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THE DISRUPTION OF THE PROCESSES OF SEX DIFFERENTIATION BY PROGESTATIONAL STEROIDS

by

WILLIAM R. GRIFFO

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1976

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

THE DISRUPTION OF THE PROCESSES OF SEX DIFFERENTIATION BY PROGESTATIONAL STEROIDS

by

William R. Griffo

Adviser: Professor Ching tse Lee

A series of experiments were performed to assess the effects of perinatal progestins on the processes of sex differentiation in inbred mice. Progestins altered anatomical, gonadotrophin, and behavioral systems of differentiation in a manner specific for genetic sex. Neonatal progestins relative to oil controls produced a defeminization and a demasculinization in genetic females and males, respectively. The defeminization syndrome appeared to be associated with the 5α reduction of progestins to 5α pregnane derivatives, while the anti-androgenic effects observed in the genetic male appeared to be partly the result of the inhibition of neonatal testosterone secretion. Biochemical tracer techniques demonstrated that progestins will cross the placental barrier and display a high affinity for neural tissue. Furthermore, the acyclic defeminization syndrome was positively correlated with a diminished hypothalamic and pituitary sensitivity to ^{3}H progesterone in adulthood.

The data supported an orthogonal model of sex differentiation since masculinity and femininity did not lie on opposite poles of a single continuum. Defeminization was not always *a priori* associated with masculinization although comparisons of progestin treated genetic females and intact genetic males are necessary in future research. A model for the physiological role of progestins in males and females is proposed and the need for caution in clinical obstetrical and gynecological practices is emphasized.

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This dissertation is dedicated to the memory of my grandparents, who played such a positive role in my life, whose love and respect I will always cherish.

It is difficult to single out a small number of special people who have helped to guide me through these four years of graduate study. Above all, to my parents, who have sacrificed so much and asked for so little in return, I extend my gratitude and love. I hope that I can some day repay them for their generosity.

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I'm sure that this dissertation would still be in draft form if it were not for my friends who have so generously

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To all of the above, I hope that I will someday repay their selfless generosity. However, any errors contained herein are my sole responsibility.

As I complete this thesis, I am saddened by the death of my old friend Mr. Stephen A. Cybolski, who knew me since childhood and who graciously helped to defray the production costs of this dissertation. I only regret that he did not live to see the final product.

W. R. G.

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INTRODUCTION

Objective and Significance

The concept of sexual differentiation by perinatal androgens has gradually been extended to include a wide variety of sexually dimorphic characteristics, anatomical, physiological, and behavioral. For example, within the last decade, a great deal of research has concentrated on the early androgen syndrome. Unfortunately, the clinical applicability of this syndrome for developmental theories remains in doubt. In a sense, our concentration with this syndrome may have been in error. Other steroid hormones besides androgens are known to be critically involved throughout the gestation of the organism exerting important roles in fetal development. Likewise, while seldom are androgens prescribed clinically, other steroid hormones, particularly the progestational compounds, have been used in a wide variety of therapeutic settings. Yet we understand very little either of the effects or the mechanism of action of these compounds. A long-range goal of this dissertation is to elucidate the variety of effects inducted by prenatal and/or postnatal exposure to progestogens in mice. This objective will be approached by examining in detail the possible behavioral, anatomical, and biochemical alterations produced by such hormone administrations.

Specifically, the proposed experiments are designed to clarify the effects of perinatal and/or neonatal administrations of progestogens on the regulation of social behavior of inbred mice. Previous research has only in a few instances attempted to examine this question--investigating the role of such progestins in the genetic female rat.

While the primary goal of this research project is to understand the effects of endocrine manipulations on the social behavior of the rodent, its findings will have implications for human fetal endocrinology, and for developmental theories of sex differentiation. Various authors, for example, have recently discussed the importance of fetal endocrine conditions on human psychosexual development (Money, 1968; Yalom, Green, & Fisk, 1973; Young, Goy, & Phoenix, 1964).

Background

The now classic studies of neonatal endocrine manipulations and alterations in sexually dimorphic behavior patterns are important today. Testosterone propionate administered early in life results in permanent alterations of the reproductive functions of females of several mammalian species: rats (Barraclough & Gorski, 1962), hamsters (Crossley & Swanson, 1968), guinea pigs (Phoenix, Goy, Gerall, & Young, 1959), laboratory mice (Edwards, 1970; Lee & Griffo, 1973), dogs (Beach & Kuehn, 1970), and

rhesus monkeys (Goy, 1970). In rodents this treatment has generally resulted in failures of ovulation in the adult, decreased behavioral responsiveness to exogenous ovarian hormones, and the masculinization of external morphological features as well as changes in behavior and urinary cues.

Similarly, a critical role for androgens in the differentiation of adult male sexual behavior has been demonstrated. For example, when male rats are deprived of androgens prenatally by the administration of an anti-androgenic compound, cyproterone acetate, or neonatally by castration on the day of birth, they display a reduced incidence of male copulatory behavior and an increased potential for female lordotic patterns over that of normal males.

Although these findings are robust, relatively little is known as we depart from the study of androgenic compounds. A review of the literature reveals that the effects of endocrine manipulations on social behavioral differentiation have not been adequately explored, nor are the physiological and biochemical correlates adequately understood.

Concept of Differentiation

Throughout the animal kingdom, we have come to recognize the importance of the diversity of cells, tissues, organs, and systems which blend harmoniously within the organism to insure homeostatic regulation. This cellular

diversity insures a division of labor, thereby enhancing the efficiency of the organism in meeting environmental complexities. The existence of this diversity and accompanying division of labor necessitates a degree of differentiation, for all cells arise from a common progenitor and differentiate through a genetic blueprint, thereby expressing themselves with specificity via biochemical functional intermediates. This concept of differentiation has been studied chiefly by the embryologist, yet it has continued to fascinate both physiologist and psychologist. The physiologist has been attracted to the complex regulation of organogenesis and accompanying enzymatic developmental sequences. The psychologist, on the other hand, has become interested in the concept of differentiation of gender dimorphic attributes either in animals or in man.

The following literature review will focus on this latter aspect of the differentiation process in mammals. Consider for a moment the concept of sexual differentiation which attempts to discuss the sexually dimorphic responses of animals. How could such a theory explain sex differences both in sexual and in nonsexual behaviors? We will be chiefly concerned with the hormonal factors operating during the early critical stages which appear to modulate or at least initiate a triggering action on this so-called sexual differentiation process.

However, before attempting to demonstrate the influences operating on such a differentiation process, it is incumbent to first document several features of such a process. For example, are sex differences reliably found in nature? Can physiological actions alter such differences?

At both the human and infrahuman level, studies of sex differences have been reported (Anastasi, 1958; Mead, 1961; Young, 1961). For example, at the infrahuman level, sex differences in mating behavior have been found on both qualitative and quantitative dimensions. In the male rodent, reproductive behavior can be described as the sequence of three distinctive activities, rear mounting of the female, intromission, and ejaculation. On the other hand, the prominent aspect of the female rodent's mating repertoire is the so-called lordosis posture in which she raises her hindquarters, exposing her genitalia to the male.

While we may, indeed, expect such gross motor differences in mating behavior between the sexes, such qualitative differences are not unique. Sex differences in nonsexual behaviors have been described for activity and exploratory patterns in mice and rats (Hitchcock, 1925; Simmel, Cheney, & Landy, 1965), aggression in both avian and rodent species (Guhl, 1961; Scott, 1966), learning ability in rats (McNemar & Stone, 1932; Scouten et al., 1975), play behavior in rhesus monkeys (Goy, 1974; Harlow, 1965), foraging behavior in birds (Selander, 1966), elimination patterns in dogs

(Doty & Dunbar, 1974; Martins & Valle, 1948), migratory behavior in birds (Howell, 1953), vocalization in many species (Armstrong, 1963; Whitney et al., 1973), and marking behavior in gerbils (Thiessen, Friend, & Lindzey, 1968).

Although the utility of such a division of labor may appear doubtful, it has been suggested that, particularly in those species which develop pair bond formations, the need for such a sexually specific delegation of responsibility may indeed have offered selection advantages.

Assuming that such differences have evolved in response to a selection advantage, how can the mechanism for such a dimorphism be explained? An obvious answer would include the pervasive difference in genetic chromosome patterns between the two sexes. In a sense, we must emphasize the word "pervasive," for a genetic difference can be manifested through any number of mechanisms. Let us review what seems to be the primary, albeit not sufficient, physiological mechanism for the manifestation of such differences and then examine more precisely the developmental factors influential in the latter expression of these differences.

Sex differences in the dependence of a behavioral repertoire upon the endocrinological state of the animal have been well documented for the mating behavior of several mammalian species. Although no simple answer exists as to the hormonal control of sex differences, a multitude of studies have established the importance of androgen for the

performance of sex behavior by the male and estrogen and progesterone for female sex behavior. Castration of the male rat (Beach & Holtz, 1946), guinea pig (Grunt & Young, 1953), mouse (McGill, 1962), cat (Rosenblatt & Aronson, 1958), and dog (Beach, 1952) results in a gradual decline of male sex behavior. Although preoperative factors will influence this castration syndrome in the male, the case for the hormonal control of female sex behavior is more clear-cut. Ovariectomy of the female results in the virtual absence of the lordosis posture in the rat (Beach, 1945), cat (Michael, 1961).

Evidence has begun to accumulate that the hormonal state may likewise exert an activational effect on other social behaviors. Aggression in rodents appears to be sexually dimorphic and androgen dependent in the male mouse (Edwards, 1970; Scott, 1966). Maternal behavior appears to require the synergistic action of estrogen, progesterone, and prolactin to reach threshold (Moltz et al., 1969).

It is clear that at least at the infrahuman level sexually dimorphic behavior patterns are common and in most cases require activational hormonal priming for full expression. These behavioral patterns are not unique to mating repertoires but extend to many social behaviors along both qualitative as well as quantitative dimensions. While the above observation is well accepted, a complete understanding is not as yet available. In a sense, we have discussed

sexually dimorphic behavior patterns as if sexually appropriate behaviors were indelibly "determined" at birth by genetic inheritance and merely brought to expression by the endocrine state in adulthood.

This genetic approach as a process of "sex determination" is challenged by an alternative view heralded by the developmental physiologist--that of a "sexual differentiation" process. This process can be defined for our purposes as a developmental physiological modification occurring at a crucial stage of ontogeny whose end result is the channeling of later adult behavior along sexually dimorphic pathways. This view of a sex differentiation process assumes that although the genome is the first intermediary in any cellular-biochemical mechanism, genes do not indelibly imprint a sexually dimorphic behavioral pattern. Rather, genes are the triggers for the crucial chemical processes necessary for sex differentiation.

Evidence for the role of developmental physiology and for this sexual differentiation process is striking. Although it is true that, in mammals, fertilization of the ovum by a sperm carrying an X chromosome will lead to subsequent female genital differentiation, while fertilization by a Y chromosome-coded sperm will ensure male anatomy, the case for genetics as being the indelible imprinter for sex and sexually dimorphic behavioral determination is not supported by physiological manipulations. Witschi (1965),

for example, was able to successfully reverse the genetic sex of tadpoles by grafting a mature testis into genetic female tadpoles. Furthermore, if estrogen is introduced into the water medium of genetic male tadpole hatchings, a complete reversal of sex will be noted, with mature ovaries as the anatomical marker. These studies on the anatomical level indicate that the genetic sex can be contradicted by endocrine manipulations in the young animal.

For many researchers this anatomical sex differentiation process had been the sole interest. Yet evidence has accumulated that this process of sex differentiation is not limited to morphological characters. The remaining segment of this literature review will examine several other components of this differentiation process besides the anatomical--that of gonadotrophin regulation, sexual behavioral differentiation, social behavior development, and lastly a relatively new area, biochemical differentiation.

Anatomical Differentiation

We may subdivide the differentiation process for anatomical structures of mammals into three stages, that of (a) a relatively undifferentiated state, (b) development of the internal reproductive structures, and (c) differentiation of external morphology. In the undifferentiated first stage, the gonads have not as yet developed and both males

and females possess both Wolffian and Mullerian duct systems. During the second stage, as a result of differences in the chromosomal constitution of males and females, the gonads of XY individuals differentiate into testes, while those of XX individuals develop into ovaries. While it is known that the fetal ovary secretes no substances into the bloodstream, the embryonic testis releases a so-called fetal morphological testicular substance which exerts effects upon the associated duct systems. Thus triggered by endocrine stimuli, the differentiation of the reproductive tract of mammals is predominantly or totally completed at the conclusion of gestation.

The early reproductive tracts are not bipotential but, instead, exist as two distinct entities. The potential male system, termed the Wolffian ducts, exists as substructures of the embryonic urinary system, while the corresponding potential female system (Mullerian ducts) is formed by enfoldings of epithelium alongside this system. The classic studies by Jost (1953) have conclusively proven that the development of the Wolffian duct system required the presence of a functional testis during rabbit embryogenesis. In the castrated male or in the female, differentiation of these structures was phenotypically female. Subsequently, over the last fifteen years, the work of Jost has been extended and several interesting conclusions have emerged (Bloch, 1967; Grumbach & Kaplan, 1973; Schultz &

Wilson, 1974; Wilson & Siiteri, 1973).

In vivo and *in vitro* preparations have demonstrated that the release of two testicular factors regulate ductal development: a C19 steroid,more than likely testosterone, stabilizes the Wolffian duct system, thereby inducing the maturation of the seminal vesicles and ventral prostate. This C19 steroid is known to initiate the differentiation of the urogenital sinus and external genitalia. A protein, on the other hand, is known to induce Mullerian duct regression. While a functional testis is a sufficient condition for male ductal differentiation, it is not a necessary one for testosterone,and other C19 steroids can effectively replace the testis in inducing male differentiation.

For both rats and mice the developmental changes of the reproductive tract in males and females have recently been charted. The indifferent gonad differentiates in fetal rats into testes at approximately 14 days post-coitum. Testosterone synthesis increases rapidly, reaching a plateau at 16.5 days. The Wolffian ducts,which are complete by day 13, are "stabilized" by testicular androgens during the 16th and 17th day of gestation. Prostatic and seminal vesicle spoutings appear on day 19, while by day 21 both structural and spatial differentiation is accomplished, as evidenced by epididymal coiling, testicular descent, and the appearance of male external genitalia. On the other hand, regression of the Mullerian duct is initiated by

testicular activity on day 15 post-coitum.

The course of anatomical differentiation in mice is analogous to that in the rat except for an earlier temporal sequence of 2 days in the mouse (Fisher & Steinberger, 1968; Noumura et al., 1966; Resko, Feder, & Goy, 1968; Wilson & Lasnitzki, 1971).

Next, the biochemical processes are set in motion which trigger the development of external morphological sexually dimorphic characteristics. At birth, mouse, rat, and hamster neonates possess a so-called genital tubercle, which is not as yet completely masculine or feminine. By the normal action of androgens released from the neonatal rodent testes, the genital tubercle of the male undergoes differentiation to form a penis and a scrotum, while in females lacking such androgenic stimulation, the tubercle gives rise to a clitoris and external vagina, including the major and minor labia.

While we do know the details of this normal sequence of sexual differentiation, relatively little is known concerning the influence of the various hormone systems. Neumann et al. (1966) and Neumann and Elger (1965) have reported that the anti-androgenic compound, cyproterone acetate, when administered to pregnant female rats will produce viable offspring with the external genitalia resembling that of a genetic female. When this anti-androgenic treatment was continued after birth, genetic male

rats with structurally intact vaginas were produced. The male Wolffian duct was not completely differentiated since it is dependent upon androgens which were biochemically repressed by the anti-androgen, cyproterone. However, anti-androgens could not antagonize the inhibiting substance (presumably a protein) which is responsible for regression of the Mullerian ducts (Elger, 1966).

The role of other gonadal steroids in this anatomical differentiation process has likewise been studied. It has been reported that the female sex hormone, estradiol benzoate, produced an anti-masculinizing effect on genetic male features. Failure of the development of male external genitalia was pronounced (Kincl & Maqueo, 1965).

