THE EFFECTS OF THE ANTIANDROGEN, CYPROTERONE ACETATE, ON THE BEHAVIOR AND MORPHOLOGY OF MALE AND FEMALE GUINEA PIGS

by

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Chapter I

INTRODUCTION

Current advances in the field of reproductive physiology have indicated that certain behavioral measures are useful and legitimate endpoints for the investigation of processes underlying the differentiation of the male and female of a species. In the last three decades, it has become increasingly apparent that the expression of dimorphic behavioral phenomena emitted by opposite sexes in mammalian species reflects biologically determined developmental processes as surely as do the anatomical differences of the genotypes. Further, the mechanisms involved in the determination of behavioral differences appear to be closely related to those operating on the morphological substrates of the developing embryo.

Sex determination is based on three separate events: the genetic state of the zygote at fertilization; the differentiation of the primordial gonad; and the differentiation of the genital and neural tissues controlling sexually dimorphic phenomena. The onset of gonadal differentiation, controlled by unknown mechanisms in mammalian species, precedes the differentiation of accessory sex structures, and, for this reason, considerable interest has been directed towards the possible role played by secretions of the fetal testis and ovary in this third phase of differentiation.

Morphological Differentiation

All vertebrate embryos pass through a phase of development in which the primordia for both male and female accessory sex structures are present (Burns, 1961). After the differentiation of the gonad occurs, the homotypical primordia differentiate into accessory sex organs while the heterotypical primordia regress. A variety of embryological investigations have revealed that in mammalian species the differentiation of male accessory structures is dependent upon the presence of the fetal testis, but that the development of female accessory structures is independent of the ovary (Burns, 1961). Thus, for the few placental mammals in which surgical removal of the fetal testis has been possible, primordia for male accessory sex organs have failed to differentiate in rabbit (Jost, 1961), mouse (Raynaud & Frilley, 1947), and rat (Wells, Cavenaugh & Maxwell, 1954). Further, in rabbit, Jost (1953; 1961) has demonstrated that the Müllerian duct system persists and develops into female accessory organs in castrated male fetuses.

Testosterone propionate has been shown to have all of the effects of the fetal testis in determining the survival and differentiation of male accessory sex organs. When administered during specific periods to developing fetuses, testosterone propionate has directly influenced the development of penis and/or seminal vesicle and prostate in female hamster (Bruner & Witschi, 1946), mouse (Raynaud, 1942, as cited in Burns, 1961), rabbit (Jost, 1961), rat (Greene, 1942), guinea pig (Dantchakoff, 1936; 1938), and rhesus monkey (Wells & van Wagenen, 1954; Goy, 1966), but has had only minimal effects on the normal differentiation of male

fetuses. However, in mammalian species testosterone propionate has failed to inhibit the differentiation of Müllerian duct structures.

Administration of estrogens to developing fetuses, in turn, has caused varying degrees of accelerated development of Müllerian duct derivatives in females, and regional development of oviduct, uterus and vaginal canals in males (Greene, 1942; Jost, 1961; Moore, 1941), but these findings have been somewhat complicated in that direct damage of testicular tissue by the exogenous estrogen has also been found (Burns, 1961).

In vitro investigations have also supported the conclusion that normal differentiation depends entirely upon the presence or absence of the fetal testis, and have additionally demonstrated that placental hormones do not intervene to maintain or influence the development of accessory sex organs. In the absence of the fetal testis, explanted rat Wolffian ducts have regressed whereas the Müllerian ducts have differentiated normally regardless of the genetic sex of the donor embryo (Jost & Bergernand, 1949). In the presence of the fetal testis, the Müllerian ducts have regressed whereas Wolffian duct derivatives have persisted and differentiated into seminal vesicle and male duct systems (Price & Pannabecker, 1956).

Behavioral Differentiation

Behavioral data have not only supported these findings, but have further clarified and strengthened the position that the presence or absence of fetal testicular secretions determines the course of sexual differentiation. Dantchakoff (1936; 1938) exposed fetal female guinea

pigs to testosterone propionate on the twenty-second day of gestation, and periodically during the first two weeks after birth. At adulthood, under the influence of more exogenous androgen, the modified female ".... appeared like a male" and "..... copulated with normal females in an absolutely normal fashion." (translation, mine).

Phoenix, Goy, Gerall and Young (1959) exposed female guinea pigs to testosterone propionate prenatally and found that if the androgen was injected between day 24 and 67 of gestation, genetic females were masculinized in terms of morphological and behavioral criteria. These masculinized females had an increased capacity to execute male patterns of mounts, intromissions, and ejaculations when given injections of testosterone propionate at maturity. In addition, these females did not display the lordosis reflex in response to ovarian hormones, a characteristic posture of sexual receptivity assumed by estrous females when suitably stimulated. Goy, Bridson and Young (1964) verified these results, and specified the fetal period of maximal susceptibility for the effects of testosterone propionate to be from day 30 to 35 of the guinea pig's 68 - 72 day gestation period. In the same study, these investigators also found a high positive correlation between the degree of behavioral masculinization and the degree of morphological masculinization. Experiments with immature female rhesus monkeys exposed to testosterone propionate during gestation also revealed a behavioral masculinization of this species for several measures of social behavior (Goy, 1966).

Only one mammalian species, the rat, has been assessed for behavioral anomalies after castration during a critical period of sexual differentiation. Grady, Phoenix and Young (1965) castrated neonatal rats at various ages and discovered that males castrated on or before the 5th day after birth were behaviorally feminized in that 1) they would respond to treatments of estradiol benzoate and progesterone by displaying estrous behavior when properly stimulated, and 2) they would not respond to testosterone propionate treatments by displaying all of the normal components of masculine behavior, such as intromission or ejaculatory patterns. In contrast, Grady et al. (1965) and Beach and Holz (1946) demonstrated that males castrated on day 10 or later did display complete patterns of copulation under adult TP treatment, but were unable to display lordosis when given estrogen and progresterone. Thus, the critical period of development for sexual differentiation of behavioral potential would appear to extend postnatally to at least the fifth day after birth, i.e., to day 27 post-fertilization for the rat.

Finally, Feder (1967) and Feder and Whalen (1965) demonstrated that a single injection of estradiol or testosterone administered to 1-day-old female rats rendered them incapable of displaying lordosis at adulthood, even when suitable exogenous hormones were supplemented. Male rats were unaffected by androgen injections at 1 day of age, but were incapable of achieving intromissions and ejaculations at adulthood if they received estrogen on the day of birth. That either estradiol or testosterone can render a female physiologically and behaviorally

anestrous suggests again that the absence of these steroids during critical periods of development insures normal female development.

