10 offspring was replicated in each experiment and was found to be statistically significant ($p < 0.001$).

Although an occasional pattern of female sexual behavior was observed in the day 10 treated offspring, these were not consistent enough to warrant serious consideration.
AVERAGE FIGHTING LATENCY IN MALE OFFSPRING
90 TRIALS/GROUP OF 10 MICE

<table>
<thead>
<tr>
<th>Norethynodrel + Mestranol</th>
<th>Day of Mother Treatment (time in seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 7 10 12 15 17</td>
</tr>
<tr>
<td>Experiment</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>83 103 379 80 95 20</td>
</tr>
<tr>
<td>2</td>
<td>63 80 365 63 45 88</td>
</tr>
<tr>
<td>3</td>
<td>80 93 400 74 65 59</td>
</tr>
</tbody>
</table>

**TABLE 1**
3. Gross anatomical examination:

Gross examination of male and female offspring in both the control and treated groups revealed that all members of a given sex possessed genital structures which were of uniform size and morphology.

The width of the perineum was consistently greater in the male animals than in the females as shown in Table 2 where anogenital measurements have been tabulated. The male offspring of treated mothers were no different from control male offspring in this respect.

Examination of treated offspring did not reveal the presence of a vagina in the male animals and upon opening the abdominal cavity it was easy to distinguish between male and female animals by the appearance of the gonads.

Since histological examinations were not performed in this study one cannot preclude the presence or absence of corpora lutea in the ovaries or spermatogenesis in the testes.
ANOGENITAL DISTANCE*

<table>
<thead>
<tr>
<th></th>
<th>CONTROLS - NO TREATMENT</th>
<th>NORLETHYNOREL + MESTRANOL (Day 10 Offspring)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female:</td>
<td>Mean = 1.5mm ± 0.03</td>
<td>Female: Mean = 1.4mm ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Range = 1.3mm - 1.6mm</td>
<td>Range = 1.3mm - 1.6mm</td>
</tr>
<tr>
<td>Male:</td>
<td>Mean = 2.1mm ± 0.02</td>
<td>Male: Mean = 2.0mm ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Range = 1.9mm - 2.3mm</td>
<td>Range = 1.8mm - 2.3mm</td>
</tr>
</tbody>
</table>

* 20 females and 20 males/group

TABLE 2
1. Aggressive behavior in female and male offspring:

In order to determine which of the two components in Enovid (norethynodrel + mestranol) is responsible for the decreased aggression seen in the male offspring of day 10 treated mothers, the experiment was expanded and norethynodrel alone was administered on a single dose on each of the designated days of pregnancy.

Female offspring of treated mothers again showed no tendency for aggressive behavior and results were similar to those illustrated in Figure 4.

In Figure 6, however, the effects on the male offspring are shown. Here again, the ordinate represents the day of mother treatment following conception and the abscissa represents the average latency to fight of 90 trials per group of 10 mice.

The average fighting latency of control male offspring was 82 seconds which was not significantly (p>0.5) different from the fighting latencies of day 7 offspring (98 seconds), day 12 (78 seconds), day 15 (82 seconds) and day 17 (98 seconds).

However, the male offspring of mothers receiving 0.1mg/kg of norethynodrel on day 10 of pregnancy showed an average latency to fight of 263 seconds which was significantly (p<0.001) greater than that of the control offspring. These mice were, therefore, much less aggressive than control male offspring.
AGGRESSIVE BEHAVIOR IN MALE MICE OFFSPRING OF MOTHERS TREATED DURING PREGNANCY

TIME (Sec.)

FIGHTING LATENCY (Average of 90 Trials/Group)

10/GROUP

- OFFSPRING OF CONTROL
- OFFSPRING OF INJECTED
(Norethynodrel 0.1 mg/kg)

Day of treatment after conception:

FIGURE 6
Similar results were obtained in day 10 offspring in subsequent experiments with norethynodrel (Table 3). This would appear to confirm the fact that norethynodrel is responsible for the decreased aggression observed in day 10 male offspring of treated mothers.
### AVERAGE FIGHTING LATENCY IN MALE OFFSPRING

**90 TRIALS/GROUP OF 10 MICE**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Norethylnodrel</th>
<th>Day of Mother Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
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<td>(time in seconds)</td>
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<tr>
<td>1</td>
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<td>2</td>
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<td>94</td>
</tr>
<tr>
<td>3</td>
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<td>110</td>
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</tbody>
</table>

**TABLE 3**
2. Gross anatomical examination:

Gross anatomical examination of both male and female offspring of treated and untreated mothers again failed to reveal any morphological abnormality.