Experimentally produced anatomical alterations in reproductive structures have their clinical parallels in the case reports of human beings born with various spontaneously occurring discrepancies of external genitalia and internal sex organs. The cyproterone effect with animals is paralleled by an androgen insensitivity syndrome, also termed the testicular feminization syndrome. Humans with this syndrome display a 46 XY chromosome pattern but appear morphologically as 46 XX females (Money & Erhardt, 1972).

Animal studies have also experimentally studied the masculinization of the genetic female fetus. Masculinization of the female can be induced by either prenatal administration of androgens to the pregnant female or by

neonatal injections within a critical time after birth in rodents (Dantchakoff, 1938; Phoenix, Goy, Gerall, & Young, 1959). Human genetic females may also clinically display similar tendencies toward masculinization. In the adrenogenital syndrome, androgenic hormones are secreted in excess. A similar masculinization of the genetic female may be produced by abnormal secretion rates of maternal androgens. This may be caused by a virulent hormonally dependent tumor or simulated by clinically prescribed exogenous administration of steroids to the mother during her pregnancy (Money & Erhardt, 1972). These two syndromes are characterized by masculinization of the external genitalia dependent upon the amount and timing of androgenic exposure. Characteristic features in these human clinical cases include hypertrophy of the clitoris and a degree of labioscrotal fusion. These studies have called our attention to the role of hormones in the differentiation of anatomical structures necessary for reproduction and species survival. However, this concept of differentiation has been extended to other areas as well.

Up till now, we have been using the data concerning the action of testosterone on anatomical loci in the tradition of the early embryologist. For example, early studies have established the fact that tissue grafts could alter the normal developmental sequence of embryology. Cells destined to become bone cells could conceivably be transplanted so

as to differentiate in another direction--that of neural tissue. A theory was thereby developed which postulated that certain regions of the developing embryo synthesize chemical products capable of acting as "organizers," so-called due to their ability to foster the organization of specialized structures from previously unspecialized tissues irrespective of the normal genetically determined functional role for that tissue. The view that androgenic compounds may act as "organizers" in this classical sense has been promoted by the studies of the differentiation of gonadotrophin regulation.

Hypothalamic-Pituitary Differentiation

Genotypically, masculinity and femininity by definition are determined by the distribution of sex chromosomes at the time of fertilization. However, as in the case of morphological sex, the phenotype of such animals may or may not parallel such genetic determination in terms of gonadotrophin secretion. As early as 1936, Pfeiffer observed that exchange of ovaries and testes between newborn male and female rats resulted in gonadotrophin patterns which in males (with implanted ovaries) resembled the female, whereas hormonal levels in females with implanted testes resembled the male. In such females the ovulatory discharge of gonadotrophins was absent. If male rats are castrated within a day or so of birth and adult ovarian transplantations are undertaken,

repeated follicular ruptures, corpus luteum formation, and cyclic running activity are apparent. These characteristics are shared by the normal ovulating adult female. Pfeiffer (1936) had concluded that early androgen exposure induced the sexual differentiation of the pituitary. However, approximately 20 years later, Harris concluded that the essential difference between pituitary gonadotrophin function in male and female rats was based upon differences in the hypothalamus. The establishment of a cyclic pattern of hormone secretion depended upon the exposure of the hypothalamus to androgenic stimulation during the first few days of life. To quote from Harris (1955), "It seems likely that some neural structure in the male animal becomes *differentiated* [italics added] and fixed in its function under the influence of androgens in early life." In mice and rats the critical ages during which perinatal exposure to androgen permanently alters hypothalamic control of gonadotrophin secretion has been outlined (Barraclough, 1967, 1961). Furthermore, in a precocial species like the guinea pig, this gonadotrophin differentiation process is most sensitive to exogenous steroid hormones during the prenatal period.

The administration of testosterone propionate to female guinea pigs during early postnatal life does not produce the early androgen syndrome described in mice and rats (Goy, Bridson, & Young, 1964). This fact suggests that the period

of hypothalamic responsiveness to androgens occurs earlier in this species, perhaps in fetal life. Brown-Grant and Sherwood (1971) observed that the administration of testosterone propionate (to pregnant guinea pigs) between days 33-37 of gestation produces an anovulatory syndrome in 50% of the treated females. As adults, vaginal cytology is characterized by numerous cornified cells, uterine and ovarian hypertrophy, and ovaries devoid of luteal tissue.

In the rat some effort has been made at determining the neural locus for this early androgenization process. Earlier studies postulated that a deleterious action by testosterone at the pre-optic suprachiasmatic area (Barraclough, 1968; Gorski, 1971) or anterior hypothalamus (Flerko et al., 1969) was responsible. Other studies utilizing direct implantation of androgen in the brains of neonatal females indicated that the basal hypothalamus was the critical loci (Nadler, 1968; Wagner et al., 1966). However, later studies stressed the role of the ventromedial arcuate nucleus area, and especially the arcuate nucleus, as the critical site for inducing the syndrome (Nadler, 1969, 1971). Unfortunately, all of these neonatal brain implantation studies can be criticized on procedural grounds, such as the absence of reliable verification of chemical placement.

Other more indirect sources of information have described the general anatomical boundaries for this early androgenic exposure. Barraclough and Gorski (1961) found

that stimulation of the preoptic suprachiasmatic area in animals treated with low doses of testosterone as neonates failed to produce ovulation unless primed with progesterone. Electrical stimulation of the arcuate ventromedial region was effective in one-half of the animals. In animals receiving high doses of testosterone as neonates, electrical stimulation of the preoptic area failed to cause ovulation even in animals treated with progesterone, whereas stimulation of the arcuate-ventromedial region was ineffective unless treated with progesterone.

Based in part on these data, Barraclough (1967) has advanced a mechanism of the action of androgenic steroids in the rat brain. A dual mechanism in the hypothalamus controls pituitary gonadotrophin release. The arcuate-ventromedial region is responsible for the tonic discharge of gonadotrophins, electrical stimulation releasing LH into the systemic circulation, while destruction of the area attenuates LH discharge. The midline preoptic suprachiasmatic area is responsible for the cyclic release of LH in amounts sufficient to trigger ovulation. Stimulation of this area causes ovulation and destruction of it eliminates estrous cyclicity. According to Barraclough, small doses of androgen interfere with the normal regulatory nature of this center only, making it refractory to activation by internal stimuli. Therefore, a tonic release of gonadotrophins occurs, as is the case in the normal intact

male.

Recently, however, several new experiments have stimulated a reevaluation of these views. Barraclough and Turgeon (1974) have studied the alterations produced in the hypothalamo-pituitary-gonadal axis of prepubertal male and female rats at critical postnatal developmental ages (6, 15, 20 days). The effects of early exposure to sterilizing doses of testosterone or estradiol on the responsiveness of the pituitary were also studied. The question of interest was in fact could estradiol or testosterone influence the pituitary as well as the hypothalamus. Indeed, this was the question, although now approached from a different vantage point, that initially attracted the attention of Pfeiffer in 1936. Neonates treated at birth with either 1.25 mg of testosterone propionate, 5 mg estradiol or oil were compared as to their hypophyseal responsiveness to synthetic LH-RH (leutenizing hormone-releasing hormone) at 6, 15, or 20 days of prepubertal development. Plasma LH release was high in females treated at 6 days of age and showed decreases in concentrations by age 20 days. In contrast, male plasma LH values remained relatively constant. The control female pituitary was much more responsive to LH-RH as compared to the control male and this responsiveness persisted throughout development into adulthood. When the pituitary response of the androgenized female rat was tested to LH-RH 24 hours after

treatment, LH plasma concentrations were noticeably depressed. Additionally, even when the early androgenized female is ovariectomized and given replacement estradiol, hypophyseal responsiveness to LH-RH relative to controls remains depressed. These studies, therefore, demonstrate that exposure of neonatal female rats to androgen not only produces anovulatory sterility but in fact the sites of such early androgen exposure are multiple. Follicular responsiveness to endogenous LH release triggered by either exogenous or endogenous LH-RH is suppressed and ovulation will be supported only in cases where plasma LH levels exceed 200 ug/ml. Lastly, the most damaging malfunction produced by early androgen treatment is the inability of the preoptic area of the hypothalamus to initiate those spontaneous events essential for the release of LH-RH into the portal blood. Actually, as Barraclough and Turgeon (1974) suggest, the loss of the essential preoptic neural trigger alone would explain the anovulatory syndrome.

As we depart from the early effects of androgens on gonadotrophin regulation, our knowledge is meager. Administration of estrogens produces similar sterilizing effects on genetic females (Barraclough, 1967) as well as producing grave morphological and histological changes in genetic males. Estrogens have been found to provoke a retardation of testicular development as well as an inhibition of spermatogenesis in males, while in females such an abnormal

hormonal milieu has been associated with tumorogenesis (Pantic, 1974). When given neonatally to intact male rats, estradiol benzoate causes atrophy of the testis, ventral prostate, and seminal vesicles as well as infertility. The seminiferous tubules of the testes are necrotic and the Leydig cells undergo degenerative changes (Kincl & Maqueo, 1965).

With regard to the action of progestins, almost nothing is known with certainty about gonadotrophin regulation. Whalen, Peck, and LoPiccolo (1966) reported ovarian cyclicity disruption by synthetic progestins.

The significance of the early androgen syndrome is unclear. On the one hand, it may represent an ideal model from which generalizations can be made concerning the effect of steroid imbalances in fetal life. On the other hand, a prolonged emphasis on this syndrome with other steroid induced imbalances remaining uninvestigated may be misguided both for a full theoretical understanding and for an intelligent rationale for practices in clinical endocrinology and obstetrics. For example, recently a scattered array of studies have reported the effects of synthetic progestogens on human female morphology and carcinogenic sensitivity (Herbst et al., 1975; Yalom et al., 1973). Yet no suitable animal model exists to explain the mechanism of action of such progestogens. Our knowledge of such a mechanism acquires an even greater degree of ambiguity in view of the

fact that the action of such progestogens appears to be sex-specific with an androgenic effect being exerted in the genetic female but a corresponding anti-androgenic effect being exerted in the genetic male. Before considering such data, first it is necessary to analyze the broad parameters of the differentiation of sex behaviors.

Differentiation of Copulatory Behavior

A great deal of recent data has been interpreted to suggest that particular sexually dimorphic patterns of copulatory behavior result from a dual system of hormonal regulators. One such regulator exerts an organizational role during a circumscribed perinatal period, while the other serves to trigger or activate sexual performance at the time of adult testing.

Generally, a "pure" organizational theory for sexual differentiation assumes certain fundamental background information. First, the presence of androgen during a critical period of acute neural responsiveness sensitizes the animal to the presence of androgenic steroids in adulthood. Thus, in response to the proper stimulus conditions, the probability of male copulatory behavior will be high. Secondly, the absence of androgen during such a period increases adult neural sensitivity to the ovarian stimulation provided by estradiol and progesterone. Thus, in such

an individual animal an increased probability of lordotic responding will be characteristic. The first condition can be stimulated in genetic females exposed to exogenous androgenic stimulation, while a similar condition can be produced in varying degrees in genetic males either by chemical or surgical orchidectomy.

A great deal of work has assessed both the validity and the species generality of such an organizational theory. In large part, the majority of the studies are supportive both of proposition 1 concerning the restricted ability of organisms exposed to testicular hormones to display female behavior (Pfaff & Zigmond, 1971) and of proposition 2, which maintains the necessary role of perinatal androgens for adult male sexual behavior (Nadler, 1971; Ward, 1972). In an effort to provide such a broad perspective of studies in this area of sexual behavioral differentiation, first a review will be made of the supportive studies for this organizational theory.

A wide variety of studies have documented the fact that testosterone propionate administered early in life permanently alters the reproductive behaviors of females of several mammalian species. In the guinea pig, Phoenix, Goy, Gerall, and Young (1959) found that genetic female offspring whose mothers were injected with testosterone propionate during days 20-55 of pregnancy were defeminized and masculinized. In adulthood the pseudohermaphroditic

(morphology was also altered in the male direction) females were ovariectomized and given standard replacement dosages of estradiol and progesterone. As compared with spayed control females (89%), lordosis in the experimental group was at a low of 22%. Additionally, it was noted that the average duration of heat was 5.7 vs. 2.54 hours for the two groups, respectively. Goy, Bridson, and Young (1964) determined that the sensitive period for this early androgenization effect extended from day 30 to day 55 of gestation. Similarly, Goy found that the highest frequency of mounting was also displayed in females whose mothers were injected during this gestational period.

In the rat (Barraclough & Gorski, 1962; Feder, 1967; Gorski & Wagner, 1965; Grady et al., 1965; Harris & Levine, 1965; Mullins & Levine, 1968; Ward, 1969) similar effects have been produced, with the exception that such treatment was most effective during the first five days after birth. In these studies, androgenized females were tested at maturity and again after ovariectomy and female sex hormone replacement therapy.

On the other hand, prenatal administration of testosterone propionate to pregnant rats will also adversely affect the coital behavior of the treated offspring. Less than half of the exposed females displayed lordotic responsiveness after estradiol and progesterone priming.

In such treated rats, an opposing process of sexual differentiation had developed under the influence of androgens. It was reported that prenatal or early postnatal androgen exposure increases mounting behavior shown by female rats subsequent to testosterone priming in adulthood (Harris & Levine, 1965; Koster, 1943; Nadler, 1968). Gerall and Ward (1966) demonstrated that injections of testosterone to pregnant rats produced females more likely to mount than oil-injected controls. However, the data are not completely convincing in view of the fact that while a normal male would mount approximately 12 times per test session, the prenatally androgenized female only mounted on the average of 2.8 times per test.

In female hamsters treated at birth with androgens, the results are supportive although not unanimous. Coniglio, Paup, and Clemens (1973) have reported that estrogen-progesterone-induced lordosis was suppressed in female hamsters treated neonatally with testosterone propionate, while no suppression was noted in females exposed to testosterone, androsterone, or control substances. However, another study by Nucci and Beach (1971) demonstrated that despite extensive morphological masculination, female hamsters exposed to perinatal hormonal stimulation exhibited mating responses indistinguishable from those of normal females. Similarly, Swanson and Crossley (1970) injected 2-day-old female hamster pups with either 10 ug or 300 ug

of testosterone propionate or the vehicle alone. After ovariectomy, responsiveness of the females to estradiol-progesterone stimulation was noted. Like the Nucci and Beach (1971) data, despite peripheral masculinization of the genital tubercle, after ovariectomy and replacement therapy, their receptive behavior paralleled that found during natural estrus. A partial resolution of this conflict may concern the dosage-time parameters. While Swanson and Crossley (1970) administered the maximum dose of 300 ug on day 2, Coniglio, Paup, and Clemens (1973) extended the injection regimen over postnatal days 2-4 administering 25 ug per day. Perhaps the mechanism or mechanisms responsible for the defeminization induced by androgenic steroids in hamsters require a period of extended exposure for optimal effects. The hamster is unique from the rat in the sense that while testosterone propionate administered prenatally defeminized the rat (Ward, 1969), it failed to exert such an effect in the hamster (Nucci & Beach, 1971).

In mice the few studies which exist support the "pure" organizational theory. Testosterone propionate administered to neonatal female mice effectively suppressed hormone-induced receptivity in adulthood (Edwards & Burge, 1971). One difference in mice concerns the ability of the non-propionate form of testosterone to induce such defeminization (Edwards, 1970)--an exception to the rat (Luttge &

Whalen, 1971) and the hamster (Coniglio, Paup, & Clemens, 1973). For example, Luttge and Whalen (1971) have reported that even as large as 800 ug of testosterone could not suppress lordosis in adult rats.

Vale et al. (1972), in a study with inbred mice, confirmed such a defeminizing action by neonatal androgens. Lee and Griffo (1973) reported an anovulatory syndrome and an alteration in olfactory cues in inbred mice exposed to 500 ug of testosterone propionate on day 1.

In another rodent species, the mongolian gerbil, relatively little is known either in terms of gonadotrophin or behavioral differentiation. Whitsett (1970) reported that neonatal androgens did masculinize morphological characters, a fact which our laboratory in unpublished work has recently confirmed.