Organization and Activation

Young (1961) and associates (Goy, 1966; Phoenix et al., 1959) have constructed an extremely useful model of hormone action which serves to consolidate the facts discussed thus far. Androgens are considered to have both organizational and activational actions which determine the regulation of mating behavior. The organizing influence of androgens occurs during critical periods of embryological development, modifying the morphology of the individual, and determining the masculine or feminine character of response patterns that can be elicited from the animal in adult life under a given adult hormonal background. The behavioral organizing actions are thought to 1) facilitate the capacity of animals to display intromissions and other male sex behavior, and 2) suppress the ability of animals to display lordosis and other associated female sex behaviors. These are believed to be irreversible modifications, such that once established, the organized neural substrates can only be modified to the extent that the vigor of the behavior, not the character, is partially susceptible to hormonal and/or other alterations. This role is to be contrasted with the activational action of androgens seen at puberty and during adulthood. Gonadal hormones at this time are thought to influence the individual to respond in accordance with the character of the substrate already established.

The study of activational properties of androgens has been easily accomplished by depriving adult males of endogenous androgens by surgical castration. To deprive males of testicular secretions during organizational periods of differentiation by surgical means is most formidable in the fetus, however, and is an entirely impractical procedure in species with relatively long gestation periods.

Cyproterone Acetate

1. Morphological Effects

The synthesis of a potent antiandrogen, cyproterone acetate* (CA), has allowed investigators to approach the problem of sexual differentiation with a promising new technique. Although many substances are antiandrogenic, CA is by far the most powerful agent yet developed. Hamada, Neumann and Junkmann (1963) administered up to 30 mg CA to pregnant rats from day 16 - 19 of gestation and obtained morphological evidence of profound feminization of male fetuses. Caesarian section was performed on the 22nd day of gestation: fetuses were killed and prepared for microscopic examination. Anogenital distances and urethral lengths of genetic males both showed dosage-dependent shortening. The development of prostatic tissue was almost entirely suppressed, and penes were clitoris-like, and often hypospadic. The characteristic male sigmoid flexure of the urethra was absent, and from the dorsal aspect of the endodermal sinus, a solid "vaginal" epithelial cord was observed. In addition, the cranial aspect of the sinus urogenitalis was separated

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* 1,2α-methylene-6β-chloro-pregn-4,6-diene-3,20-dione-17α-hydroxyacetate. Also referred to as SH-714 in some of the literature cited.

by a septum resulting in a dorsal vaginal orifice and a ventral urinary tract. When animals were allowed to live, however, this vaginal orifice regressed and finally disappeared. Testes appeared normal, with the exception that interstitial cells were somewhat hypertrophic with high dosages. These findings were confirmed by subsequent studies (Neumann & Elger, 1966; Neumann, Elger & Kramer, 1966) and expanded to include treatments of gravid rats from the 13th to 21st day of gestation with 10 mg CA, and with daily treatments of the fetuses with 0.3 mg CA until day 21 post partum. With this procedure, Neumann and Elger (1966) brought about the development of a permanent vagina in male rats. The vagina was narrower and shorter than the vagina of normal females, but the epithelium responded to ovarian hormones in a normal fashion.

Junkmann and Neumann (1964) presented evidence that the probable mechanism of antiandrogenic activity of CA was one of competitive inhibition for androgen receptors within target organs, since no evidence could be found for estrogenic actions (negative uterine growth test and negative Allen-Doisy test), nor was there any evidence for anti-gonadotrophic actions, since testes of treated fetuses and treated juveniles appeared normal (Neumann & von Berswordt-Wallrabe, 1965; 1966). The recognized progestational actions of CA were not felt to be causally related to the antiandrogenic actions since the free alcohol, cyproterone, was also highly antiandrogenic, but not at all gestagenic. In addition, 19-nor 17-hydroxyprogesterone, chemically related to CA, was strongly gestagenic, but had no antiandrogenic effects whatsoever.

Subsequent studies established that CA could suppress the inhibitory effect of testosterone on gonadotrophin release (Neumann, Elger & von Berswordt-Wallrabe, 1967). In addition, males treated with CA prenatally and for 3 weeks postnatally could successfully support immature ovaries transplanted from young females. The presence of corpora lutea proved that ovulation had occurred after the transplant. Further, vaginal smears indicated that approximately normal sexual cycles were occurring (Neumann & Elger, 1965). These findings demonstrated that hypothalamic-pituitary functions of these males were feminized, since only females or males castrated during the critical period of sexual differentiation secrete gonadotrophins in a manner which allows cyclic ovulation to occur (Harris, 1964; Gorski & Barraclough, 1963).

Exogenous testosterone was also shown to be inhibited by CA, as evidenced by the following studies: 1) Growth of prostatic and seminal vesicle tissue in the castrated adult rat given testosterone propionate was inhibited when CA was administered simultaneously with the androgen (Neumann & Kramer, 1964); 2) Testosterone masculinization of fetal female rats was prevented with simultaneous injections of CA (Neumann, Elger & von Berswordt-Wallrabe, 1966).

2. Behavioral Effects

Relatively little work has been reported concerning the effects of CA on behavioral phenomena. Neumann and Elger (1965) reported that rats exposed to CA during fetal and early postnatal development elicited the lordosis response when stimulated by a male, provided that an ovarian transplant had been made. The behavioral responses were cyclical, and correlated with estrogenic changes of the vaginal epithelium.

Swanson (1966) employed open field ambulation and emergence tests in hamsters to evaluate the effects of cyproterone and CA. Normally, male and female hamsters have characteristically different patterns of behavior in these test environments. CA given at the day of birth to both sexes had no effect whatsoever on ambulation or emergence scores for either sex. Large doses of cyproterone, given from day 10-16 of gestation, resulted in 1) no genital modifications, 2) no changes in ambulations scores, and 3) emergence patterns of males resembling those of normal females.

Zucker (1966) administered 10 mg CA to adult, intact male guinea pigs daily for five weeks, but found no significant inhibition of intromission or ejaculatory behaviors. Increasing the dosage of CA to 30 mg for the next 3 weeks also had no disruptive effects upon mounting, intromission, or ejaculation frequencies, although fertility was definitely affected.