Table 4 shows that anogenital distance measurements were within the normal range for both males and females thus making it possible to determine sex with ease.

Necropsy of the mice produced no evidence of macroscopic abnormalities of the gonads or accessory organs, therefore, sexual differentiation was considered to be essentially normal.
ANOGENITAL DISTANCE*

CONTROL - NO TREATMENT

Female: Mean = 1.6mm ± 0.03
Range = 1.3mm - 1.6mm

Male: Mean = 2.1mm ± 0.02
Range = 1.9mm - 2.3mm

NORETHYNODREL - 0.1mg/kg
(Day 10 offspring)

Female: Mean = 1.45mm ± 0.03
Range = 1.3mm - 1.6mm

Male: Mean = 1.96mm ± 0.07
Range = 1.9mm - 2.1mm

* 20 females and 20 males/group

TABLE 4
1. Aggressive behavior of female and male offspring.

The offspring of pregnant mice treated with a single dose of mestranol on one of the designated days of pregnancy were also observed for alterations in aggressive behavior.

Female mice once again failed to give any indication of either increased irritability or increased aggressive behavior. Therefore, the results of treatment with mestranol were similar to those with norethynodrel alone or in combination with mestranol (Fig. 7).

In Figure 8 however, the male offspring of mothers receiving no treatment showed an average latency to fight of 120 seconds which was not significantly (p > 0.5) different from the average latency to fight of the male offspring of mestranol treated mothers on day 7 (135 seconds), day 12 (115 seconds), day 15 (132 seconds) and day 17 (93 seconds).

In contrast, however, the male offspring of mothers treated with a single dose of mestranol on day 10 of pregnancy showed an average latency to fight of 29 seconds which was significantly lower (p < 0.001) than that of the control male offspring.

In this instance aggressive behavior was so severe that serious wounds were inflicted on several of the mice during the staged encounters. Although the mice were usually separated immediately after the first attack this was at times
AGGRESSIVE BEHAVIOR IN MALE MICE OFFSPRING OF MOTHERS TREATED DURING PREGNANCY

Range of values in seconds:-- Control (92-136); Day 7 (98-141); Day 10 (12-34); Day 12 (92-126); Day 15 (102-140); Day 17 (81-102).
Aggressive Behavior in Female Mice Offspring of Mothers Treated during Pregnancy

(10/Group)
C = Control - No treatment
N = Norethynodrel 0.1 mg/kg
M = Mestranol 0.0015 mg/kg
N & M = Norethynodrel & Mestranol 0.1 mg/kg

(No fighting occurred within the 10 minute observation period)

FIGURE 8
difficult to achieve due to the aggressivity of the mice and the forcefulness of the attacks.

Similar results were obtained in one of two subsequent experiments (Table 5).
AVERAGE FIGHTING LATENCY IN MALE OFFSPRING
90 TRIALS / GROUP OF 10 MICE

<table>
<thead>
<tr>
<th>Mestranol Experiment</th>
<th>Control</th>
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<td>96</td>
<td>21</td>
<td>105</td>
<td>99</td>
<td>94</td>
</tr>
</tbody>
</table>

TABLE 5
2. Gross anatomical examination:

Male and female offspring of mothers treated on designated days of pregnancy with mestranol revealed no anatomical abnormalities. The external genitalia of male mice was normal in appearance and a vagina was not found to be present in any of the offspring of treated mothers. Neither were there any visible signs of virilization of the external genitalia of the female mice offspring of treated mothers.

Measurement of the perineum in both males and females of treated and untreated mothers (Table 6) was consistent with previous observations.

Examination of the internal anatomy of all experimental mice confirmed the sex of each animal in the group and no macroscopic abnormalities of the gonads or accessory organs were apparent.