Recent studies have now begun to test the generality of this organization model in primates. In the rhesus monkey, testosterone administered to genetic females from days 40 to 100 of pregnancy androgenized both the reproductive tracts as well as sex-dimorphic behavior (Goy & Resko, 1972). It has been confirmed that prenatal androgenic exposure also delays the time of first menstruation in the primate (Goy, 1970)--a fact analogous to reports of delayed menstruation in human genetic females with a diagnosis of the adreno-genital syndrome (Money & Ehrhardt, 1972).

As we examine the effects of other steroid hormones on female sex differentiation, we are immediately confronted by two problems. First is that relatively little is known with certainty. The second concerns the issues it raises for a so-called "pure" organizational theory of sex differentiation.

In the female rat, treatment with the female sex hormone, estradiol benzoate, during early neonatal periods of development, like early treatment with testosterone propionate, suppresses the degree of receptive behavior displayed in adulthood (Whalen & Nadler, 1963) while also at times altering female reproductive physiology (Gorski & Wagner, 1965). Similar results in the female rat have been reported (Feder & Whalen, 1965; Harris & Levine, 1965; Whalen & Edwards, 1967). In the hamster, the data are ambiguous. Ciaccio and Lisk (1971) demonstrated that prenatal injections of estradiol disrupted gonadal function; however, sexual receptivity after female sex hormone replacement was at control levels. In contrast, Coniglio, Paup, and Clemens (1973), administering a nonsteroidal estrogen, diethystilbestrol (DES), on days 2-4 found a decreased lordosis duration in adult female hamsters with increases in both anal-genital distances and the diameter of the vaginal orifice. Several explanations can be offered to resolve the discrepancy between the Coniglio et al. data and those reported earlier by Ciaccio and Lisk. First, it is obvious

that the actions between the natural estrogen (estradiol) on the one hand and the synthetic estrogen (diethylstilbestrol) may not be analogous. Recent data, in rats, from McEwen's laboratory adds credence to this idea. McEwen and co-workers (personal communication) have demonstrated the existence of a so-called "baby binding protein" in the serum of neonatal rats which binds with high affinity estradiol but not diethylstilbestrol. It has been postulated that such binding properties help to insulate the fetus from maternal estrogens. Thus, due to its greater ability to reach the brain, diethylstilbestrol may be a more potent defeminizing agent. Secondly, the discrepancy may again be interpreted in view of what had been mentioned earlier concerning the sensitivity of neonatal hamsters to exogenous neonatal steroids. Like the Swanson and Crossley (1970) paper, Ciaccio and Lisk (1971) administered a single dose of estrogen, while positive reports have included repeated steroid priming during the critical period. Possibly, steroid degradation by the liver might be accelerated in this species, thus neural tissue would require a longer incubation time.

Beach (1971) has interpreted these positive neonatal estrogenic effects on the defeminization process as indicating that it may not necessarily be the role of perinatal androgen which produces changes in adult behavior but, rather, any mere endocrine imbalance initiated by other

gonadal steroids as well could trigger an abnormal differentiation process. While not necessarily disputing Beach's warning, pieces of information could be interpreted as providing an alternate view. Various studies have confirmed the necessary mechanism for the aromatization of testosterone into estrogens for the display of masculine behavior patterns. Treatment with androgens which can aromatize to estrogens can maintain or induce sexually dimorphic male behavior patterns, while steroids which cannot aromatize to estrogens are incapable of such an effect (Beyer & Komisaruk, 1971; Beyer, McDonald, & Vical, 1970; Beyer, Vidal, & Mijares, 1970; Feder, 1971; Goldfoot, Feder, & Goy, 1969; Luttge & Whalen, 1971; Whalen, Battie, & Luttge, 1972). In fact, neonatal androgenization has been interpreted as really being a neonatal estrogenization process since non-aromatizable androgens like androsterone do not alter normal sexual differentiation (Coniglio, Paup, & Clemens, 1973; Paup, Coniglio, & Clemens, 1972).

In view of the importance of Beach's (1971) admonition for a theoretical model, it is unfortunate that the effects of other important steroids have not as yet been studied. One such example is the progestational compounds, which exert a critical role during pregnancy (Novak & Woodruff, 1974), and are prescribed for clinical purposes during pregnancy and often serve as oral contraceptive strategies (Jeppsson,

Johansson, & Sjoberg, 1973). While the occasional clinical effectiveness of these compounds has been demonstrated, certain precautions must also be raised. For example, one such progestogen 17αethinyl-19-nortestosterone has been correlated with female fetal virilization (Wilkins, 1960). Jacobsohn (1965) reported that 18.5% of the female offspring of women administered this progestogen during pregnancy were virilized in contrast to only 1% of the control babies. In an experimental paper Whalen, Peck, and LoPiccolo (1966) have confirmed these virilizing effects in rats while noting that neonatal administration of such compounds does alter gonadotrophin regulation while noting no changes in female sexual behavior relative to controls. A mechanism for such effects has never been adequately explained despite the important clinical significance.

To review, in several rodent species the presence of either androgenic and to a lesser extent estrogenic steroids produces a defeminization of the genetic female. What about the effect of such steroids on a corollary masculinization process? Actually this question poses a theoretical dilemma for students of this behavioral differentiation process. In a sense, two mutually exclusive models may be formulated to explain such a process. These models are somewhat analogous to those proposed for the development of the genital tubercle and the development of the internal reproductive tracts. According to the first model,

androgenic exposure "both" defeminizes as well as masculinizes. This is in accord with a model which postulates one bipotential anlagen capable of differentiating either a male or female system depending upon the hormonal concentrations of fetal life. The second view would hypothesize that such differentiation does not originate from a common anlagen but, rather, from discrete loci or primordia. According to the latter view, defeminization and masculinization are not perfectly correlated but, rather, a potential for bisexuality does indeed exist.

With these two theoretical approaches in mind, let us examine this postulated opponent masculinization process in genetic females exposed to steroid hormones during development. In the guinea pig, such a masculinization process has been found to occur (Goy et al., 1964; Phoenix et al., 1959) which apparently parallels the opponent defeminization process. In the rat, the data are somewhat more confusing. Treating the female rat with androgen before day 5 of life will enhance the emission of masculine responses (Harris & Levine, 1965; Whalen & Edwards, 1967). These studies found, however, only incomplete male copulatory sequences with no occurrences of ejaculation. However, Ward (1969) found that when prenatal androgen injections (day 16-20 of gestation) were combined with postnatal injections, 70% of the genetic females displayed complete masculine copulatory sequences, including ejaculation.

Females, however, treated only prenatally did not terminate a copulatory sequence with ejaculations. Goldfoot et al. (1969), however, reported that while androstenedione will behaviorally masculinize the genetic female, it did not defeminize.

In hamsters, Carter, Clemens, and Hoekema (1972) reported a behavioral masculinization with androgen exposure, while Paup et al. (1972) reported an enhanced mounting response in such androgenized females, noting that such a frequency of mounting was not correlated with the degree of peripheral virilization resulting from the androgenic treatment. However, in light of the possible explanatory model systems for behavioral differentiation, certain aspects of this study should be stressed. While on the surface both this study and that reported by Coniglio et al. (1973) are consistent with a bipotential model, special details are not. For example, while a masculinization syndrome can be inducted by testosterone or testosterone propionate (Carter et al., 1972; Paup et al., 1972), defeminization on the other hand was triggered only by testosterone propionate. Since a propionate moiety is physiologically longer lasting, these data suggest that the facilitatory process on masculine behavior follows a different temporal sequence than the opposing defeminization process.
Thus, in conclusion, no clear answer as yet exists as to the type of differentiation process occurring during

perinatal stages of development.

The answer becomes even more obscure as we turn to the study of the psychosexual development in the genetic male. According to a "pure" organizational theory, in the absence of androgenic stimulation during a critical stage, the organism will develop in a manner such that during adulthood the probability of the execution of the lordosis response will be increased, while spontaneous male behavior will be depressed irrespective of testosterone replacement therapy in adulthood.

Two experimental approaches have been utilized in the study of alterations of differentiation in the genetic male. In the first, neonatal male rodents are orchidectomized under hypothermia anesthesia and tested in adulthood for sensitivity to either testosterone or estrogen and progesterone. A second approach of assessing the role of testicular androgens in such a process concerns the use of anti-androgenic compounds (cyproterone and cyproterone acetate), which induce a pharmacological castrating effect similar to surgical castration.

In the normal male guinea pig, both intromission and ejaculatory patterns require a triggering action by androgens. Riss et al. (1955) castrated male guinea pigs two days postpartum and tested for male sexual behavior in adulthood. While untreated castrates did not intromit, castrates injected with 500 ug TP/kg body weight did in 100% of the

situations.

Zucker (1966) has reported a dose-dependent demasculinization of sexual behavior after prenatal injections of the anti-androgen, cyproterone acetate. This demasculinization process was evidenced by a failure of intromission and ejaculation.

In the guinea pig at least no corresponding feminization process in the genetic male deprived of androgens has been reported. Goldfoot and Goy (1971) were unable to induce female receptivity by estrogen and progesterone in fetal males exposed prenatally to relatively high doses of cyproterone acetate.

Sexual differentiation of the genetic male rat has been extensively studied. But in this case interpretive problems arise. Gerall et al. (1967) have demonstrated that orchidectomy of male rats at birth renders them less responsive to exogenous androgen replacement therapy in adulthood. This lack of responsiveness is judged by a sharp decrease in intromission and ejaculation patterns over that of controls. The authors postulated that an insufficiently stimulated penile organ was responsible for this deficiency. To quote from the paper, "Therefore, early androgen appears to permanently affect metabolic processes in the somatic organs and may determine their capacity to provide adequate feedback necessary to exceed thresholds for evoking various response systems and consummatory reflex mechanisms." In

an interesting series of studies, Beach et al. (1969) reported a strong degree of covariance between percentage of mounts with intromission and penis size (penis weight/ body weight). Whalen and Edwards (1967) have reached similar conclusions.

Ward (1969) found that perinatal injections of cyproterone acetate reduced male copulatory behaviors. In one potentially significant study, Ward (1972) induced stress in pregnant female rats by restraining them in semi-circular tubes upon which 2150 lumens of light per square meter were directed. The prenatally stressed males displayed deficient and incomplete sequences of male copulatory behavior and elevated lordotic responding; postnatal stress alone had no effect. The results were initially interpreted by Ward as supporting the view that stress in some manner increases the weak androgen of adrenal origin, androstenedione, thereby decreasing testicular androgens. Ward has since modified her interpretation and suggested that possibly the stressful experience triggers a direct ACTH-mediated response on the fetal gonads. However, several interpretative problems exist. First, it is known that ACTH in all likelihood does not cross the placental barrier since it is a high molecular weight compound (Turner & Bagnara, 1971). Secondly, there is no evidence demonstrating a depressive effect on testicular androgen by adrenal androstenedione, nor are there any data to suggest

that stress will preferentially favor the adrenal biosynthesis of androstenedione over other metabolites. Thirdly, although this may be a remote possibility, the stressful experience (intense illumination) may have altered a pineal-gonadal interaction (Wurtman, 1968). Lastly, Ward tested such males with testes *in situ*; thus the question remains as to the endogenous concentrations of androgens at the time of testing. It is suggested that dual behavioral-biochemical experiments be initiated, testing the effects of various types of stressors on sexual differentiation while correlating any observed changes with plasma steroid concentration both in the pregnant female as well as the pup. Perhaps the Terkel and Urbach (1974) technique of blood collection may have a useful application in this regard.

To summarize, independent methods of surgical and chemical orchidectomy and prenatal stress have supported an organizational theory. The question now remains as to the opponent process of feminization in such genetic males. This is important for it pertains to the choice of possible models of sexual differentiation. Goldfoot and Goy (1971), studying the guinea pig, injected pregnant females with cyproterone acetate and examined the male offspring in adulthood for female receptive behaviors after priming with estrogen and progesterone. No support for an opponent process of feminization of the external genitalia was found.

Neonatal castration of male rats results in a potential capacity to respond to estradiol and progesterone with receptive behaviors (Beach et al., 1969; Grady & Phoenix, 1963; Harris, 1964; Whalen, 1964; Whalen & Edwards, 1967). Grady et al. (1965) found that by day 10 of neonatal life, the feminization syndrome induced by orchidectomy is prevented. However, as Harris and Levine (1965) and Whalen and Edwards (1967) found, treatment of such neonatal castrates with replacement steroids shortly after surgery also prevents such a feminization process.

In the rat, other steroids as well have been shown to alter this male differentiation process. The administration of estrogen to neonatal males is one such example (Feder, 1967; Harris & Levine, 1965; Levine & Mullins, 1964; Whalen, 1964). Diamond, Wong, & Llacuna (1973) reported a strong deficit in male sexual behavior in rats treated neonatally with 500 ug estradiol (a massive dose). Proceeding under the assumption that progesterone may also function as an anti-androgenic compound, Diamond et al. (1973) reported that neonatal progesterone in high doses inhibits male sexual behavior. Unfortunately, no attempt was made to simultaneously analyze a feminization process. One problem with this study concerns the failure to study the effects of these neonatal steroids on the sensitivity to either testosterone or estrogen and progesterone. The reported sexual deficiency in neonatally hormone-treated males may only

reflect a steroid concentration problem at the time of adult testing rather than a change in responsiveness to steroid hormones. This view is supported by recent work in our laboratory in another species, the inbred mouse. Mice treated neonatally with high doses of progesterone secrete into the peripheral circulation significantly less testosterone than controls. Testosterone concentrations were determined by a sensitive competitive protein binding assay (Griffo, Chen, Lee, & Lukton, 1974).

The above literature review indicates that the work on other steroids besides androgens is indeed minimal despite the fact that working with androgens alone has not allowed us to definitely understand the nature of this differentiation process. Also, the importance of progestogen administrations during pregnancy has attracted little attention from behavioral endocrinologists despite the obvious human application (Money & Erhardt, 1972; Yalom et al., 1973).

Differentiation of Social and Nonsocial Behaviors

Up till this point we have examined three subclasses of a major differentiation process unfolding during ontogeny. Yet no mention has been made concerning the possible mechanism or mechanisms responsible for such a major developmental process. While this topic will be more fully explored in the next section entitled Biochemical

Differentiation, a word or two now is necessary to introduce the importance of the differentiation of other non-sexual social behaviors. Beach (1971), in a very well written monograph, has forcefully argued for a reexamination of our traditional notions of differentiation using central organizational theories and language. He has suggested that perhaps the mechanisms responsible for these steroid-induced alterations in normal sexual development are due to changes in peripheral sex organ sensitivities and capacities unlike the permanent neural modification proposed for gonadotrophin differentiation. According to Beach's argument, neonatal androgenization induces male sexual behavior in genetic females not because of changes in a neural substrate but, rather, due to clitoral hypertrophy and hypersensitivity. Similarly, according to this view, neonatal castration in genetic males reduces the incidence of intromission and ejaculation due to changes in the sensitivity and dimensions of the penile organ irrespective of a neural substrate change.

The importance of this study of the differentiation processes operating upon the development of other social behaviors (themselves not dependent upon peripheral morphology) concerns an evaluation of this argument by Beach--incidentally, an argument which Beach himself does not always believe seriously (Beach, 1975).