Zucker's experiment dealt with CA's effect of blocking the activational properties of androgen at adulthood, while Swanson's parameters of antiandrogenic administration were different from Neumann and associates. Nonetheless, the possibility exists that either the effects of cyproterone acetate or the effects of androgen insufficiency could vary among mammalian species. Therefore, it is important to assess the effects of CA on laboratory animals with a longer period of gestation in which fetal gonadectomy cannot now be accomplished surgically. Should CA prove effective in other species,

its real value will be realized in a variety of potential investigations for which no experimental method now exists for depriving a fetus of its own androgenic secretions.

Thesis Problem

Pilot work done in this laboratory (Goy, Kuehn & Zucker, unpublished) indicates that CA given in concentrations of 3, 5, or 15 mg per day to fetal male guinea pigs from day 28 - 57 of gestation affects male sexual behavior of the intact adult. The results, however, suggest that inappropriate dosages of CA were used, since only limited external morphological changes occurred, and complete male patterns of copulation were accomplished by most treated males: only the increased number of intromissions preceding ejaculation separated these males from their controls.

Thus, the intent of this thesis was to evaluate more fully the effects of CA on the guinea pig by administering increased quantities of the antiandrogen prenatally and subsequently observing its effects on castrated as well as intact males for female as well as male behavior patterns at adulthood. The hypothesis was that CA would lower the probability of occurrence of full masculine sex behavior such as normal frequencies of mounting, intromission, and ejaculatory patterns while increasing the probability of occurrence of feminine behaviors, such as lordosis.

Chapter II

MATERIALS AND METHODS

Fetal Treatments

Observed matings were conducted for 18 female guinea pigs obtained from a heterogenous stock of 125 - 150 adult females maintained under approximately constant temperature and light cycle conditions (22-27°C; lights on 6:00 am - 4:00 pm). Beginning 27 days after fertilization, subcutaneous injections of 15 or 40 mg CA were administered once every 24 hours for 30 days. In addition, subcutaneous injections of 20 mg CA were given daily, either as two 10 mg injections every 12 hours, or as a single injection once every 24 hours, to separate groups. The antiandrogen was prepared by dissolving crystalline CA in a mixture of 65% castor oil, 35% benzyl benzoate, at 37°C. CA was then stored under refrigeration during the course of the injection schedule. The 40 mg/day treatment was found to be abortogenic -- 3 out of 3 animals aborted and/or died by the 42nd day of pregnancy. Therefore, additional females were mated, and 40 mg CA was given from day 28 - 39, at which time the dosage was reduced to 20 mg/day for the remaining 18 days. An additional 10 females were mated and were either treated with the castor oil/benzyl benzoate vehicle from days 28 - 57, or were left untreated for the duration of pregnancy. All animals delivered normally at days 68 - 72.

Neonatal and Juvenile Treatments

All of the male offspring from the 40 mg treatment group and appropriate numbers of vehicle and nonvehicle control groups were

castrated on the day of birth. All of the males from the 15 mg CA group and one half of the males in all other treatment and control groups were castrated 28 - 38 days post partum. All animals were weaned at 30 days of age, and were housed in groups of 8 - 10, segregated on the basis of sex, gonadal integrity, and CA treatment parameters. Table I displays the number of animals assigned to each treatment group.

At 60 - 80 days of age, 11 males and females were inspected by 2 - 3 judges for degree of external genital modification. Categories of severe, moderate, or no gross modification were employed in order to roughly gauge the extent of morphological anomalies. Presence of vaginal-like labia and/or structures resembling a vaginal membrane, external shape of vagina and/or phallus, and presence or absence of hypospadius were the main criteria considered.

Females were visually inspected each evening from days 70 - 100 for evidence of cyclic changes in the integrity of the vaginal membrane, and were manually stimulated at that time in order to determine the presence of behavioral estrus. At approximately 100 days, females were bilaterally ovariectomized, under Nembutal, four days after they were last found in behavioral heat.

Testing Procedures

1. Rationale

To determine whether prenatal CA affected adult sex behavior in males, it was necessary to compare treated and control males under identical adult hormonal and environmental conditions. Neumann and

Table I

Number of Subjects Within Each Treatment Group

Treatment	Intact Males	Castrated Males	Spayed Females
15 mg CA	0	3	3
1 x 20 mg CA	4	3	6
2 x 10 mg CA	6	4	4
40 mg CA	0	7	9
	10	17	22
Vehicle Control	5	6	5
Untreated Control	5	4	5
	10	10	10

von Berswordt-Wallrabe (1966) have presented histological evidence suggesting that testicular secretion functions are normal in CA-treated animals. Thus, it was assumed that intact CA males would produce normal plasma androgen concentrations at adulthood, and could be directly compared with intact control animals for frequencies of mounts, intromissions, and ejaculations. To test this assumption, Dr. J. Resko determined plasma testosterone and androstenedione concentrations in the intact CA and control males in this experiment at the conclusion of behavioral testing. His findings are presented in the Results section of this thesis.

Castrated CA males and castrated control males were compared for mounting behaviors in the absence of gonadal hormones to determine if mounting differences existed when the activating effects of adult androgens were not present, and during testosterone replacement therapy to measure any differences in response latency to the effects of adult androgen levels. Additionally, these males were given ovarian hormones which would normally induce estrous behavior in spayed but otherwise normal females to determine the potential for CA males to display lordosis. While lordosis would be taken as the most obvious behavioral indicant of feminization, additional behavioral measures were employed to assess this endpoint. It was known that intact females display considerable mounting behavior at estrus, and that this phenomenon is readily duplicated in spayed females with exogenous estrogen and progesterone (Young & Rundlett, 1939; Young, 1941; 1961). In contrast, mounting behaviors of normal prepuberally castrated males are known to

be affected to a lesser degree by ovarian hormones (Gerall, 1966). Thus by observing changes of mounting frequencies of CA castrates as a function of the presence or absence of estrogen and progesterone, a second measure of feminization was available. Finally, the absolute frequencies of mounting in the absence of any adult gonadal hormone was compared for castrated CA males and spayed control females for a third measure of feminization.

Spayed females exposed to prenatal CA were also tested for lordosis and mounting behavior when given estrogen and progesterone at adulthood, since it was necessary to determine whether CA disturbed normal female behavior in females. If true, interpretations of the males' behavior would be significantly affected by such a finding.

The following is a detailed description of testing methods and criteria employed:

2. Tests of Intact Males

At 120 days of age, intact males were individually adapted to a 2' x 2' observation cage for 5 minutes, and were then paired for 10 minutes with a spayed stimulus female brought into heat with injections of 0.3 μg estradiol dipropionate (ED) followed 36 hours later by 0.4 mg progesterone (P). Three such tests were given at weekly intervals. A stimulus female was rarely used for more than one test on any given week, and was never used for two consecutive weeks. Further, each stimulus female was manually stimulated immediately before a test to determine that she would display lordosis for a minimum of 6 seconds. A fourth mating test lasting for 20 minutes

and using intact females found in estrus rather than spayed females was conducted when these males were approximately 180 days old.