Therefore, the animals were considered to be sexually intact as in the previous experiments.
ANOGENITAL DISTANCE*

<table>
<thead>
<tr>
<th>Control - No Treatment</th>
<th>Mestranol 0.0015mg/kg (Day 10 Offspring)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female: Mean = 1.6mm ± 0.03</td>
<td>Female: Mean = 1.4 ± 0.007</td>
</tr>
<tr>
<td>Range = 1.3mm - 1.6mm</td>
<td>Range = 1.3mm - 1.5mm</td>
</tr>
<tr>
<td>Male: Mean = 2.1mm ± 0.02</td>
<td>Male: Mean = 2.0mm ± 0.03</td>
</tr>
<tr>
<td>Range = 1.9mm - 2.3mm</td>
<td>Range = 1.9mm - 2.2mm</td>
</tr>
</tbody>
</table>

* 20 females and 20 males / group

**Table 6**
4. COMPARISON OF STEROID EFFECTS ON MALE OFFSPRING

Figure 9 represents a comparison of the effects on aggressive behavior of day 10 male offspring of mothers treated with:

(a) norethynodrel + mestranol (N + M)
(b) norethynodrel (N)
(c) mestranol (M)

It can be readily seen that norethynodrel + mestranol (N+M) as well as norethynodrel (N) alone, significantly (p<0.001) decreased aggressive behavior in these offspring.

Mestranol (M) on the other hand, when administered alone, produced a significant (p<0.001) increase in aggressive behavior as demonstrated by the decrease in average latency to fight (controls = 120 seconds; mestranol = 29 seconds).
Aggressive Behavior in Male Mice Offspring of Mothers Treated during Pregnancy

TIME (Sec.)
390
330
300
270
210
150
90
30

FIGHTING LATENCY (Average of 90 Trials/Group)

Expt. 1 Expt. 2 Expt. 3
C N & M C N C M

Treatment on Day 10 of Pregnancy

Range of values in seconds:
- Expt. 1-Control (62-94); N & M (310-400);
- Expt. 2-Control (60-96); N (223-281); Expt. 3-Control (109-130); M (18-35)

FIGURE 9
TESTOSTERONE - 0.5mg/animal

1. Aggressive behavior in female and male offspring:

The effect of testosterone on the isolation-induced aggression of male and female offspring of mothers treated during pregnancy was studied.

The female offspring in all treatment groups failed to show any form of aggressive behavior. These results were similar to those previously illustrated (Fig. 7).

Testosterone had no effect in altering aggressive behavior in any of the male offspring of treated mothers (Fig. 10):

- controls - 114 seconds
- day 7 - 132 seconds
- day 10 - 108 seconds
- day 12 - 120 seconds
- day 15 - 112 seconds
- day 17 - 102 seconds

All male offspring, regardless of day of mother treatment were equally aggressive and there was no significant (p > 0.5) difference between groups of mice.
AGGRESSIVE BEHAVIOR IN MALE MICE OFFSPRING OF MOTHERS TREATED DURING PREGNANCY

Range of values in seconds: - Control (96 - 123); Day 7 (112-139); Day 10 (93 - 114); Day 12 (106-131); Day 15 (94-126); Day 17 (38-112).

FIGURE 10
2. Gross anatomical examination:

Neither male nor female offspring of mothers treated during pregnancy with a single dose of testosterone showed any gross genital abnormalities.

Female offspring presented no signs of virilization of the external genitalia.

Measurement of the perineum (Table 7) made differentiation of the sexes easy and subsequent necropsy revealed normal appearing gonads and accessory organs in animals whose sex had been previously determined by anogenital measurements.

As a result of these findings all treated animals were considered to have undergone normal sexual differentiation.
ANOGENITAL DISTANCE*

CONTROL - NO TREATMENT  TESTOSTERONE - 0.5mg/animal
(Day 10 offspring)

Female: Mean = 1.6mm ± 0.03  Female: Mean = 1.4mm ± 0.13
Range = 1.3mm - 1.6mm  Range = 1.3mm - 1.5mm

Male:  Mean = 2.1mm ± 0.02  Male:  Mean = 2.1mm ± 0.03
Range = 1.9mm - 2.3mm  Range = 1.9mm - 2.2mm

* 20 females and 20 males / group

TABLE 7
1. Effect of treatment on the mother:

Cyproterone acetate, a potent antiandrogen, was administered to pregnant mice in order to compare the effects of this compound on the isolation-induced aggression of the offspring, with that of other compounds previously studied.

The results presented in Figure 11 were totally unexpected following administration of this compound, and prior to this report, have never been presented in the literature.