Several studies have been reported which describe the effects of gonadal hormones present during the neonatal period on male and female nonsexual behavioral patterns. Edwards (1968, 1969) administered testosterone propionate to female mice on the day of birth and tested the animals in adulthood for isolation-induced fighting. While noting that no animals fought in adulthood before exogenous testosterone administration, more than 90% fought subsequent to such hormone priming. In view of the fact that normal female mice rarely fight even when given testosterone (Scott, 1966; Tollman & King, 1956), these findings are very significant. Bronson and Desjardins (1968) reported similar results and found that neonatal estrogens may mimic these androgenic actions. Similar findings were reported by Whitsett, Bronson, Peters, and Hamilton (1972) and a critical period was postulated for the actions of such androgens. The critical period was correlated with the uptake of the hormone into neural areas. Vale, Ray, and Vale (1972, 1973) examined the interaction between genotype and neonatal steroids in modifying aggression in female inbred mice. In the first study, androgenized females differed in defensive aggressive displays as a function of their genotype. Androgenized females of an inbred strain (BALB/C) were much more aggressive than androgenized females of A or C57 BL/6 strains, which is consistent with the behavior-genetic analyses of normal males of these three inbred strains (Scott &

Fredericson, 1951; Southwick & Clark, 1968; Vale, Vale, & Harley, 1971). In the second study, while neonatal estradiol benzoate administration produced a less dramatic reversal in genetic females, nevertheless the relative ranking of the females remained consistent. The authors concluded that genotype comparisons can be a potentially important variable for the study of neonatal gonadal steroids. This is particularly expedient in view of the conflicting reports which have concluded that perinatal androgenic exposure is a sufficient but not a necessary condition for the production of androgen priming of intraspecific aggression in mice (Svare, Davis, & Gandelman, 1974) with females and Brain, Evans, and Poole (1974) with cyproterone-treated males. Both of the above studies have utilized outbred mice of Rockland Swiss and Tuck To designations respectively. Although it should be noted that neonatal castrations in the male Mongolian gerbil actually increased adulthood displays of aggression (Anisko, Christenson, & Buehler, 1973), it is suggested that other social behaviors showing significant qualitative and quantitative genotypical variance be studied in this context, particularly as to the effects of alternate steroid hormones. With regard to another form of aggression, that of shock-induced aggression, the findings are interesting. In this aggression paradigm, two animals of the same sex are placed together in a cage and exposed to brief periods of

electric shock. With short latencies, threatening postures are noted and fighting commences. Neumann (cited in Hahn, 1973) has demonstrated that the aggressive displays are much more pronounced in genetic males than females. It was shown that this sex dimorphism may be altered by neonatal endocrine manipulations. Male neonates were treated with cyproterone acetate on days 1-10 of life and tested in adulthood. In comparison to male control animals, treated males displayed a significant reduction in fighting and threatening behavior. An interesting aspect of this study concerns the reported inability of cyproterone acetate to alter this dimorphism when given prenatally, suggesting that the organization of this type of aggressive display occurs only during the neonatal stage. The question remains as to the significance of this finding for other forms of aggression.

In our laboratory, we have reported that neonatal androgen administrations will also alter the urinary cues which in mice promote aggression. For example, male mice rarely if ever attack females, but do attack adult females treated neonatally with 500 ug testosterone propionate (Lee & Griffo, 1973). Since the androgenized female is acyclic, it was assumed that the constant estrogen levels in the urine were sufficient to promote aggression. Vandenburg and co-workers (personal communication) have found that the adult urine of neonatally androgenized females

can likewise effectively accelerate the emergence of puberty in female mice, an attribute it shares with intact male urine.

Given the aggression-promoting cues present in neonatally androgenized female urine, together with Mugford's (1974) finding that neonatally castrated male mice do not emit such cues unless primed with testosterone in adulthood, the question remains concerning a physiological explanation for such data. Additionally, we understand very little concerning either the differentiation of changes in sex dimorphism in aggression by other neonatal steroids or in alterations in adult urinary cues. One possible method to study the mechanism responsible for changes in urinary aggression-promoting cues by neonatal steroids would be to compare the aggression-promoting properties of acyclic female urine from subjects in a state of constant vaginal estrus (with constant and moderate levels of circulating estrogen) with those of females who are in a state of constant diestrus. By correlating the urinary aggression-promoting qualities with estrogen levels in the androgenized female, an understanding of the hormonal determinants of the urinary transmission of information can be facilitated.

The evidence linking adult neonatal steroids with changes in urinary aggression promotion has implications for the former studies of Edwards (1968, 1969) and Bronson and Desjardins (1968), who reported changes in aggression

itself in such androgenized females. Lee (1976) has argued that possibly the increases in the aggression scores of neonatally androgenized females may reflect the fact that they are more suitable targets for attack.

Normal female rats tend to be more active in an open field and emerge with shorter latencies from an enclosed box (Pfaff & Zigmond, 1971). Gray, Levine, and Broadhurst (1965) injected neonatal female rats with androgen and observed a diminution in grooming and open field activities while noting higher emergence latencies.

The development of maternal behaviors has also been studied since a sex-dimorphic pattern exists. Two subclasses of maternal behavior which differ in certain aspects have been postulated in rats. A short latency maternal response of retrieving and nursing upon presentation of stimulus pups is a sexually dimorphic response requiring activation by female sex hormones. This short latency response can be inhibited in genetic female rats treated neonatally with androgens or facilitated in genetic males treated with cyproterone acetate during the second half of their fetal period and in the first three weeks postpartum. Such males, when treated in adulthood with female sex hormones to induce lactation, were capable of nourishing, retrieving, and licking pups (Quadagno & Rockwell, 1972).

In a sense, these results provided indirect support for the role of perinatal androgens in the differentiation

of prolactin secretion, prolactin being a necessary requirement for lactation (Turner & Bagnara, 1971). The inhibitory influence by the hypothalamus regulates the release of hypophyseal prolactin (Nicoll & Meites, 1962; Ramirez & McCann, 1963). A reduction in this prolactin inhibitory factor (PIF) or an alteration of the hypothalamic-pituitary portal system results in an enhanced secretion of prolactin from the anterior lobe of the pituitary (Turner & Begnara, 1971). Female rats which are exposed to androgenic stimulation during their fetal period exhibit a greatly reduced mammary gland growth after appropriate estrogenic stimulation (Kumaresan & Turner, 1966). Neill (1972), in an extensive series of studies, provided a more direct understanding of this differentiation of prolactin secretion. Treating ovariectomized female rats or neonatally castrate male rats with estradiol for 2 days in adulthood produces a prolactin surge of up to 100 ng/ml as measured by radioimmunoassay. On the other hand, following treatment with estrogens, prolactin levels in adult male castrates and neonatally androgenized females approached only 10-20 ng/ml after estrogenic priming.

The second form of maternal behavior has been discussed by Quadagno and Rockwell (1972) and Rosenblatt (1967) and is not sexually dimorphic. It is studied by means of the concaveation approach through which even intact male rats can display maternal behaviors. Adult males are exposed to

fresh pups daily and after 5 days of exposure, maternal response patterns can be detected.

Unfortunately, the gender dimorphic maternal response has been examined only with regard to a small number of steroids and species. It is suggested that the inbred mouse be used as a model for (a) it too shows sexually dimorphic maternal responses (Noirot, 1964), and (b) its study will allow an extension of the Vale et al. (1973) approach of neonatal hormone alterations in inbred strains.

A variety of other sexually dimorphic behavioral patterns have been described and many likewise can be altered by neonatal gonadal steroid manipulations: the saccharine water preference of female rats (Valenstein et al., 1967), the play behavior of female rhesus monkeys (Goy, 1970; Goy & Resko, 1972; Phoenix et al., 1968; Young et al., 1964), active avoidance behavior in Holtzman rats (Scouten, Grotelueschen, & Beatty, 1975), REM and non-REM sleep patterns in rats (Branchey et al., 1973), micturition patterns in beagles (Beach, 1974), marking behavior in gerbils (Turner, 1975), open field activity in rats (Blizzard & Denef, 1973), and lastly sexual preferences in male golden hamsters (Johnson & Tiefer, 1972). All of these studies argue against Beach's hypothesis that early hormonal stimulation merely alters peripheral organ sensitivities.

Biochemical Differentiation and Possible Biochemical Processes of Differentiation

Several subclasses of a general developmental differentiation process have been reviewed. However, except for brief statements, no mention has been made of the biochemical and physiological mechanisms operative in this differentiation process, nor has any mention been made concerning alterations in biochemical processes induced by steroid hormones. The purpose of this last review segment is to assess the evidence linking perinatal hormones with differentiation of biochemical processes at both neural and peripheral levels. Lastly, evidence implicating various neural loci as the targets for neonatal steroid hormone action during ontogeny will be discussed.

The liver, during development, becomes a key site for such biochemical differentiation. A basic sex dimorphism in the liver metabolism of steroid hormones and drugs has been previously reported (Conney, Schneiderman, Jacobson, & Kuntzman, 1965; Forchielli, Brown-Grant, & Dorfman, 1958; Yates, Herbst, & Urquhart, 1958). For example, hydroxylation of several steroid hormones is more efficient in liver microsomal preparations of male rats than of females; the microsomal 5 reductase enzyme on 3-oxo-Δ^4-steroids is more efficient in female than in male rats (Yates et al., 1958). DeMoor and Denef (in an extensive series of studies) have reported that the sex-specific type of cortisol metabolism in liver homogenates from male rats is an enzymatic differentiation which is critically influenced by a testicular

androgen in early postnatal life. They subsequently found that postpubertal castration of male rats produces no significant alterations on hepatic cortisol metabolism (DeMoor & Denef, 1968; Denef & Demoor, 1968, 1969, 1972; Denef, Vandeputte & DeMoor, 1968), a finding at variance with the findings of Yates et al. (1958).

Gustaffson et al. (1974) have attempted to resolve this discrepancy. Both in vitro and in vivo studies of the metabolism of 4 - (4 - ^{14}C) androstene - 3,17-dione, 5α- (4 - ^{14}C) androstane - 3α, 17α-diol and 5α(1, 2, ^{3}H) androstane - 3α, 17 α - diol were undertaken in liver microsomal preparations. Results showed that the varying types of enzyme activities in the rat liver are each controlled by a unique regulatory system. The first category of enzymes represented by the 2α, 7α, and 18 hydroxylases which act on 5α- androstane - 3α, 17α - diol and the 6α- hydroxylase active on 5α - androstane - 3α, 17α - diol substrate and 6α - hydroxylase functioning on 4α - androstene - 3, 17-dione substrate are all more active in male than in female rats. However, this sex-dimorphism could be completely abolished by both postpubertal and neonatal gonadectomy. The enzymes showed a positive response to testosterone propionate administration and were suppressed by estradiol benzoate.

The second class of enzymes (2α hydroxylase active on 5α- androstane - 3α, 17α-diol, 16α- hydroxylase, the

17α - hydroxysteroid reductase and the 5α - reductase active on 4-androstene - 3, 17-dione together with 3α - hydroxysteroid reductase active on 5α - androstane - 3, 17-dione) were more active in male than in female rats but neonatal orchidectomy could completely abolish the dimorphism. The third class of enzymes (the 7α - hydroxylase active on the 5α - androstane - 3α, 17α - diol and 4-androstene-3, 17-dione and the 17α - hydroxysteroid reductase active on 4-androstene-3, 17-dione) are not sex dependent. Thus, three categories of liver enzymes in microsomal preparations exist which differ in the regulation of activity: Type I whose basal levels are influenced by nongonadal factors but reversibly inducible by androgens, Type II enzymes "programmed" by androgens during the neonatal period, and Type III enzymes regulated by non-gonadal factors.

The altered androgen responsiveness of the liver of animals exposed to androgens may be analogous to reported alterations in androgen metabolism of the testis. Bottiglioni, Collins, Flamigni, Neumann, & Sommerville (1971) injected genetic male rats with cyproterone acetate nine days before and nine days after birth. *In vitro* incubation of testicular tissue with ^{14}C - progesterone or pregnenolone produced less testosterone in the feminized rats than in the controls. Kincl (personal communication) has found a similar pattern in adult male rats exposed neonatally to estrogen.

These metabolic modifications in the liver and testes of neonatally hormone-treated animals may be linked with changes in the concentrations of cytoplasmic or nuclear receptor proteins and/or alterations in genetic information. Additionally, neonatal imprinting by androgen may also alter the degree and specificity of steroid binding in various tissues, thereby providing a higher concentration of testosterone receptor proteins in such imprinted rats. This point will now be discussed for it may help to explain the previously described behavioral effects in animals exposed to testosterone neonatally.

The distribution of tritiated estradiol in rat neural tissues was first conclusively studied by measuring the radioactivity in extracts of dissected brain regions. In all studies (Eisenfeld & Axelrod, 1965, 1966; Glascock & Michael, 1962; Kato & Villee, 1967, 1968), hypothalamic samples, particularly anterior areas, yielded higher levels of radioactivity than cortical samples. Similarly, high levels of radioactivity have been detected in anterior hypothalamic areas of rats treated with tritiated estradiol (Luttge & Whalen, 1972; McEwen & Pfaff, 1970; McGuire & Lisk, 1968). Using the histological approach of light microscopy autoradiography, Pfaff and Keiner (1973) reported that ^{3}H estradiol is bound in cells of a limbic-hypothalamic complex. Estrogen-concentrating cells included nuclei of the medial preoptic area, medial anterior hypothalamus,

ventromedial nucleus and ventral premammillary nucleus. Areas of the amygdala which bound estradiol included the medial and cortical nuclei.

For our purposes, the question remains regarding possible alterations in such intracellular steroid binding patterns in animals exposed to particular types of steroid hormones. Although the data are often contradictory (Green, Luttge, & Whalen, 1969) and theoretical interpretations minimal, various laboratories have demonstrated that treatment of neonatal female rats with testosterone propionate shortly after birth decreases the binding of estradiol in the hypothalamus and uterus by day 60 (Flerko et al., 1969a,b; Maurer & Wooley, 1971; McEwen & Pfaff, 1970; Tuohimaa & Johansson, 1971; Vertes & King, 1971). Furthermore, these changes not only become apparent at puberty for Clark, Campell, and Peck (1972) have demonstrated that estradiol binding on day 28-35 of life was reduced in the nuclear fraction of the anterior and posterior hypothalamus of female rats androgenized at birth and undetectable in the nuclear fraction of the hypothalamus of intact male rats. Conversely, treatment of male rats with neonatal cyproterone acetate for days 1-14 increased the hypothalamic uptake of estradiol in adulthood. The anterior hypothalamus, the median eminence, and the anterior pituitary of such feminized males retained significantly more estradiol.

In males, the pattern of steroid uptake is less well defined. Evidence is limited concerning the existence of intracellular soluble or cell nuclear binding sites for testosterone in the rat brain. McEwen et al. (1972) reported that, based upon cell fractionation studies, such binding may exist in the preoptic hypothalamus. Pfaff (1968) and Sar & Stumpf (1973a,b) have found some evidence of ^{3}H testosterone in preoptic, central, and posterior areas of the hypothalamus and the anterior pituitary of 35-day-old male rats. Retention can be decreased by pre-treatment with known androgen antagonists, cyproterone acetate or progesterone. Tuohimaa & Niemi (1972) demonstrated that the same cyproterone neonatal treatment which facilitates estradiol retention in genetic males also significantly reduces the concentration of labeled testosterone in hypothalamic areas. Estrogen administrations to newborn males appear to exert a similar effect. A newer approach has been to study the metabolism of testosterone to 5 α dihydrotestosterone (DHT), a potent metabolite that binds to prostate receptors. Based upon several studies (Naftolin et al., 1972; Sholiton et al., 1970; Shore and Snipes, 1971), it is now believed that testosterone can be converted intraneurally to 5 α DHT, 3 α androstanediol and androstanedione as well as being aromatized to estrogens. Masso, Justo, and Martini (1974) have presented evidence that such a conversion is a sexually

dimorphic process in rat pituitaries. Neonatal castration transforms the 5α reductase activity of the male differentiated pituitary to a female pattern, while administration of exogenous testosterone to neonatal females channels the 5α reducing capacity of the anterior pituitary toward the male pattern. Such data, together with the fact that luteinizing hormone-releasing factor when added to the incubation media containing anterior pituitary tissues exerts no influence on the 5α- reductase activity of the gland, begin to seriously question the early conclusions of Harris and co-workers. Rather, such data illustrating a role for the pituitary in this differentiation process support the "ancient" explanations offered by Pfeiffer in 1936. There is no doubt that we have come full circle! However, any conclusive statement regarding the effects of neonatal hormones on intraneural steroid uptake and binding would be premature. Additional studies using in vitro and in vivo approaches are necessary in future work. Research also should attempt to extend this work to the effects of other steroid hormones as well as other species. Since neonatally androgenized females and neonatally castrated males are refractory to exogenous administrations of estradiol and progesterone, and are therefore defeminized and demasculinized respectively, these steroid uptake and binding studies begin to assume theoretical importance. The very nature of such refractoriness may depend upon such

"differential" steroid uptake and concentration patterns.