The frequency of occurrence of the following behaviors was recorded for all mating tests: abortive mounts; mounts with and without pelvic thrusting; intromission patterns; and ejaculatory patterns. An abortive mount was defined as the placing of both forepaws anywhere on the back of the partner without other contact. If the male was correctly oriented towards the partner's genitalia and ventral-dorsal contact was made, a mount was scored. If pelvic thrusting accompanied the mount, this was also recorded. Intromission patterns were distinguished from mounts with pelvic thrusting on the basis of a much deeper thrust of a characteristically slower cadence, involving different hind limb and pelvic postures than that of pelvic thrusting seen in the absence of intromission. Actual insertion of the penis into the vagina was not necessary for this behavior to be scored; hence the use of the term intromission pattern. Ejaculatory patterns were characterized by a prolonged final thrust, an exaggerated drawing in and quivering of the flanks, and often an extremely slow dismount and loss of balance. Characteristically, the normal male displays "butt drag" behaviors within 1 - 5 minutes after an ejaculation (Young & Grunt, 1951). This was also scored, but was not essential in the definition of the ejaculatory pattern. The actual presence of a seminal plug was also not taken to be an essential component for the behavior to be scored.

3. Tests of Castrated Males

At 120 days of age, castrated males were tested for mounting behavior once a week, for 4 weeks, with receptive, spayed females. On alternate weeks, however, these males were given 0.3 μg ED followed 36 hours later by 0.4 mg P, and were tested for mounting behavior 4 - 6 hours after the P injection. A counterbalanced design was employed such that one half of each group was given this hormone treatment on tests 1 and 3, while the remaining animals received the hormones on tests 2 and 4. The identical scoring system was used for mounting behavior as that already described for the intact males. In addition, on test in which estrogen and progesterone were used, castrated males were manually stimulated hourly for 12 hours after the P injection in order to determine if lordosis could be elicited. Lordosis, the primary component of the receptive posture of the estrous female, is characterized by a stereotyped spinal extention and an elevation of the perineum. Young, Dempsey, Hagquist and Boling (1937) demonstrated that by stroking the back and perineal regions, this response was readily displayed by estrous females, and actually served as a more reliable index of heat than vaginal smears in guinea pig. Thus, by "fingering" animals in this manner, the duration of lordosis was measured with a stopwatch and was timed from the onset of the complete posture being assumed until any deviation in that posture was taken. This included actual movement of any limb or any indication of decreased perineal elevation. Each animal was fingered three times in succession each hour while in an isolated observation cage; the highest of the three scores was recorded as the lordosis duration for

that hour. The number of hours for which lordosis was displayed was termed duration of heat.

Two to 4 weeks after the final mounting test, 25.0 μg ED followed 36 hours later by 0.4 mg P was given to all castrated males, so that an additional test for lordosis could be conducted. In addition, at 9 months of age, androgen replacement therapy, consisting of daily subcutaneous injections of 1 mg testosterone propionate, was administered to 40 mg CA castrates and control castrates for 28 days. Mounting tests were conducted on the 13th and 22nd day of injections.

4. Tests of Spayed Females

At 120 days, approximately 2 - 3 weeks after ovariectomy, all females were tested in exactly the fashion just described for the castrated males, with the exception that a lordosis test was not made under the higher ED dosage and TP replacement therapy was not initiated. Exactly the same criteria for mounting patterns were employed, and the same regimen of hormone administration was followed, so that each female was evaluated for mounting twice while being exposed to no exogenous hormones and twice while having received estrogen and progesterone.

5. Histology and Physiology

Ovaries taken 4 days after heat were fixed in Hollande-Bouin's solution, cut at 10 μ, and stained with hemotoxylin and eosin. At 9 months of age, intact males were sacrificed, blood was collected for gas chromatographic determinations of testosterone, and testes, prostates, and seminal vesicles were weighed and prepared for histological study.

Dr. J. Resko examined 20 mg CA and control castrated males at 9 months for testosterone uptake and metabolism in hypothalamus, cerebellum, seminal vesicles and prostate, using a double isotope determination technique. In addition, at 9 months, growth responses of seminal vesicle and prostate were compared for 40 mg CA and control castrates after 28 days treatment of 1 mg/day testosterone propionate.

Chapter III

RESULTS

Morphological Effects of Prenatal CA in Males and Females

1. Males

The external genitalia of males exposed to CA prenatally were severely modified at birth. A dosage dependent hypospadius was observed, and with doses greater than 15 mg, the penis assumed a leaf-like appearance, completely failing to become tubular (Figure 1). This modification occurred in 22 out of 29 cases for males receiving either 20 mg or 40 mg CA per day during prenatal development. Four out of 7 males treated with 40 mg CA from day 28 - 39 developed a membrane immediately below a hypospadic phallus which strongly resembled a vaginal membrane (Figure 3). However, no evidence of the retention of Müllerian derivatives or distal vaginal structures were found.

Accessory sex organs in CA males were severely modified. Seminal vesicles were visibly smaller in intact CA males than in intact control males, and were completely devoid of seminal fluid. A total absence of secretory fluid was also found in seminal vesicles of CA castrated males given 28 days of testosterone propionate replacement therapy, whereas control castrates given this treatment produced large quantities of seminal fluid. Prostates in intact CA males were also visibly smaller than prostates of intact control males.

Figure 1. Hypospadic phallus of an intact adult male guinea pig treated with 20 mg CA from day 28 - 57 of gestation.

Figure 2. Penis of an intact adult control male guinea pig.