In Experiment 1 all of the ten mothers in every treatment group mutilated and killed their offspring immediately or within one day following delivery. When this act of cannibalism did not occur immediately the offspring were able to be observed and appeared morphologically normal.

Bearing in mind the possibility that some extraneous factor might have produced these results the experiment was carefully duplicated using a new lot of pregnant mice. The results of Experiment 2 (Fig. 11) show that this effect appeared to be drug related since none of the offspring in the control groups were killed by the mothers.

Using smaller groups of pregnant mice and injecting them randomly with a single dose of cyproterone acetate an attempt was made to confirm the fact that the mice, in utero, just prior to delivery, were alive and fully developed. This was indeed the case in every instance when caesarian section was
Effect of a Single Dose of Cyproterone Acetate to Pregnant Mice

% Offspring Killed at Birth

Day of Treatment

Expt. 1 - offspring mortality = 100%; Expt. 2 - Day 7 (82%) Day 10 (100%); Day 12 (89%); Days 15 and 17 (100%).

FIGURE 11
performed on days 19 or 20 of gestation.

In only one instance was the situation different, but this was not reproducible.
SUMMARY OF RESULTS

OFFSPRING OF DAY 10 TREATED MOTHERS

Norethynodrel + Mestranol - males - decreased aggression
- females - no change

Norethynodrel
- males - decreased aggression
- females - no change

Mestranol
- males - increased aggression
- females - no change

Testosterone
- males - no change
- females - no change

Cyproterone Acetate
- cannibalistic effect in all treated mothers

TABLE 8
CHAPTER VI

EXPERIMENTAL DATA FOLLOWING TREATMENT
OF
ADULT MALE MICE
NORETHYNODREL + MESTRANOL - 0.1mg/kg

Aggressive behavior in adult male mice:

When fifty-day old normal adult male mice were isolated for twenty-one days and were then subjected to round-robin fighting without prior drug treatment they showed an average latency to fight of 209 seconds (Fig. 12). Since these mice were to be used as their own controls it was necessary to establish that they were indeed aggressive.

On day 2, these mice were treated with a placebo which consisted of the vehicle minus the active component. One hour after the treatment the animals were exposed consecutively to each of the mice in their experimental group. The average latency to fight was 162 seconds which was not significantly (p>0.5) different from that of the previous day when no treatment was given.

On day 3, a single injection of norethynodrel plus mestranol (Enovid) was administered one hour prior to the encounters. The average fighting latency increased to 595 seconds, which was a significant (p<0.001) decrease in aggression as compared to control values. Instead of fighting, the mice usually sat quietly in a corner of the cage or engaged in brief exploratory activity.

However, on day 4, twenty-four hours later, after receiving no treatment, these same mice showed an average latency to fight of 218 seconds, essentially the normal control value.
AGGRESSIVE BEHAVIOR OF ADULT MALE MICE FOLLOWING INJECTION OF NORETHYNODREL with MESTRANOL

Range of values in seconds: - Control (C) -(175 - 221); Diluent (D) - (155 - 170); N+M (552 - 601); Control (C) -(201 - 227).

FIGURE 12
COMPARISON OF EFFECTS: NORETHYNODREL, MESTRANOL AND NORETHYNODREL + MESTRANOL:

Aggressive behavior in adult male mice:

Figure 13 represents a comparative study of the effects of norethynodrel, mestranol and norethynodrel + mestranol on three different groups of adult male mice.

Each group consisted of 10 mice who served as their own controls in that particular group. Following a period of twenty-one days of isolation, the ten mice in each group were subjected to staged encounters, as in the previous experiments, without prior hormone treatment in order to establish their base level of aggressivity.

The average latency to fight was as follows:

Control Group 1 = 40 seconds
Control Group 2 = 32 seconds
Control Group 3 = 71 seconds

On day 2, each mouse received the hormone treatment designated for its group, one hour prior to exposure to the other mice within its group. The average latency to fight following treatment was as follows:

Norethynodrel Group 1 = 92 seconds
Mestranol Group 2 = 40 seconds
Norethynodrel + Mestranol Group 3 = 150 seconds
Aggressive Behavior of Adult Male Mice
One Hour after Treatment

(10/Group)

C = Control trials — Day 1
N = Norethynodrel 0.1 mg/kg
M = Mestranol 0.0015 mg/kg — Day 2
N & M = Norethynodrel & Mestranol

Range of values in seconds:— Group 1 (C) 37-43; (N) 85-97;
Group 2 (C) 29-36; (M) 34-48; Group 3 (C) 62-78; (N&M) 142-163

FIGURE 13
The most significant alterations \( (p<0.001) \) in aggressive behavior appeared in Groups 1 and 3, norethynodrel and norethynodrel + mestranol treated mice.
Aggressive behavior in adult male mice:

A behavioral comparison of cyproterone acetate treatment of normal adult male mice with the male offspring of treated mothers was impossible in this study due to cannibalism on the part of the treated mothers.