Other biochemical changes also have been reported to be predetermined at birth and may or may not be causally related to the gonadatrophin and behavioral changes induced by various neonatal hormones. For example, in an earlier paper Ladosky and Gaziri (1970) noted a sex-specific surge in 5-hydroxytryptamine (serotonin) (5 HT) concentrations in the female rat brain by day 12 of life. Guillian, Pohorecky, and McEwen (1973) have examined more closely this sex dimorphism and found that levels of 5 HT are higher in the female brain on postnatal days 10, 12, and 14, while either androgenization of the female or ovariectomy of the female reduces the forebrain and midbrain concentrations of 5 HT on day 12. These findings may have potential significance in view of the fact that serotonin has been considered to be a putative transmitter of the central nervous system (Cooper et al., 1970) as well as being implicated in the regulation of gonadal function (Labhsetwar, 1972).

Studies have also linked a central cholinergic mechanism with gonadotrophin secretion (Everett, 1964; Markee et al., 1952; Taubenhaus & Soskin, 1941). Libertun et al. (1973) examined sex differences in a putative hypothalamic cholinergic system in Long-Evans rats. The activity of ACh (Choline acetyltransferase) and AChE (acethylcholinesterase) were simultaneously assayed in the

anterior preoptic suprachiasmatic area and the posterior arcuate mammillary area of the hypothalamus. Obtained results demonstrated differences in the biosynthetic and hydrolyzation cholinergic mechanisms in the hypothalamus of male and female rats both before and after puberty. In females androgenized at birth a reduced activity of the preoptic suprachiasmatic cholinergic enzymes was noted. However, these results must be reevaluated since reductions were also apparent in genetic males at birth.

Other biochemical changes triggered by perinatal hormones include: histological changes in the hypothalamus (Field & Raisman, 1973), incorporation of ^{3}H lysine in the Purkinje cells of the rat cerebellum (Litteria & Thorner, 1974a,b), and lipid metabolism in rats (Hacik & Palkovic, 1973). It remains a task of the future to establish the possible behavioral implications of these biochemical changes.

Rationale and Specific Aims

While considerable progress has been achieved in the analysis of the nature and scope of effects influenced by neonatal hormones, our understanding of the processes of sex differentiation remains incomplete. The attention of much of the previous research has concentrated on the early androgen syndrome and has attempted to relate such findings to either of two models of sex differentiation. Yet a

review of the findings produces no clear answers either for theoretical models or for clinical practices. Given the importance of understanding the mechanism of action of the possible therapeutic gestational steroids, our future task seems to be to develop reasonable and empirically testable animal models. By examining the action of these compounds in light of morphological, gonadotrophic, behavioral, and biochemical theories of differentiation, our understanding of the normal and pathological conditions of psycho-sexual development will be enhanced. Furthermore, by adopting several novel techniques to this problem, a much finer analysis of these differentiation processes, particularly at the biochemical level, can be achieved.

The specific objectives of the proposed experiments are:

1. to examine the differentiation processes of genetic females neonatally exposed to progesterone and to examine the dose-time parameters (Exp 1).
2. to examine the effects of progestin metabolites in genetic females (Exp 2).
3. to explore possible opposing anti-androgenic actions of progestins in the genetic male (Exp 3).
4. to determine the role of progestin metabolites as anti-androgenic agents in the neonatal genetic male (Exp 4).

5. to examine the effects of neonatal hormone treatment on olfactory cue production in adult male mice (Exp 5).
6. to determine the role of prenatal progestins as masculinizers and defeminizers in genetic females (Exp 6).
7. to study the ability of tritiated Progesterone to cross the placental barrier while examining the time and site parameters (Exp 7).
8. to examine the processes of biochemical differentiation by studying the effects of neonatal steroids on the uptake of sex steroids in adult females (Exp 8).
9. to provide a rudimentary account of the possible sites for neonatal hormone action (Exp 9).

Methods of Procedure

1. General Methods

Subjects. The subjects in all experiments were C57BL/10J or C_3H/HeJ mice descended from stock purchased from R. Jackson Memorial Laboratories, Bar Harbor, Maine.

Animal housing. All subjects were either singly or collectively (2-4 animals) housed in standard polypropylene cages and maintained on *ad lib* Wayne Lab Box and tap water. Food was available in food hoppers and water was provided through a metal drinking tube protruding into the cage.

Morphological analyses. In adulthood, genetic males were checked for anatomical male differentiation--anal-genital distances, testicular descent, and penile deformities. On the other hand, in genetic females, vaginal diameters, anal-genital distances, presence or absence of scrotal folds, and clitoral hypertrophy were assessed.

Gonadotrophin analysis. Gonadotrophin regulation was evaluated in genetic females by means of cyclicity tests. During designated times, vaginal smears were taken by means of a probe technique. Cells were stained with .5% solution of methylene blue. Stages of the cycle were recorded (Turner & Bagnara, 1971).

Hormonal administrations. Unless otherwise indicated, all hormones, recrystallized before use, were dissolved in sesame oil and injected subcutaneously at the nape of the neck. Control injections provided the oil vehicle only. Afterward, all injection sites in neonates were sealed with flexible colloidon to prevent leakage.

Surgery. Adult orchidectomies were made by a trans-scrotal incision under nembutal anesthesia. Adult ovariectomies were performed using a double incision method and suturing of the muscle wall.

EXPERIMENT 1

The preceding literature review has focused attention on the role of neonatal and prenatal hormones in the various differentiation processes of experimental animals. The purpose of the present experiment is to provide a more comprehensive understanding of the role of progestogenic compounds during ontogeny. Although not widely studied, progesterone administered to prenatal rats does produce disruptions in the genetic female (Revesz et al., 1963; Whalen, Peck, & Lo Piccolo, 1966). This proposed experiment will compare the actions of two progestogens, Progesterone (P) and medroxyprogesterone acetate (MPA) in mice. The action of these two compounds will be viewed with respect to three of the differentiation processes, namely, anatomical, gonadotrophin, and behavioral. In order to more fully understand the actions of these two progestogens, dose-response as well as time-response curves will be established.

Method

Subjects. The original breeding stock of C57BL/10J and C3H/HeJ mice were purchased from Jackson Memorial Laboratories, Bar Harbor, Maine, at six weeks of age and exposed to colony mating conditions at 10 weeks of age.

Procedure. Female pups of both C57BL/10J and C3H/HeJ strains were injected on day 1 of life with one of the

following hormonal concentrations dissolved in sesame oil: a) 500 ug P (N = 6), b) 250 ug P (N = 5), c) 125 ug P (N = 5), d) 50 ug P (N = 5), e) 500 ug MPA (N =6), f) 320 ug MPA (N = 13), g) 160 ug MPA (N = 9), h) 80 ug MPA (N = 7), i) 500 ug testosterone propionate (TP) (N = 11), or j) oil (N = 25). All neonatal injections were provided in .03 cc of oil solvent and administered at the nape of the neck. Flexible colloidoin was used to seal each injection site. Earlier pilot work suggested that 500 ug P and 320 ug MPA were sufficient to defeminize over 80% of genetic female pups. Thus, litters were also randomly assigned to one of the following temporal conditions: a) day 1--within 24 hours of birth, b) day 4, c) day 6, d) day 10, or e) day 20. At these ages pups were injected in the usual manner with either 500 ug P, 320 ug MPA, or the oil vehicle alone.

Estrous cyclicity determinations. Genetic female pups from all conditions were weaned by approximately day 28 and housed collectively in unisex groups of 3-5 with animals of the same age and treatment condition. On approximately day 45 animals were singly housed and a 30-day sequence of vaginal smears was initiated. Both C57 and C3H pups were defeminized to an identical extent, thus the data from both strains were pooled.

Anatomical analyses. After such a series of smears, each subject was anesthetized and various anatomical measures were recorded. Body weight to the nearest .01 gram, anal-

nose lengths, and anal-genital distances were calculated. Color photographs were taken and the degree of clitoral hypertrophy was noted. Virilization was defined as an enlarged clitoris extending from the urethra. Even moderate degrees of hypertrophy were sufficient for females to be classed as virilized. Such judgments as well as all behavioral testing were conducted in a double blind fashion. At this time also, subjects were bilaterally ovariectomized, at which time ovarian and uterine hypertrophy was noted.

Behavioral analysis. Following a one-week recovery period subjects were first exposed to two consecutive weekly injection regimens of 10 ug estradiol benzoate (EB), followed 42 hours later by 500 ug P. After these two pre-testing administrations, subjects were injected with the same concentrations and tested for female sex behavior 6 hours after the P injection. All females were exposed to proven sexually vigorous males of their respective inbred strains. All interactions occurred within the home cage of the stud male (13 x 8 x 15 in.). Latency to mount, frequency of pelvic thrusts, and lordotic responses were recorded. A lordotic response was operationally defined, during a 10-minute session, as arching of the back and raising of the genitalia. The lordosis quotient (LQ) was defined as frequency of lordosis/frequency of mounts x 100. A resistance quotient (RQ) was defined as frequency of active kicking or biting of male/frequency of attempted male mounts x 100. All subjects

were tested once a week for four consecutive weeks.

Approximately ten days after the last induced estrous test, subjects were injected daily for 12 days with 500 ug TP dissolved in sesame oil in a volume of .1 cc. Injections were administered at 10 a.m., with behavioral testing commencing 5 hours later. On the day of the 5th, 7th, 9th, and 11th injections, femals were exposed in their home cage to an ovariectomized female of their respective inbred strain which had been previously brought into behavioral receptivity by EB + P therapy. During a fifteen-minute test interval, latency to the first mount, total frequency of mounts, frequency of intromissions and ejaculations were recorded for each genetic female subject. Ejaculation was assessed both behaviorally as well as by an examination of the vaginal tract of the female.

Results

Anatomical differentiation. Females treated with Protesterone or MPA on day 1 of life displayed a dose-dependent alteration in female anatomical differentiation of the genital tubercle. Although anal-genital distances did not differ among the groups, P and MPA at the highest dosages administered produced extensive clitoral hypertrophy. The hypertrophy was apparent at the time of weaning and persisted throughout the adult life of the subject.

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[Insert Figure 1 about here.]

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Figure 1 depicts this extensive peripheral masculinization in subject 1A treated with 320 ug MPA and subject 1B treated on day 1 with 500 ug P. Females treated with 500 ug TP, 500 ug P, and 320 ug MPA show an enlarged phallus and reduced vaginal diameters. Controls never displayed such abnormalities of the genital tubercle. A chi-square analysis demonstrated that the percentage of animals displaying somatic virilization was not independent of concentration and structure of the neonatal steroid (X^2 = 49.76, df = 9, p .001).

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[Insert Figure 2 about here]

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Figure 2 demonstrates that peripheral virilization is a dose-dependent phenomenon in that 500 ug MPA resulted in clitoral hypertrophy in 77% of the females, whereas 80 ug MPA did not result in any such abnormality.

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[Insert Figure 3 about here].

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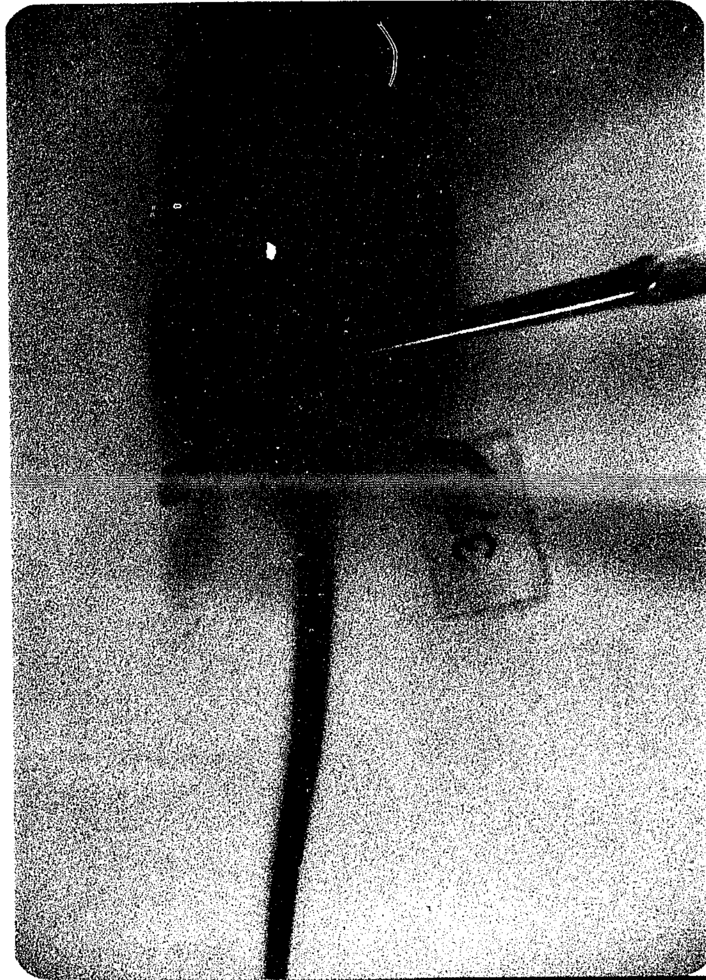
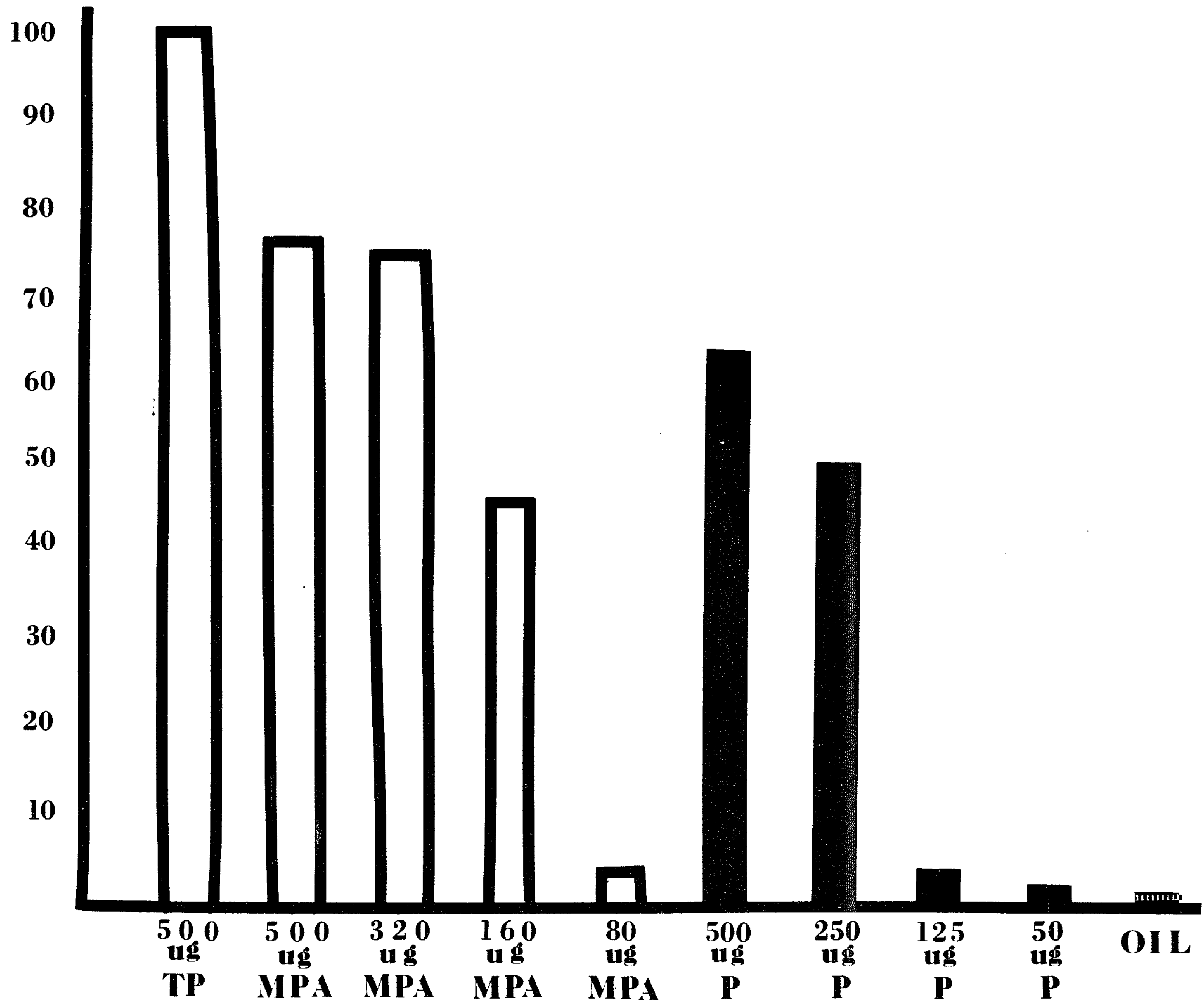
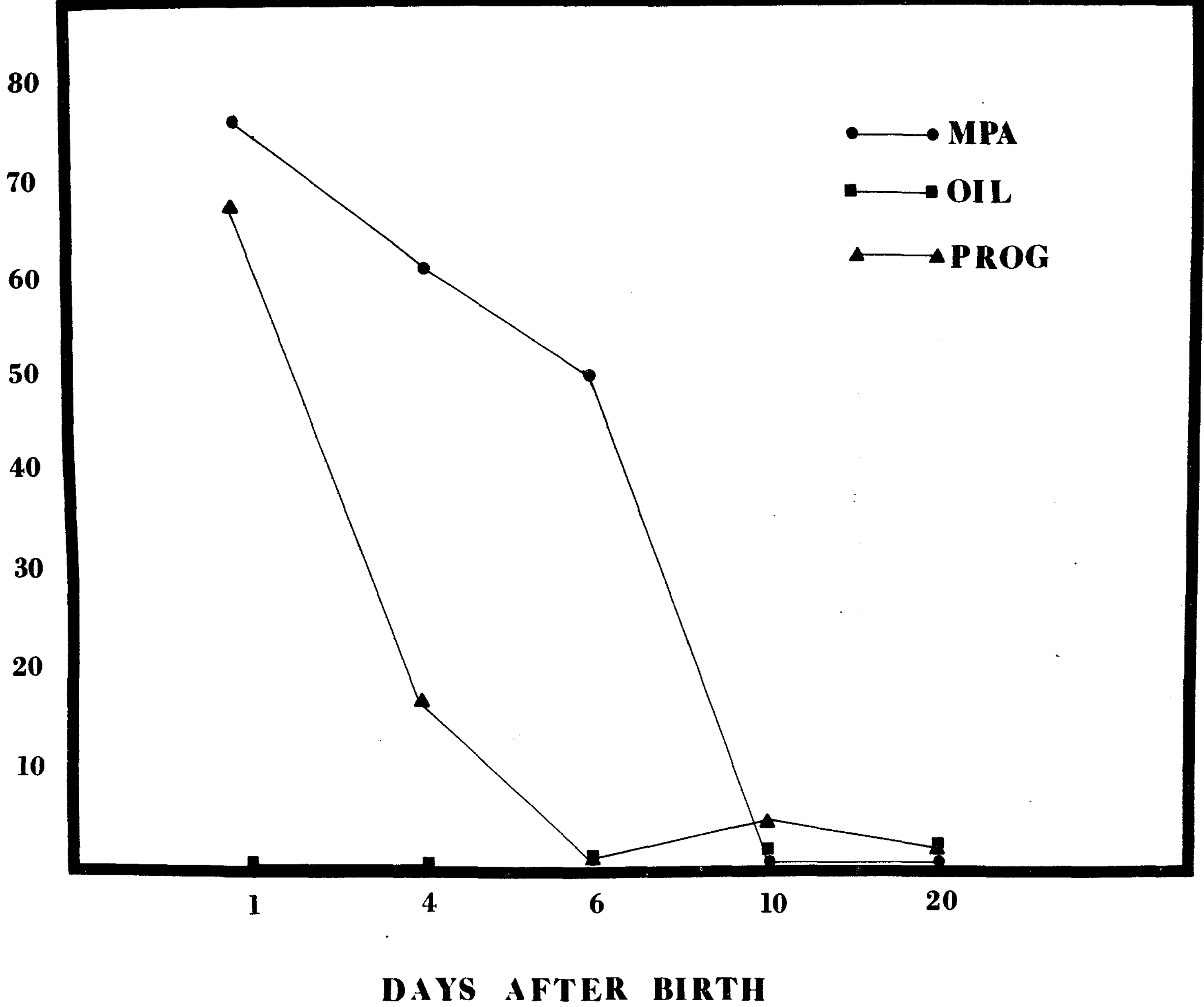
Another important consideration concerns the changes in the sensitivity of the anatomical differentiation process with time. Figure 3 depicts this sensitivity. Both 320 ug MPA and 500 ug P produced maximal virilization on