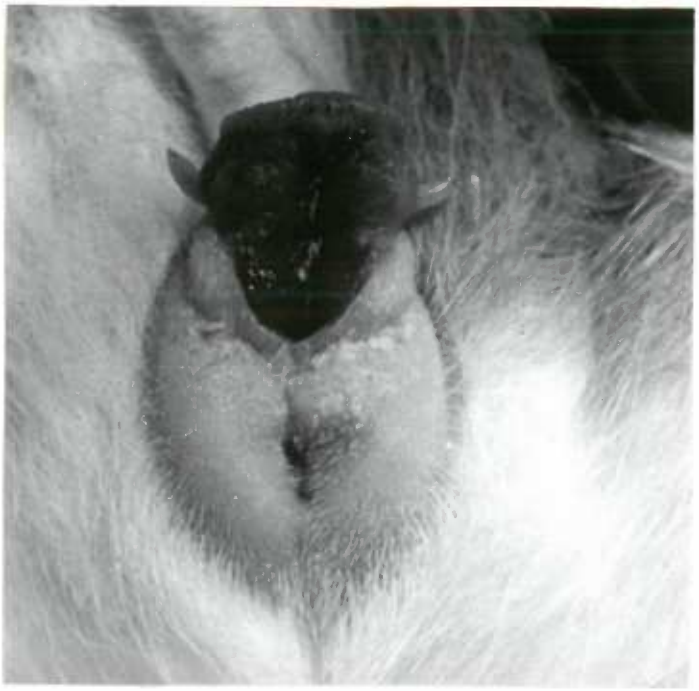

Figure 1

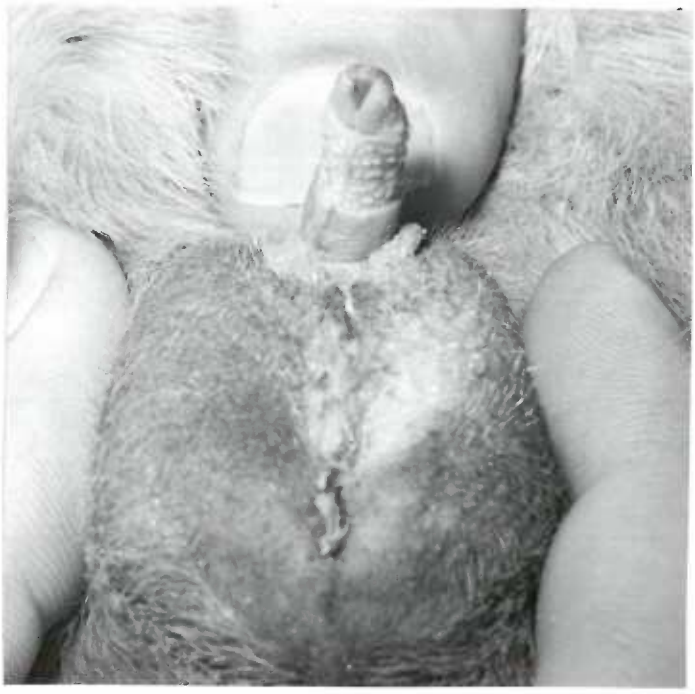

Figure 2

Organ weights of seminal vesicles and prostates were determined in intact CA and control males, and in castrated CA and control males either given 28 days of testosterone propionate immediately before sacrifice, or left untreated. All seminal vesicles were weighed after their contents were emptied. In addition, seminal vesicles of castrated and control CA males given testosterone propionate were weighed both before and after the seminal fluid was emptied. The results, summarized in Tables II and III, indicate that seminal vesicles and prostates of intact CA animals were both significantly lighter than their controls, but were significantly heavier than corresponding structures of control castrates and CA castrates not given testosterone propionate. The latter finding suggests that accessory sex structures in animals exposed to prenatal CA were structurally modified, but were nonetheless capable of limited growth in response to endogenous or exogenous androgens. The comparison of seminal vesicle weights before and after expressing the contained fluid in testosterone-treated CA castrates further illustrated the non-secretory state of this structure. Seminal vesicles of castrates contained a median of 1.0 g of fluid, while CA castrates contained less than 0.02 g per animal (Table III).

Microscopic examination of seminal vesicles and prostates in intact CA males and in testosterone-treated CA castrates revealed that the entire glandular structure of these accessory sex organs was severely affected. Convolutions of basement membranes which support the columnar epithelium were markedly reduced; epithelial cells were shorter and less dense, and were often modified to the extent that

Table II

Organ Weights of CA and Control Males

(g tissue/g body wt) 10^3

Treatment	N	Mean Prostate	Mean Sem. Vesicle	Mean Both Testes
Control	7	1.55	1.74	3.75
Intact				
CA	10	0.82	0.82	3.64
t		6.36**	9.39**	0.41
Control	4	0.26	0.20	--
Castrated				
CA	5	0.15	0.16	--
Mann-Whitney U		0*	6	

** $p < .001$. All tissues collected at nine months of age.

* $p < .008$.

Table III

Organ Weights of Castrated Males Given Testosterone at Adulthood

Group	N	Sem. Vesicle with Contents	Sem. Vesicle without Contents	Sem. Vesicle Fluid	Prostate
Control	3	2.61	1.56	1.05	1.29
CA	6	0.30	0.28	0.02	0.49
Mann-Whitney U		0	0	0	0
$\underline{p} <$		.01	.01	.01	.01

All tissues collected at 9 months of age. One mg/day testosterone propionate was given for 28 days prior to sacrifice. Weights are median values expressed in g tissue/g body weight X 10^3.

columnar organization was lost. In addition, a definite encroachment of muscular and connective tissue into epithelial borders was noted. These observations confirmed the fact that these structures were definitely non-secretory.

Testes taken from intact CA males were not different in weight or structure from testes of intact controls (Table II). Leydig cell development was not different from controls and, in addition, definite evidence of spermatozoa in testes of CA males was obtained. This observation confirmed that of Neumann and von Berswordt-Wallrabe (1965, 1966), and suggests that androgen production was normal in intact CA animals during adulthood, and that prenatal CA was not antagonistic to spermatogenesis in adulthood.

2. Females

Genetic female phallic development was also affected by CA treatments. Hypertrophied clitorides were seen in 52% of females treated with 20 mg or less CA, and in 100% of females treated with 40 mg CA during part of embryonic development. In addition, vaginal orifices were narrower than control females. By comparing genital photos of a CA female with that of a severely modified castrated CA male, it can be seen that external genital structures are superficially, remarkably similar (Figures 3 & 4).

Microscopic evidence revealed that ovaries were entirely normal for CA females. Development of corpora lutea indicated that ovulations had occurred. No abnormal cystic follicular development was observed

Figure 3. Genitalia of an adult male guinea pig castrated at birth and treated with 40 mg CA from day 28 - 39 of gestation, followed by 20 mg CA from day 40 - 57 of gestation.

Figure 4. Genitalia of an adult female guinea pig spayed at 100 days and treated with 40 mg CA from day 28 - 39 of gestation, followed by 20 mg CA from day 40 - 57 of gestation.