It seemed important, however, to investigate the action of this compound on the isolation-induced aggression of normal adult male mice in an attempt to determine whether such treatment would adversely affect the behavior of these mice.

From Figure 14 it can be seen that there was no noticeable change in the aggressive behavior of these mice following treatment with cyproterone acetate. During the control run the average latency to fight was 160 seconds as compared with 140 seconds one hour after drug treatment.
Aggressive Behavior of Adult Male Mice Following Injection of Cyproterone Acetate

Range of values in seconds: - Control = 147 - 171; Cyproterone Acetate = 126 - 150.

FIGURE 14
SUMMARY OF RESULTS

NORMAL ADULT MALES - ONE HOUR AFTER TREATMENT

Norethynodrel + Mestranol - decreased aggression
Norethynodrel - decreased aggression
Mestranol - no change
Testosterone - not studied in adults
Cyproterone acetate - no change

TABLE 9
CHAPTER VII

DISCUSSION OF EXPERIMENTAL DATA
If there is a particular time, a critical stage, during which a given treatment has a maximal effect, then this can serve as a tool for further research of interest and importance. Examples of such research might be:

(1) a comparison of the developmental stages at which apparently similar treatments are reported to have similar effects in other species,

(2) investigation into how the treatment produces its effects,

(3) elucidation of the underlying physiological, neurological or humoral mechanisms by considering what developing changes are taking place in particular organs or processes during this critical period. Such thinking characterizes many investigators in behavioral science (Scott, 1951; King, 1958; Thompson & Schaefer, 1961; Scott, 1962.)

The results of this investigation clearly show that norethynodrel + mestranol, and norethynodrel alone, when administered in a single dose to mice on the 10th day of pregnancy significantly inhibited isolation-induced aggression in the male offspring but did not noticeably affect the behavior of female offspring similarly treated on that day. It would appear at first glance, that at the "critical period" of day 10 of pregnancy the brain responds to the presence of sex hormones by differentiating irreversibly into a pattern which is behaviorally characteristic of the sex
corresponding to the hormones of prenatal exposure.

That this is not necessarily so is demonstrated by the fact that mestranol (an estrogenic synthetic steroid), when administered alone, produced a significant increase in the aggressive behavior of day 10 male offspring. Furthermore, testosterone administration during the so-called "critical period" of development did not alter the behavior of either male or female offspring.

The changes that were observed with norethynodrel and/or mestranol were restricted to aggressive behavior and did not extend to sexual differentiation. Separation of the sexes according to anogenital distance measurements and postmortem examination was relatively easy. The presence of testes in the males as well as an adequately developed phallus lends support to the concept that the behavioral alteration observed in these animals may not be the result of male hormone suppression with a resultant castration effect, or male hormone stimulation, which may in turn modify behavior.

Possibly there is some direct, permanent neuronal modification of specific circuits in the brain which are related immediately to a secondary sex-related behavioral characteristic such as aggression. The regulatory influence of hormones on central nervous system development is dependent upon the stage of maturation of the neural tissue at the time of hormonal administration or deficiency and on the
affinity of specific CNS structures for specific hormones.

There is biochemical, neurophysiologic and behavioral evidence that progestational compounds enter the brain and affect brain function (Lisk, 1960; Hamburg, 1966). What is not known, however, is whether or not the behavioral effects observed are a result of metabolic transformation of the steroids or a direct effect of the steroid itself on specific receptors.

Data on the effects of norethynodrel on the aggressive behavior of mice offspring of treated mothers is non-existent. Therefore, it is impossible to theorize on the basis of previous investigations. One can only conclude from these experiments that there is a period during embryonic development when neural tissue is particularly sensitive to this compound and during which time an adult pattern of behavior is irreversibly determined. That this pattern does not always coincide with the phenotypic sex of the animal is clear from the data presented.