1A



1B


% SOMATIC VIRILIZATION
100
90
80
70
60
50
40
30
20
10
500 ug TP
500 ug MPA
320 ug MPA
160 ug MPA
80 ug MPA
500 ug P
250 ug P
125 ug P
50 ug P
OIL


% SOMATIC VIRILIZATION
80
70
60
50
40
30
20
10
MPA
OIL
PROG
1
4
6
10
20
DAYS AFTER BIRTH

day 1 with minimal virilization by day 10. An analysis of the percentage of subjects displaying somatic virilization with respect to time produced a significant effect (X^2 = 78.94, df = 4, $p < .0001$). Although MPA appeared to produce a greater degree of hypertrophy, the differences did not reach an acceptable level of statistical significance.

Gonadotrophin differentiation. This type of differentiation process was indirectly assessed by means of a daily examination of the vaginal smears. The percentage of subjects which cycled at least 80% of the time was recorded. In our laboratory, inbred mice typically cycle either every 4, 5, or 6 days. Thus, an animal was considered to cycle if three, four, or five days of diestrus preceded an estrus day. Figure 4 depicts the percentage of cyclers as a function of dosage and chemical structure of the steroid. Testosterone propionate as well as all dosages of MPA and P produced a greater abnormality in cyclicity patterns as compared to oil controls (X^2 = 29.87, df = 9, $p < .001$). Note that equivalent dosages of MPA, P and TP produced equivalent decrements in cyclicity patterns.

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[Insert Figure 4 about here.]

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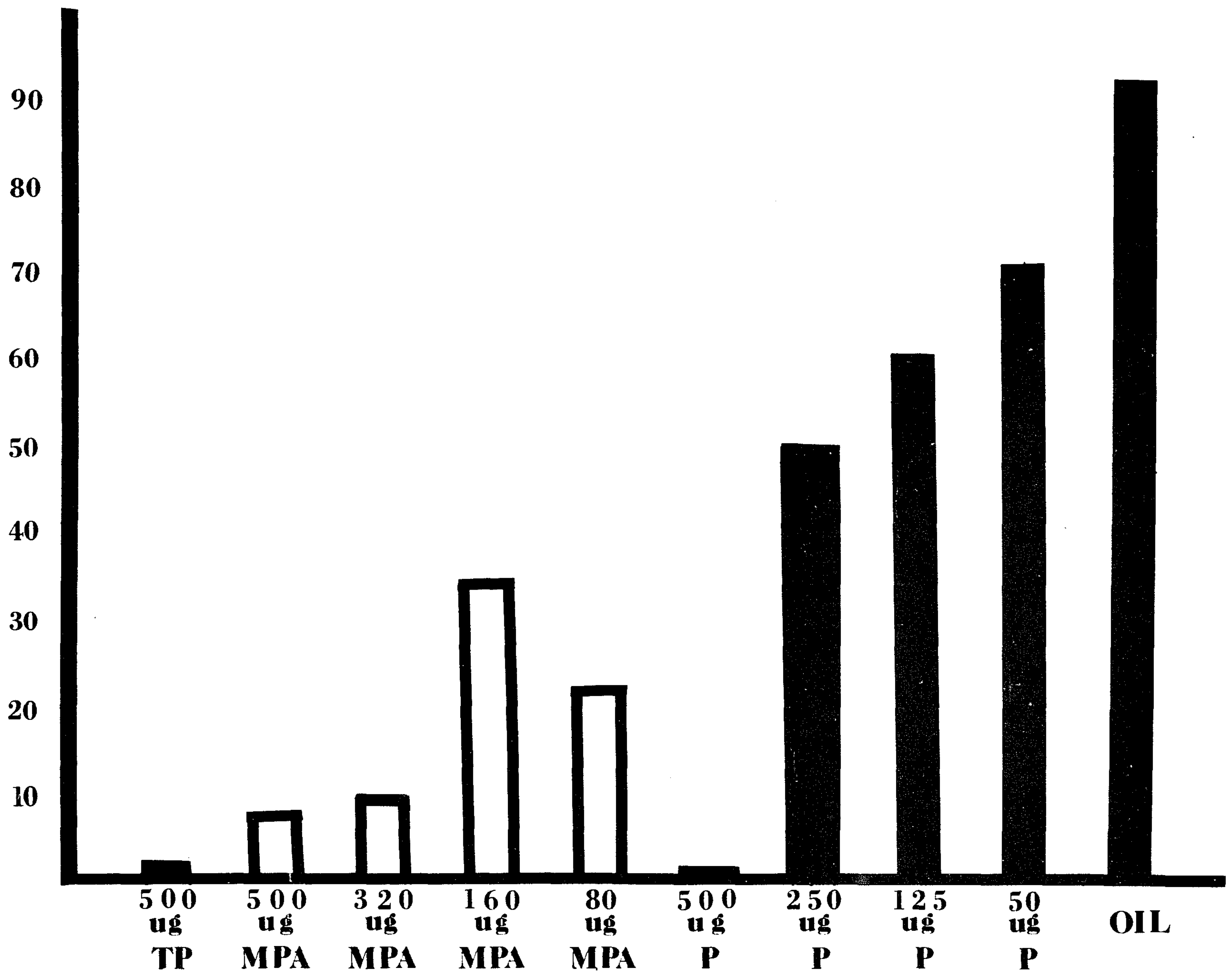
Additionally, the percentage of cyclers was assessed in females receiving MPA or P on either neonatal days 1, 4, 6, 10, or 20.


% SUBJECTS CYCLING
90
80
70
60
50
40
30
20
10
500 ug TP
500 ug MPA
320 ug MPA
160 ug MPA
80 ug MPA
500 ug P
250 ug P
125 ug P
50 ug P
OIL

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[Insert Figure 5 about here.]

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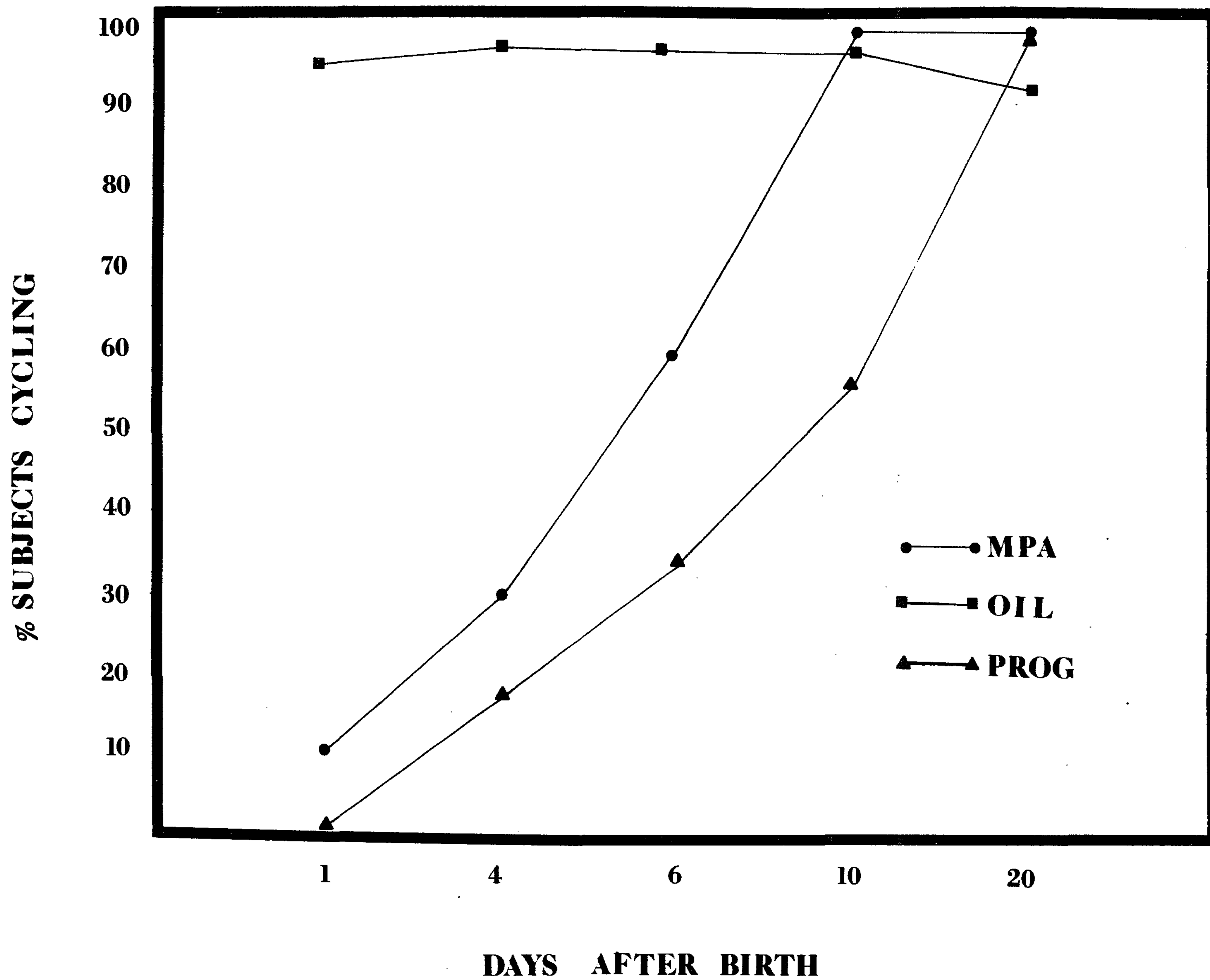
Figure 5 demonstrates that the neonatal age at which the progestogenic compound is administered influences the degree of cyclicity disruption. Progesterone was more disruptive than MPA at ages 6 and 10. By day 20, the effects of progestogens were minimal. Over-all, an analysis of the ages demonstrated a significant time effect ($X^2 = 17.96$, df = 4, $p < .01$).

The degree of gonadotrophin differentiation was also indirectly assessed by comparing the various groups with respect to the percentage of possible cycles. Since the majority of the control subjects as well as the usual colony population of our laboratory are 5-day cyclers, five days was considered the criterion. Thus the percentage of possible cycles was calculated as follows: number of established cycles/number of possible cycles (number of days of smears/5) x 100. Using this measure, Figure 6 depicts the effects of various steroids and steroid concentrations. A one-factor analysis of variance with unequal samples was calculated according to Winer (1962, p. 98) . A significant treatment effect was found among the 10 treatments ($F = 14.35$, df = 9/74, $p < .0001$). A posteriori Tukey tests demonstrated that all dosage conditions were significantly different from the oil control condition ($p < .01$). Furthermore,


100
90
80
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10
% SUBJECTS CYCLING
MPA
OIL
PROG
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DAYS AFTER BIRTH

testosterone was more disruptive than P at 50 ug and 125 ug with MPA at 500 and 320 ug more disruptive than P at 125 ug ($p < .01$).

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[Insert Figure 6 about here.]

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Using the same measure, percentage of possible cycles, the sensitivity of the females at various neonatal ages was assessed. A 3 x 5 analysis of variance (3 drugs x 5 time conditions) with unequal sample sizes was calculated according to Winer (1962, p. 242). Progesterone and MPA did not differ among themselves as disruptors of the estrous cycle ($F = 1.20$, $df = 2/93$, $p > .50$). However, as noted in Figure 7, a significant time effect was noted ($F = 16.98$, $df = 4/93$, $p < .01$) with no significant interaction ($F = .30$, $p > .50$). Tukey _a posteriori_ tests demonstrated that MPA on day 1 was significantly more disruptive than MPA on day 20 or P on either days 10 or 20. Furthermore, the percentage of possible cycles was significantly less in females treated with P on day 1 than those treated with either P on day 20 or MPA on days 10 or 20.