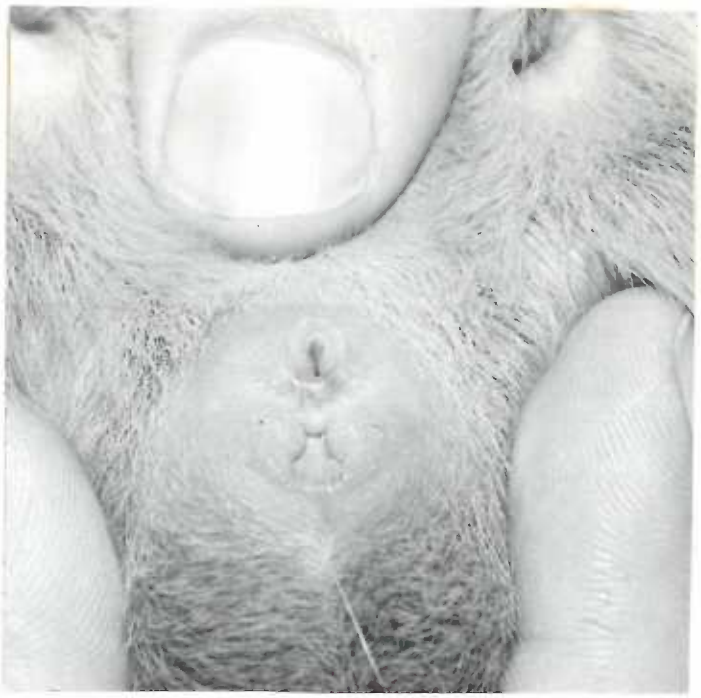

Figure 3

Figure 4

Figure 5. Genitalia of an adult control female guinea pig.

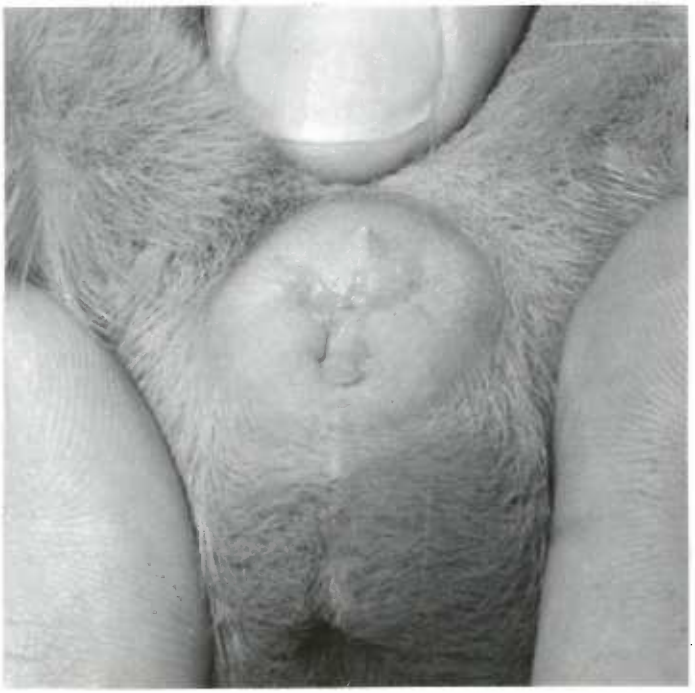

Figure 5

in 7 pairs of ovaries taken from 20 mg and 40 mg CA females four days after estrus.

Physiological Effects of Prenatal CA in Males

Data contributed by Dr. J. Resko established that plasma testosterone levels in intact CA males at 9 months of age were not different from intact controls (Table IV). Thus, adult hormonal levels were adequate for normal sex behavior to have occurred. In addition, no differences in free tritiated testosterone concentrations between 1X20 CA and control castrated groups were found in tissue samples from hypothalamus or cerebellum. However, significantly more free tritiated testosterone was recovered in seminal vesicles and prostates of CA-treated castrates, suggesting that the metabolic functions of these organs were modified (Table V). Testosterone did not appear to be effectively metabolized to normal conversion products in concentrations normally expected, although the same concentrations of androstenedione, the principle metabolite of testosterone, were found in both CA and control groups for both brain tissues and secondary sex structures. These results suggest that normal conversion of testosterone to androstenedione occurred in CA animals, but that abnormal processes governing testosterone metabolism to other products were taking place in seminal vesicle and prostate. Thus, at the doses employed, CA had the effect of inhibiting the functional properties of secondary sex structures to the extent that secretion was never observed in the presence of normally adequate amounts of endogenous or exogenous testosterone, and yet allowing some growth and normal testosterone-to-androstenedione metabolism to occur.

Table IV

Plasma Testosterone Concentrations in Intact Males

Treatment	N	Mean µg Testosterone/100 ml Plasma	t
Control	10	0.269	0.85*
CA	10	0.190	

* non-significant

Data collected by Dr. J. A. Resko

Table V

Uptake and Metabolism of Tritiated Testosterone

in CA and Control Castrated Males

Mean ratio $\frac{\text{tissue}}{\text{plasma}}$ dpm/100 mg

Treatment	N	Seminal Vesicle / Plasma	Prostate / Plasma	Cerebellum / Plasma	Hypothalamus / Plasma
			Zone A		
Control	4	0.65	0.52	1.74	2.39
CA	4	1.12	0.81	1.68	1.97
Mann-Whitney U		2*	1**	6	5
			Zone B		
Control	4	12.79	7.47	3.49	5.71
CA	4	13.91	8.13	3.78	6.24
Mann-Whitney U		7	7	7	8

* $p < .057$; ** $p < .029$. Data contributed by Dr. J. Resko, for uptake and metabolism of testosterone H^3 (Zone A) to androstenedione H^3 (Zone B) in castrated male guinea pigs. Tissues were obtained 1.0 hr after injection of 10μcuries testosterone H^3. Significantly more testosterone recovered in accessory organs of CA animals indicated that metabolic processes had been disturbed.

Behavioral Effects of Prenatal CA in Males and Females

1. Mounts, intromissions, and ejaculations of intact males.

Intact males treated with 20 mg CA prenatally, regardless of injection schedule, did not differ from controls in frequency of abortive mounts, and mounts with and without pelvic thrusting, but failed to display normal patterns of intromissions and ejaculations. The frequency of intromission patterns per minute of activity*, demonstrated by intact CA males in 40 tests, was significantly less than for controls ($t = 2.82$, $p < .02$), although a total of 5 out of 10 CA males displayed intromission for at least one test.