The increased aggressivity of male offspring of mothers treated with mestranol tends to derive more support from the studies of other investigators. Bronson and Desjardins (1968) have reported that estrogens, depending upon the time and dose of their injection may mimic some of the effects of endogenous androgens in the male.
A number of studies have shown that certain steroid hormones affect the development of the central nervous system (Heim & Timiras, 1963; Vernadakis & Woodbury, 1963; Vernadakis & Timiras, 1963; Vernadakis & Woodbury, 1964; Heim, 1966). Estradiol dipropionate given to intact female rats for four consecutive days accelerated brain development and increased brain excitability. This increase in brain excitability was observed for six months after estradiol administration.

It was also seen (Curry & Heim, 1966; Casper et al, 1967) that early postnatal injection of estradiol influenced the rate of brain myelinization in the developing rat and thereby hastened functional brain maturation. A similar effect was not reported for testosterone.

The permanent effect of estradiol on brain excitability in developing rats was believed to be due to irreversible cellular changes induced by the hormone (Heim & Timiras, 1963). These authors suggested that the critical age period during which estradiol is most effective in altering brain function is between the 8th and 12th days of age.

One might suggest, therefore, that mestranol, administered to female mice on day 10 of pregnancy affected brain development of the offspring in such a way as to lower the threshold of responsiveness to stimuli in adulthood which provoke aggressive behavior. That this was not likewise true
in the female offspring of mothers treated on day 10 may be due to the inadequacy of this particular parameter for measuring behavioral changes in female mice.

The fact that synthetic steroid hormones have important effects on the functioning of the central nervous system is currently established and in certain cases these nervous effects may be expressed in the triggering of specific behaviors.

Unquestionably, the mechanism of action of steroid hormones on the developing central nervous system is still unclear. It has been implied that the septal nuclei in connection with the amygdala, take part in the emotional reactions of animals (King, 1958; Zeman & Innes, 1963) probably by inhibiting the emotional behavior. In fact, according to Delgado (1961) stimulation of septal areas depresses both emotional reactions and motor activity. It is interesting to speculate, therefore, some relationship between the steroid hormones studied here and the embryonic development of these specific areas of the brain.

The fact that cyproterone acetate reversed the maternal behavior of female mice to such an extent that all of the offspring were destroyed at the time of birth, was indeed significant. Cyproterone acetate is a progestational, anti-androgenic compound which has been investigated in rats at a much higher dosage than that employed in this study. It has
been shown that the action of cyproterone acetate is one of competitive inhibition with testosterone in peripheral tissues. It has little effect on testosterone uptake by neural tissues (Whalen, Luttge & Green, 1969).

If one were to postulate, for the sake of argument, that increased amounts of testosterone are secreted during pregnancy, then cyproterone acetate could raise the blood titre of testosterone by inhibiting its uptake in peripheral tissues. Since cyproterone acetate has a prolonged duration of action, and since neural uptake of testosterone is not inhibited, the possibility presents itself that increased amounts of testosterone entering the brain tissue might account for the alteration in maternal behavior. The fact that exogenous testosterone administration did not produce a similar effect could be explained by the difference in exposure time of the brain to increased levels of testosterone or to uptake of testosterone by peripheral tissues.

Estrogen and prolactin interaction have been implicated in the maternal behavior of mice toward their newborn pups. (Moltz, Levin & Leon, 1969). Administration of progesterone several days before delivery (by caesarian section) was believed to be responsible for altering the hormonal balance of these hormones, thereby resulting in destruction of 50 percent of the litters by the mothers. Since cyproterone
acetate is a progestational compound it is conceivable that the cannibalistic behavior observed in the mothers following treatment with cyproterone acetate was a result of some drug-induced hormonal imbalance.

There are, of course, other possible hypotheses to account for this type of cannibalistic behavior of mice toward their newborn pups. Instinctual recognition on the part of the parent mouse of congenital malformations in the offspring which may not be readily discernible to the investigator might well have resulted in the destruction of the pups. There is also the possibility that cyproterone acetate interfered with mammary gland function at the time of delivery thereby resulting in an inability to care for the pups.

Further investigations are necessary to clarify the underlying mechanism of this particular aspect of abnormal behavior.

The results obtained following treatment of adult male mice with sex steroids warrants some consideration.