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[Insert Figure 7 about here.]

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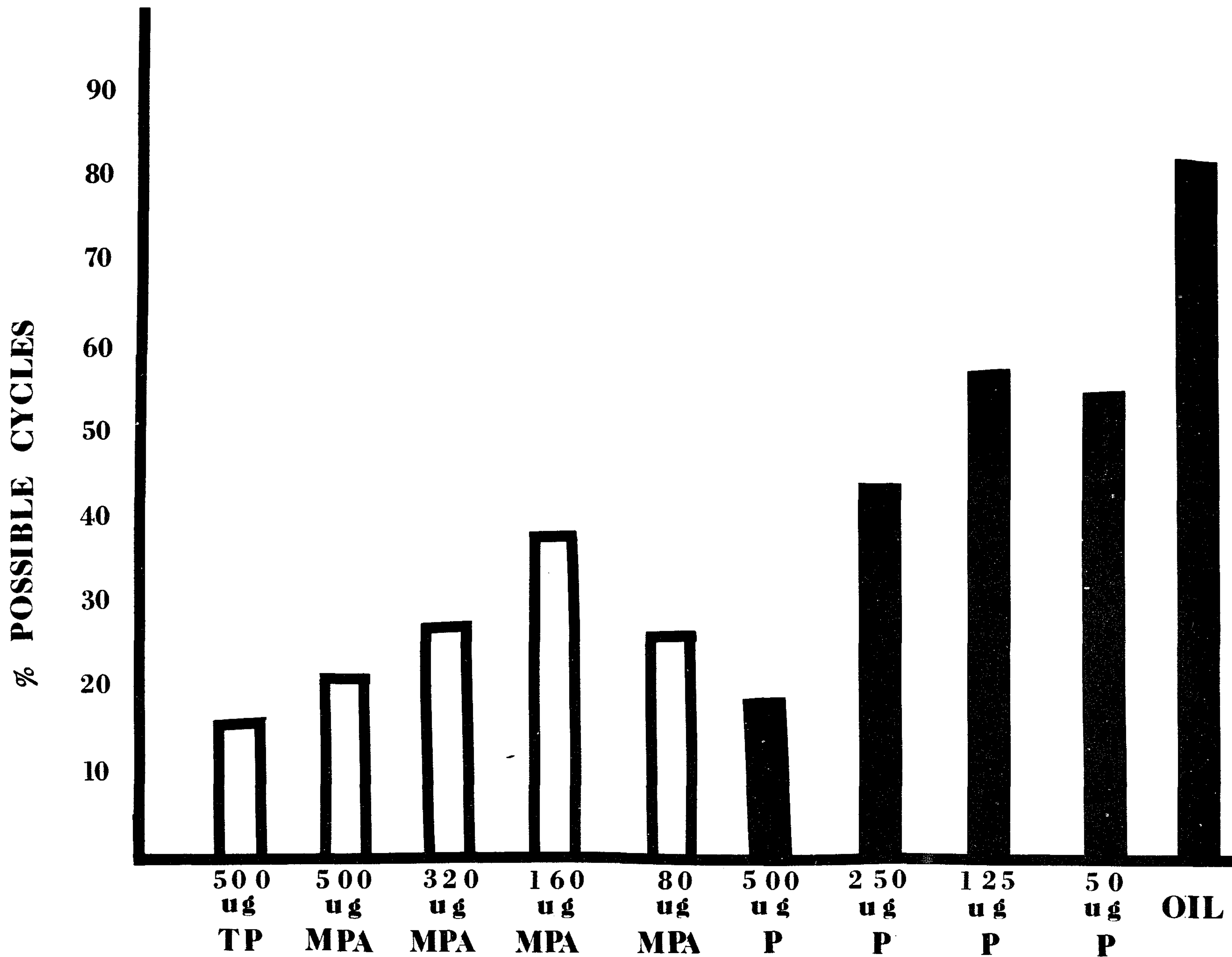
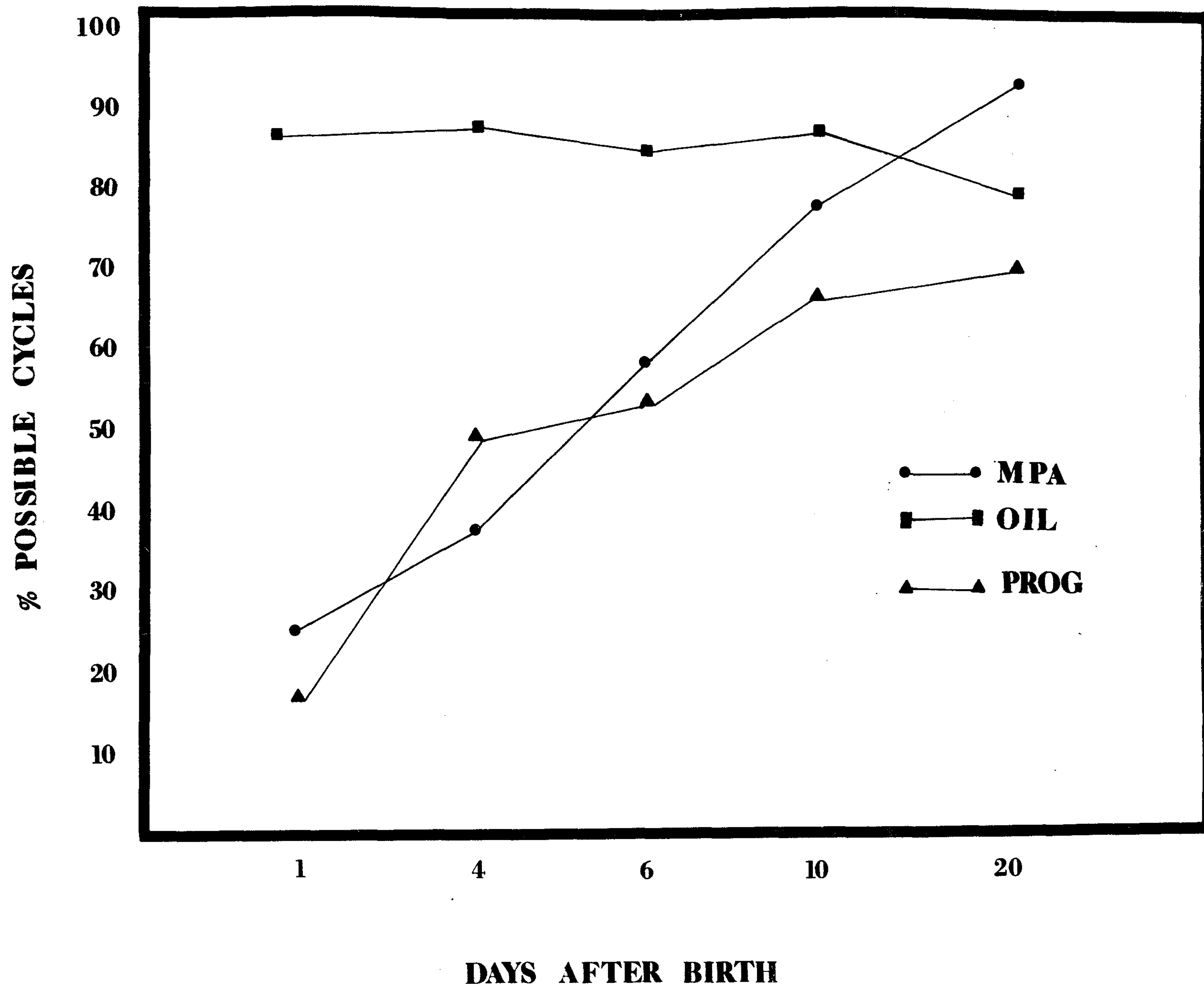
Female sex behavior. Progesterone, MPA, and TP produced sharp dose-dependent modifications in female sex


% POSSIBLE CYCLES
90
80
70
60
50
40
30
20
10
500 ug TP
500 ug MPA
320 ug MPA
160 ug MPA
80 ug MPA
500 ug P
250 ug P
125 ug P
50 ug P
OIL


100
90
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10
% POSSIBLE CYCLES
MPA
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1
4
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10
20
DAYS AFTER BIRTH

behavior as judged by lordotic quotients. All dosages of P as well as MPA produced significant deficiencies in lordotic receptivity as compared to oil controls. A 4 x 9 factorial analysis of variance with repeated measures on one factor and unequal sample sizes produced no significant replication effect ($F = .50$, $p > .50$) and no significant treatment by replication effect ($F = 1.03$, $p < .40$). Therefore, a total lordotic quotient score was calculated by summating each of the four lordotic quotients. A one-factor analysis of variance with unequal sample sizes was calculated and a significant treatment effect was found ($F = 43.89$, $df = 9/74$, $p < .0001$). An a posteriori Tukey test determined that all treatments were significantly different from oil ($p < .01$); however, none of the treatments differed among themselves.

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[Insert Figure 8 about here.]

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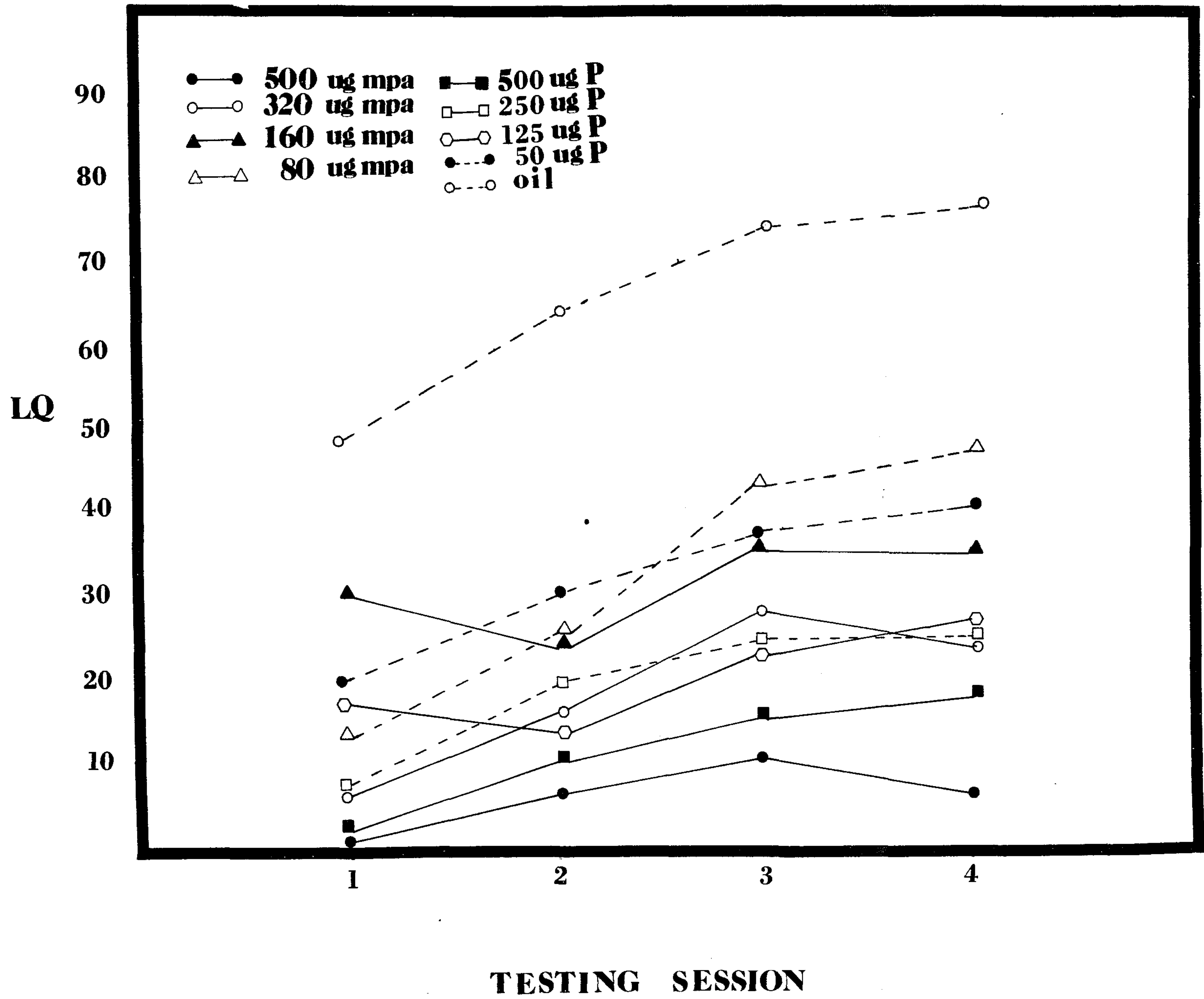
With respect to a critical period hypothesis for the defeminization of genetic female mice by neonatal hormones, a 3 x 5 anova with unequal sample sizes was calculated (3 drugs x 5 time periods). The results depicted in Figure 9 illustrate that a significant drug treatment effect was found with MPA producing a greater decrement in female receptivity than P ($F = 62.07$, $df = 2/93$, $p < .0001$). Similarly, a significant time effect was found ($F = 43.95$, $df = 4/93$, $p < .0001$), with the drug x time interaction also


500 ug mpa
320 ug mpa
160 ug mpa
80 ug mpa
500 ug P
250 ug P
125 ug P
50 ug P
oil
90
80
70
60
50
40
30
20
10
LQ
1
2
3
4
TESTING SESSION

being significant ($F = 29.35$, $df = 4/93$, $p < .01$). MPA at day 20 produced a significantly stronger decrement in adult female sex behavior than did P at day 20. Yet MPA at day 20 and P at day 20 did not differ from oil controls. Interestingly enough, MPA at day 10 did produce a stronger decrement than oil controls ($p < .05$).

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[Insert Figure 9 about here.]

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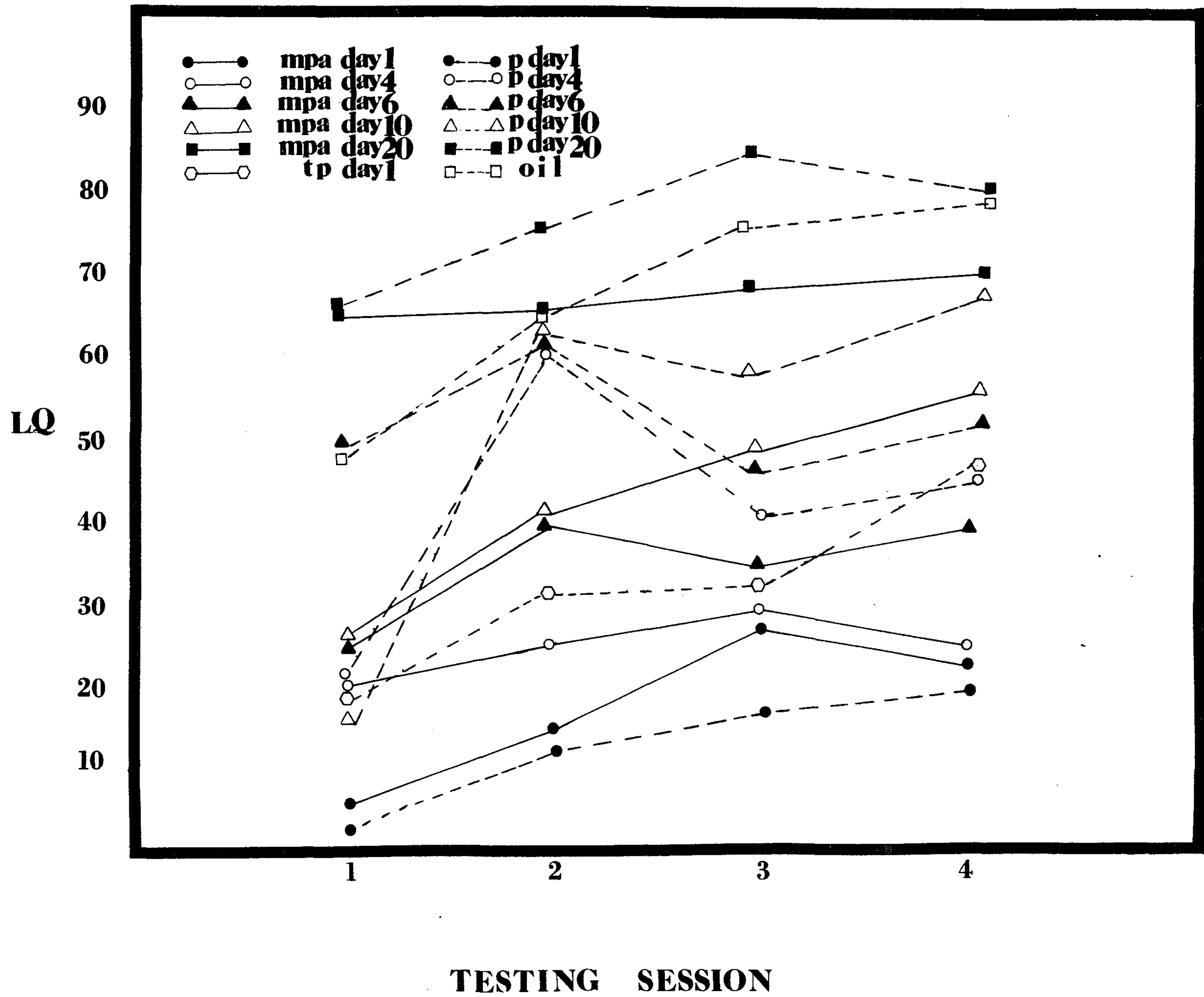
Male sex behavior. Table 1 illustrates that neonatal treatment with either TP or P results in a greater adult masculine behavior display than controls. However, MPA treatment did not produce such an increase.