Only three ejaculations were scored out of 40 tests by intact CA males: one 2X10 mg CA animal achieved one ejaculation; and one 1X20 mg CA animal achieved ejaculations on two separate occasions. This is in contrast to a total of 29 ejaculations seen during 40 tests for controls; 9 out of 10 control animals ejaculated on at least one test, and 7 out of 10 animals ejaculated on two or more occasions. The ejaculatory patterns observed for the two CA males were totally complete, however, except for the absence of a seminal plug, and were followed by genital cleaning, prolonged refractory periods, and the characteristic butt drag in each case. No ejaculation was achieved by CA males when tested for 20 minutes with intact estrous females at 180 days of age, whereas 90% of the control animals achieved ejaculations on this test within 8 minutes. A single control male failed to mount on every test.

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* Tests were terminated at ejaculation or after 10 minutes. To compare frequencies of various types of mounts between groups, a ratio of mounting frequency to time observed was constructed.

2. Mounts of castrated males.

The mounting abilities of castrated CA males also reflected behavioral effects of prenatal antiandrogen administration. In the absence of gonadal hormones, CA males displayed mounting on significantly fewer tests than control castrates. Eight out of 17 males failed to mount in any fashion on at least one test, whereas only one out of 10 castrated controls failed to mount during a test (χ^2 = 21, df = 1, $p < .001$). Castrated CA males also failed to achieve as many correctly executed mounts with pelvic thrusts as controls on tests for which no hormone was administered (Tables VI and VII), but mounted at much higher rates than spayed females under identical conditions (t = 6.65, df 47, $p < .005$). Thus, in the absence of adult androgen levels, CA males were poorer than control males for mounting behavior, but were definitely superior to ovariectomized females.

Mounting rates did not significantly change for either CA or control castrated groups when given exogenous estrogen followed by progesterone (Tables VI and VII), indicating that prenatal CA did not alter the normal mounting response tendencies of males to the administration of ovarian hormones.

CA castrated males were less sensitive than control castrates to the behavioral activating effects of androgens in adulthood. When tested for mounting after 13 and 22 days of injections of testosterone propionate, CA males obtained significantly fewer mounts with pelvic thrusting than control castrates on the 13th day of injections (Table VIII)

Table VI

Mounting Rates of Gonadectomized Animals per Minute of Activity

Group	N	Without ED+P (mean for 2 tests)	With ED+P (mean for 2 tests)
Control Males	10	1.15	1.08
CA Males	17	0.52	0.47
Control Females	10	0.04	0.24
CA Females	22	0.05	0.22

Table VII

Mounting Frequencies for Gonadectomized Males:

2 x 2 Unweighted Means Analysis of Variance

Source of Variation	SS	df	MS	F
Between Subjects				
A (Cyprot. Acet.)	1928.41	1	1928.41	31.75**
Subj. within Gps.	1518.54	25	60.74	
Within Subjects				
B (ED+P)	18.89	1	18.89	
AB	12.59	1	12.59	
B x Subj. within Gps.	997.40	25	39.89	

** $p < .01$. This statistical comparison indicates that prenatal CA significantly reduced mounting of males in adulthood, and that ED+P had no effect on mounting rates for CA or control castrates.

Table VIII

Median Mounts per Minute of Castrated Males During Testosterone Replacement

Group	N	13th Day	22nd Day
Control	3	2.5	1.7
CA	6	1.2	1.4
Mann-Whitney U		1**	4

** $p < .024$

and failed to achieve intromissions on any test. Two of three control castrates achieved at least one intromission on the 22nd day of testosterone treatments. There was no significant difference between mounting scores for the two groups on the 22nd day of injections, but this observation was complicated by the fact that the mounting rate of the control group was lower on this test than at 13 days, most probably because of an interaction between mounting rates and the display of intromissions. These results suggested one additional modification of these males attributable to the actions of the prenatal antiandrogen.

3. Mounts in castrated females.

No significant differences existed between spayed CA females or spayed control females for mounting behaviors, either in the absence of ovarian hormones or when given ED and P (Tables VI and IX). Furthermore, both groups of animals displayed the expected significant increase in mounting when given ED + P, thus demonstrating that although CA modified the external genitalia of females, its effects did not extend to a modification of mounting response tendencies.

4. Lordosis behavior of castrated males and females.

One hundred percent of the CA females were found in spontaneous behavioral estrus before ovariectomy, and 100% of these females responded to exogenous ovarian hormones by displaying lordosis. Control females and CA females ovariectomized and given ED followed by P did not differ for measures of latency to heat onset, mean maximum lordosis, or duration of heat. In contrast, no control castrated male or CA castrated male

Table IX

Mounting Frequencies for Gonadectomized Females:

2 x 2 Unweighted Means Analysis of Variance

Source of Variation	SS	df	MS	F
Between Subjects				
A (Cyprot. Acet.)	0.12	1	0.12	
Subj. within Gps.	731.73	30	24.39	
Within Subjects				
B (ED+P)	45.33	1	45.33	15.11**
AB	0.01	1	0.01	
B x Subj. within Gps.	89.87	30	3.00	

** $p < .01$. This analysis reveals that CA and control females were not significantly different from each other for mounting behavior, and that ED+P significantly increased both groups' mounting rates.

ever displayed lordotic postures, even when given seven times the amount of estrogen used for the female groups described above. Thus, CA as used in this experiment was not an effective agent for the establishment of lordosis in male animals, but was not antagonistic to the display of this behavior in the female.

Chapter IV

DISCUSSION

The results of the several comparisons made in this thesis have indicated that prenatal administration of CA modified critical neural and/or somatic tissues involved in normal male patterns of behavior. The observations most clearly indicating that the induced modifications led to deficient male patterns of behavior are as follows:

1) Castrated CA males displayed lower mounting rates than castrated controls in the absence of gonadal hormones, and 2) they responded to exogenous androgens more slowly and less completely than control castrates; 3) intact CA males did not display normal intromission frequencies relative to intact control males, and 4) they only rarely displayed an ejaculatory pattern.

Other observations indicated that the deficiencies in the development of traits normally characteristic of the male were only partial. Mounting rates of CA castrated males in the absence of gonadal hormones were significantly greater than ovariectomized control females, illustrating that although prenatal CA suppressed adult levels of mounting in males, the behavior was not reduced to levels seen in ovariectomized females in the absence of gonadal hormones.