The observation that norethynodrel + mestranol, as well as norethynodrel alone, completely suppressed isolation-induced aggression in normal adult male mice one hour after drug administration is quite clear. Since this non-aggression was unaccompanied by psychomotor activity or patterns of
female sexual behavior and was completely dissipated within 24 hours it may be concluded that these compounds do not irreversibly alter aggressive behavior patterns in normal adult male mice.

One cannot eliminate the possibility that these compounds, by a direct action on specific centers in the brain, were able to suppress aggressive behavior until the compounds had undergone metabolic degradation.

There is, however, considerable evidence to support the concept of a sedative or anesthetic effect of progestational steroids. Selye (1941) found that in several species, including the rat, progesterone and a variety of other steroids brought about a state of general anesthesia when administered in high dosages by the intraperitoneal or intravenous route. Kuntzman et al (1965) showed that control rats given progesterone lost their righting reflex for greater than 150 minutes. A comparison of the anesthetic effect of different progestins (Meyerson, 1967) revealed that norethynodrel at a dose of 10 mg/animal produced general anesthesia in rats which lasted approximately 10 minutes.

Although the righting reflex was never lost in any of the mice undergoing treatment prior to staged encounters for aggressive behavior in the present investigation, this difference may simply be a reflection of the difference in dosage used. In this study the dosage was much lower and
corresponded with the low dosage form of the trademarked oral formulation (Enovid). The quiescent behavior of the animals following norethynodrel + mestranol and norethynodrel alone would seem to be indicative of some degree of sedation.

Testosterone was not studied in this series of experiments since it has undergone extensive investigation and its effects on aggressive behavior have been previously discussed. It would also seem unlikely that cyproterone acetate, through its antiandrogenic action would have an effect on aggression. Although the uptake of testosterone by peripheral tissues is inhibited by cyproterone, thereby increasing neural uptake, increased amounts of testosterone administered to intact, adult male mice has not been shown to produce a super-aggressive animal.
CONCLUSION
The data presented in this dissertation indicates the need for further investigations of early, prenatal hormone treatment. The fact that a single dose of compounds such as those used in these studies, administered at a "critical" period of development could produce an alteration in behavior which is apparent at a time far removed from the time of administration is, in itself, of great importance.

These studies were conducted on a pure strain of Swiss-Webster mice. It is possible, therefore, that the observations made during these studies are peculiar to this particular strain, for it is well known that many drug effects are species specific as well as strain specific. This does not diminish the value of this work but merely provides a starting point for future investigations.

The threefold purpose of this investigation has been accomplished:

(1) Synthetic progestational and estrogenic compounds have been shown to alter, irreversibly, the adult behavior of male offspring of mothers treated on the tenth day of pregnancy,
(2) As a result of this method of treatment, it was possible to obtain a normally organized genital tract in the presence of deviated development of the brain and,
(3) Norethynodrel and mestranol did produce an acute effect on the behavior of normal adult male mice which was relatively
short in duration.

Finally, some mention should be made of the unexpected and, heretofore unpublished, effects of cyproterone acetate on pregnant mice. Further investigations are required to determine whether or not the cannabalistic effect produced by this compound is a dose-related phenomenon or whether it does, in fact, alter the normal hormonal balance in the mother at or near the time of delivery.
ADDENDUM

THE EFFECTS OF THE VEHICLE (PROPYLENE GLYCOL + DISTILLED WATER) ON THE AGGRESSIVE BEHAVIOR OF MALE OFFSPRING OF MICE TREATED DURING PREGNANCY

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<tr>
<td>S.D. 9.7</td>
<td>6.8 13.2 4.7 7.1 6.8</td>
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<tr>
<td>S.E. 12.9</td>
<td>±2.06 ±4.0 ±1.4 ±2.1 ±2.06</td>
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</tbody>
</table>

*Average latency to fight - time in seconds - 90 trials/group of 10 mice


Beach, F.A., (1941) Female mating behavior shown by male rats after testosterone propionate, Endocrinol., 29:409 -412.


Harris, G.W., (1964) Sex hormones, brain development and brain function, Endocrinol., 75: 627 - 648.


The dissertation submitted by Sister Elvera Abbatiiello, O.P. has been read and approved by five members of the faculty of the Graduate School of Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 12, 1969  Signature of Advisor

Date  Signature of Advisor