The development of behavior normally characteristic of the genetic female was not accomplished by the administration of this chemical antiandrogen in the dosages used. CA castrated males

neither increased mounting rates nor displayed lordosis in response to injections of estradiol followed by progesterone. The failure of these behaviors to develop could not be attributed to interfering effects of CA per se, since littermate females also exposed to CA displayed lordosis and increased mounting rates in response to ED and P in a fashion indistinguishable from that of normal females. Thus, prenatal CA, as employed in this study, was found to disrupt mechanisms in the genetic male guinea pig controlling the display of normal levels of male sex behavior, but was not effective in allowing the expression of female behaviors to occur in these same males. These results suggest that there exist neural mechanisms determining the display of lordosis which are separate and independent from mechanisms controlling male patterns of mounting, intromission and ejaculation.

The results obtained with respect to the development of behavioral characteristics were strictly paralleled by the results for morphological characters. Males treated with the largest amounts of CA prenatally developed marked deformities of the phallus and stunted and nonfunctional accessory glands. Certain female characters, in contrast, were undeveloped, and no treated male showed formation of a distal vagina. Nevertheless, prenatal CA did block the masculine development of one derivative of the unogenital sinus. All males treated with the higher dosages displayed severe hypospadius, and the normal male-type urethra was prevented from forming.

The morphological and behavioral modifications obtained in this study were not as severe as those reported by Neumann and associates,

in spite of the fact that as much or more CA per gram body weight was administered to guinea pigs as to rats in Neumann's studies. In fact, clitoral hypertrophy, regularly found in female guinea pigs exposed to the higher dosages of CA, was not obtained by Neumann's laboratory in rat except at dosages of CA much higher than that necessary to obtain excellent pseudohermaphroditism in male rat (unpublished Schering report, 1963).

Several possible mechanisms might account for this apparent species difference, including differential actions of the so-called blood-brain barrier, metabolism of CA by the pregnant guinea pig, or the possibility that hypersecretion of androgens might have occurred in guinea pig in the presence of CA due to the negative feedback mechanism of the hypothalamic-pituitary-gonadal axis. If CA acted as a competitive inhibitor at hypothesized hypothalamic cells sensitive to androgen levels, more gonadotrophins would be released from the pituitary, resulting in higher than normal concentrations of plasma testosterone. Bloch and Davidson (1967) demonstrated that crystalline CA implanted into hypothalamic tissue of prepubertal male rats has exactly this effect. This suggestion is supported by the observation of clitoral hypertrophy found in CA-treated female guinea pigs. This morphological change might also be reflecting a mechanism which brought about increased levels of steroids in fetal females exposed to CA.

The single most important theoretical finding of the present study may well be represented by the statement that it is possible experimentally to produce sexual types which show marked deficiencies in the formation

of male characters without a corresponding augmentation of female characters. The dissociation of these two behavioral patterns suggests that independent biological processes control their development. Considerable evidence has been presented, however, which demonstrates that exogenous testosterone propionate and endogenous testicular secretions modify both of these response tendencies during specific periods of prenatal and/or neonatal life. The conclusion of an independence of mechanisms subserving lordotic and male-like mounting patterns is not discordant with these facts, however, in that threshold or sensitivity differences for androgenic actions could exist for tissues mediating these responses.

Phoenix, Goy and Young (1967) have suggested that animals exposed to varying androgen dosages and the time of prenatal treatment could be fitted into a continuum from normal male to normal female, with the intertypes in this schema demonstrating relatively less mounting and more lordosis behavior than the normal male provided that appropriate hormonal conditions were met at adulthood. This concept of a graded organization of sexuality implies that 1) the organization of sexual behavior is not an "all or none phenomenon," 2) the display of male and female patterns of behavior vary inversely as a function of prenatal concentration and temporal exposure to androgens, and 3) sexuality can be graphically represented as a unidimensional variable.

The results of this thesis, however, provide an example of sex-type formation which cannot be fitted into such a framework. While

showing deficient male patterns of response, CA males failed to show increased feminine patterns of behavior. There is also evidence, for example, that low testosterone titers during development were sufficient to block the display of lordosis without facilitating tissues controlling male patterns of mating. With either low doses of TP or exposure to TP at specific periods of fetal development, Phoenix et al. (1959) and Goy, Bridson and Young (1961) produced female guinea pigs which did not display lordosis in response to ovarian hormones, and which showed only minimal mounting response facilitation. With higher doses of TP or with different temporal periods of administration, however, lordosis was suppressed and mounting was facilitated. In addition, the genetic male rat has been treated hormonally during early postnatal development so that the same male behaves in adulthood either as a "female" or as a "male" depending upon the specific hormones supplied (Goldfoot, Feder and Goy, work in Ms.).

These findings indicate that an inverse relationship between male and female patterns of behavior does not satisfactorily describe several behavioral patterns which have been observed. They suggest, rather, that for behavioral characters, as for morphological characters (Burns, 1961), separate and independently developing primordia exist for masculine and feminine characteristics, and that to some extent the fate of these separate primordia can be independently controlled.

Chapter V

SUMMARY AND CONCLUSION

The basic guiding hypothesis for this thesis was that the development of dimorphic behavioral potentials are to a great extent under the control of prenatal hormonal conditions, and that specifically, the presence or absence of prenatal androgens determines the character of mating patterns demonstrated by the organism at maturity. The prediction was made that male guinea pig fetuses exposed *in utero* to a chemical antiandrogen during the second and third trimesters of pregnancy would display typical feminine patterns of sexual behavior at adulthood in response to appropriate hormone administrations. The behavioral criteria employed in the evaluation of the behavior of male animals given prenatal treatments of CA included an assessment of the animal's potential to display the lordosis response and differential mounting frequencies in response to ovarian hormones, and its potential to display normal frequencies of mounts, intromissions and ejaculations under androgenic and non-androgenic conditions of stimulation.

The results confirmed the hypothesis that CA interfered with normal processes of male organization. These effects were seen in behavioral deficiencies of intromission and ejaculatory patterns in the intact male, and in mounting deficiencies in the castrated male. The behavioral deficiencies were not complete, however, in that castrated CA males displayed significantly more mounts per minute

than castrated control females. Morphological deficiencies of penis, seminal vesicle and prostate also clearly indicated that organizational processes during embryological development had been severely affected.

Nonetheless, additional behavioral evidence indicated that CA males as treated in this study did not display typical female responses of estrus in response to injections of ovarian hormones. There was also no morphological evidence for the development of female accessory structures, including Müllerian derivatives or distal vaginal structures.

It was concluded from these results that there exist separate biological mechanisms which control male and female mating behaviors, and which are relatively independent of one another. Additional experimental evidence was offered to support the view that the concept of sexuality should be considered to be multidimensional rather than a single functional inverse relation between male and female behavioral potentials.

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