- - - - - - - - - - - - - - -

[Insert Table 1 about here.]

- - - - - - - - - - - - - - -

An analysis of variance with unequal sample sizes was calculated. A significant treatment (dosage and steroid structure) effect was found with regard to average mount frequency across the four post-operative TP replacement sessions ($F = 21.58$, $df = 8/75$, $p < .001$). Although 500 ug TP females intromitted in 36% of the sessions, oil females never achieved an intromission. A chi-square analysis on the percentage of animals intromitting found a significant independence between intromission probability and steroid structure and concentration ($X^2 = 15.51$, $df = 8$, $p < .05$).



mpa day1
mpa day4
mpa day6
mpa day10
mpa day20
tp day1
p day1
p day4
p day6
p day10
p day20
oil
90
80
70
60
LQ
50
40
30
20
10
1
2
3
4
TESTING SESSION

Table 1

Masculine Behavior Potential in Genetic Females

Masculine Behavior	Oil	500 ug TP	320 ug MPA	160 ug MPA	80 ug MPA	500 ug P	250 ug P	125 ug P	50 ug P
			Concentration of Progestin						
Females									
N	25	11	13	9	7	6	5	5	5
% Mounting[a]	64	82	84	77	71	66	60	80	60
$\bar{X}$ Mount Freq.[b]	4	10	5	4	3	14	6	6	6
% Intromitting[a]	0	36	23	11	0	16	0	0	0
% Ejaculating[a]	0	10	0	0	0	0	0	0	0

[a]The percentage of total number of tests the animals within a group performed the behavior.

[b]Score represents the mean of four testing sessions.

However, as shown in Table 1, there was no correspondence between the percentage of animals ejaculating and neonatal steroid treatment.

Although significant, the phenomenon of progestin-induced masculinization of the genetic female is a fairly fragile phenomenon. When the maximum dosages of progestins and TP were administered on day 4, the tendency for masculine like sexual vigor was virtually abolished. Furthermore, only mounting frequencies and percentage of intromissions were higher in neonatal progestin groups than in oil groups ($p < .01$); ejaculatory frequencies or percentage did not vary as a function of neonatal treatment. Additionally, this type of masculinization was very much time dependent in that MPA and P were only slightly and nonsignificantly more masculinizing than oil with regard to mounting behavior. Similarly, the percentage of animals intromitting and ejaculating, although higher in MPA and P groups, was not significantly higher than oil-treated subjects for day 4 treatments.

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[Insert Table 2 about here.]

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Discussion

Prior to this experiment, relatively little had been known concerning the action of progestins during development. Revesz et al. (1963) reported that 1 mg P disrupted

Table 2

Effect of Time of Progestin Treatment on Masculine Behavior Potential in Genetic Females

	Day of Injection														
Masculine Behavior	1 MPA	1 P	1 Oil	4 MPA	4 P	4 Oil	6 MPA	6 P	6 Oil	10 MPA	10 P	10 Oil	20 MPA	20 P	20 Oil
Females															
N	13	6	25	5	5	5	5	8	5	7	9	5	9	5	5
% Mounting[a]	84	66	64	75	63	65	65	60	80	71	66	60	65	60	60
$\overline{X}$ Mount Freq.[b]	5	14	4	9	7	3	4	3	4	5	4	4	4	4	5
% Intromission[a]	23	16	4	13	8	0	0	0	0	0	0	0	0	0	0
% Ejaculating[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]The percentage of total number of tests the animals within a group performed the behavior.

[b]Score represents the mean of four testing sessions.

the estrous cycles and produced peripheral virilization in rats. Whalen, Peck, and Lo Piccolo (1966), also working with rats, found that a synthetic progestin, Norlutin, was capable of virilizing the genetic female offspring of treated pregnant females. In humans, also, the virilizing side effects of synthetic progestins have been reported (Jacobsohn, 1965; Money & Ehrhardt, 1972 Wilkins, 1960).

The present experiment confirms in mice the previously reported rat data in that extensive peripheral virilization resulted from either MPA or P treatment on postnatal days 1-4. This peripheral virilization was a dose-dependent phenomenon in that 320 ug MPA produced the maximum virilization, while 80 ug MPA did not virilize any of the five subjects. Differentiation of the genital tubercle appeared to be complete by day 6.

This experiment likewise has demonstrated that gonadotrophin differentiation develops up till day 10 of neonatal life in that approximately 50% of animals treated with 500 ug P on day 10 were acyclic in adulthood. Furthermore, the percentage of possible cycles was depressed in such animals. Although slight variations did exist among experimental subjects, the majority of acyclic females exhibited a pattern of constant vaginal cornification. However, in some cases a pattern of constant diestrus was noted, while other subjects would exhibit an estrus smear for a certain variable period followed by another period of diestrus of

variable duration. A third category was also detected. In this case an animal would be estrus for 4-5 consecutive days followed by a 1-2 day period of diestrus. This pattern would continue for 30 days.

Progestin treatment also produced disruptions in the ability of the ovariectomized female to respond to adult estradiol and progesterone treatment. This is reflected by the depressed lordotic receptivity scores. All progestin treatments were significantly different from oil, but the intensity of effect diminished with decreasing dosage across both progestin groups. A critical period was established for this defeminization effect in that by day 20 progestin treatment did not lower either receptivity scores or the quality of lordotic behavior. Yet a significant time by drug interaction was found in that while MPA on day 10 produced a significantly stronger decrement compared to oil controls, no such effect was noted with P on day 10. Thus, it appears that the developing neural systems for gonadotrophin differentiation are most sensitive to progesterone till day 10; systems for behavioral differentiation are sensitive only to MPA by day 10. Whether this difference in sensitivity warrants the idea that there are two distinct neural systems, one for gonadotrophin differentiation and another for behavioral differentiation, remains a question for future empirical inquiry.

It should be stressed that the process of behavioral defeminization observed in the present experiment was not merely a passive absence of lordotic responding. Rather, both receptivity quality scores as well as behavioral observations indicated that the progestin-treated females actively and vigorously resisted and in certain cases retaliated against the advances of the stud males. This retaliation often took the form of intense backward kicking and biting of the male, a display similar to that observed in diestrous normal females.

Although masculine behavior potential in genetic females was evident relative to oil controls, absolute levels of performance were rather disappointing relative to normal genetic males, at least the typical colony males of our laboratory. No one female, irrespective of experimental condition, was ever observed to ejaculate. This was also true of somatically virilized females who possessed a phallic structure capable of insertion. Thus, it appears that neonatal progestin is not sufficient to completely masculinize the genetic female mouse. One other study by Edwards and Burge (1971) demonstrated that masculinization was not completely fostered in genetic females even by neonatal testosterone. Therefore, it appears that phallic development, although a necessary condition for the display of the advanced components of male sexual behavior, is not a sufficient one.

EXPERIMENT 2

The results of Experiment 1 have clearly demonstrated that early progestin administration is capable of producing grave disruptions in the three processes of differentiation in genetic female inbred mice. One remaining question concerns the physiological mechanisms underlying such defeminization. Previously, virilization resulting from progestin treatment was interpreted as being due to the fact that Progesterone and related synthetic progestins could be a link in the metabolic pathway from pregnenolone to testosterone. Thus, exogenously administered progestins would be converted to androgens either peripherally or centrally and, thereby, act to disrupt the respective processes of female differentiation (Revesz et al., 1963; Whalen, Peck, & Lo Piccolo, 1966). Although a plausible explanation, certain features of the syndrome appear to question it. First, if Progesterone or MPA acts via the androgenization route, then it would follow that a given dose of progestin would be less disruptive than a corresponding pure androgenic concentration. Experiment 1 disputes this hypothesis. Secondly, there is very little evidence that Progesterone's principal metabolic product in neural regions is an androgen. Rather, various studies have demonstrated that exogenously administered ^{3}H Progesterone is chiefly

converted into one of two pregnane derivatives in rats, namely, 5 α pregnane, 3,20 dione and 3 α hydroxy 5α pregnane, 3,20 dione (Karavolas et al., 1976).

Likewise, on the behavioral level, evidence is beginning to suggest that the functional role for Progesterone in the adult rodent may be due to its rapid conversion to these two metabolites. Whalen has demonstrated that 5 α dihydro-progesterone (5 α pregnane 3,20 dione) can be substituted for Progesterone when inducing lordosis in ovariectomized female mice, although this finding is genotype specific (Gorzalka & Whalen, 1976).

The purpose of this experiment is to ascertain whether this "early progestin" syndrome could be due to the reduction of Progesterone to 5 α reduced derivatives. In other words, is the neonatal action of Progesterone related to its eventual androgenic synthesis or can the syndrome be explained by postulating a unique role for progestins themselves?

Method

Subjects. All subjects were either C57BL/10J or C3H/HEJ females born to progenitors purchased from the Jackson Memorial Laboratories.

Procedure. Entire litters were randomly assigned to one of seven treatment groups as follows: a) 300 ug TP, b) 320 ug MPA, c) 500 ug P, d) 20 ug diethylstilbestrol (DES),

f) 500 ug 5 α Pregnane 3,20 dione, g) 500 ug 5 α Pregnane 3 β-ol-20-one. All steroids were first dissolved in a small quantity of acetone, resuspended in sesame oil, then heated to remove the volatile acetone. Each pup of the litter was injected within 24 hours after birth with a .03 cc solution. The results of these treatments in genetic males will be deferred until Experiment 4.

Females were weaned at day 28 and collectively housed until the initiation of daily vaginal smearing, which commenced on day 50. On approximately day 85, bilateral ovariectomies were performed and subjects were then allowed a one-week recovery period. Following this period, females were experimentally manipulated with regard to female and male sex behavior as described in Experiment 1. As in Experiment 1, strain differences did not appear so the data were pooled from both inbred strains.

Results

Experiment 1 demonstrated that two progestins, natural progesterone and medroxyprogesterone acetate were capable of disrupting the processes of female anatomical, gonadotrophin, and behavioral differentiation. Experiment 2 confirms the basic findings of Experiment 1 and extends them by examining the neonatal actions of two progestin metabolites.

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[Insert Figure 10 about here.]

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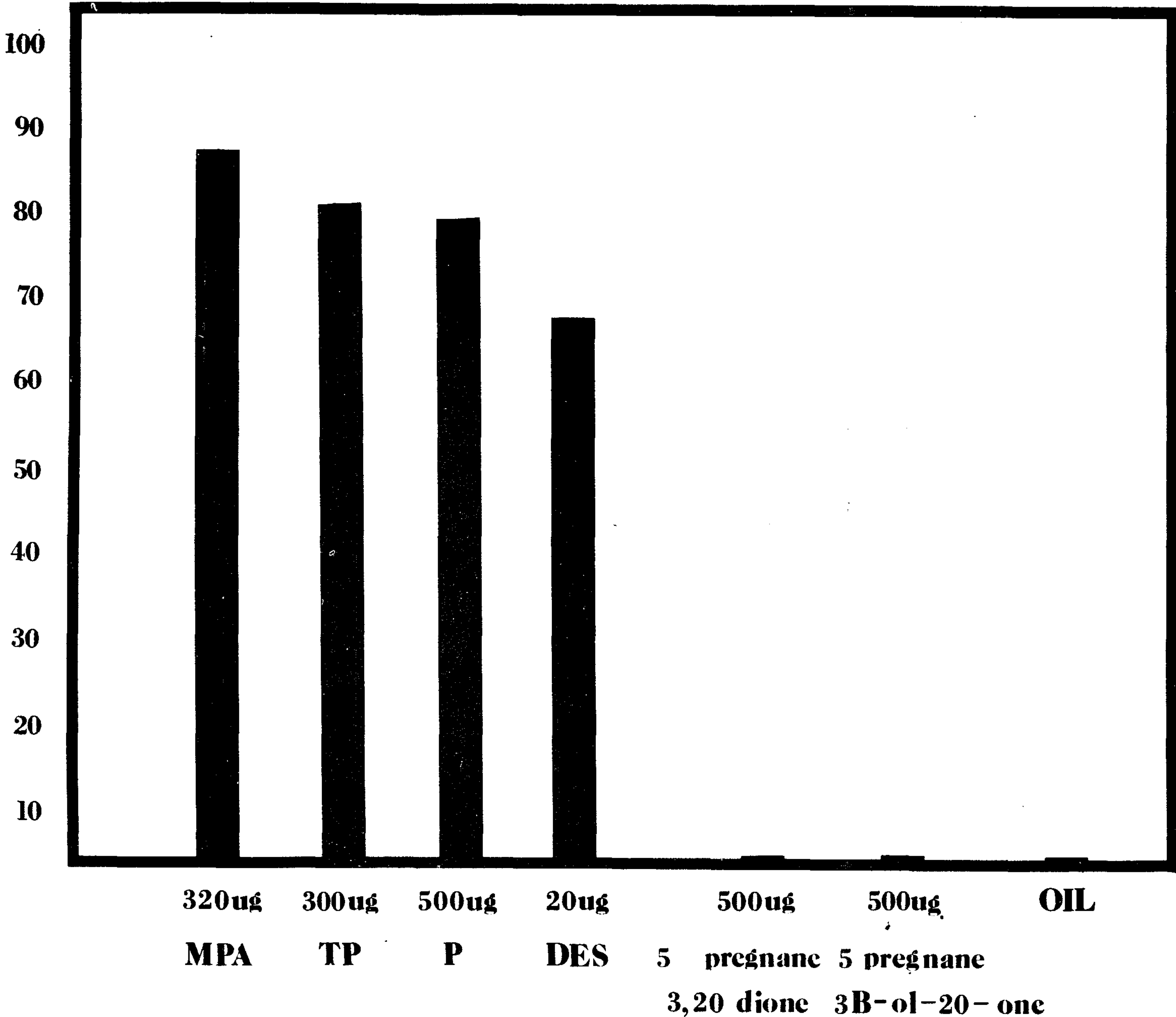

% SOMATIC VIRILIZATION
100
90
80
70
60
50
40
30
20
10
320ug MPA
300ug TP
500ug P
20ug DES
500ug 5 pregnane 3,20 dione
500ug 5 pregnane 3B-ol-20-one
OIL

Figure 10 illustrates the high percentages of clitoral hypertrophy in female mice who received either MPA, TP, or P within 24 hours of birth. In contrast, 20 ug DES produced a slightly lower anatomical virilization. Interestingly, the two progestin metabolites, 5 α pregnane 3,20 dione and 5 α pregnane 3 β -ol-20-one, even at dosages of 500 ug did not virilize the somatic structures of the genetic females. No observable morphological characteristics could distinguish these two metabolite groups from the oil vehicle group. An over-all analysis was significant (X^2 = 40.46, df = 6, $p < .001$).

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[Insert Figure 11 about here.]

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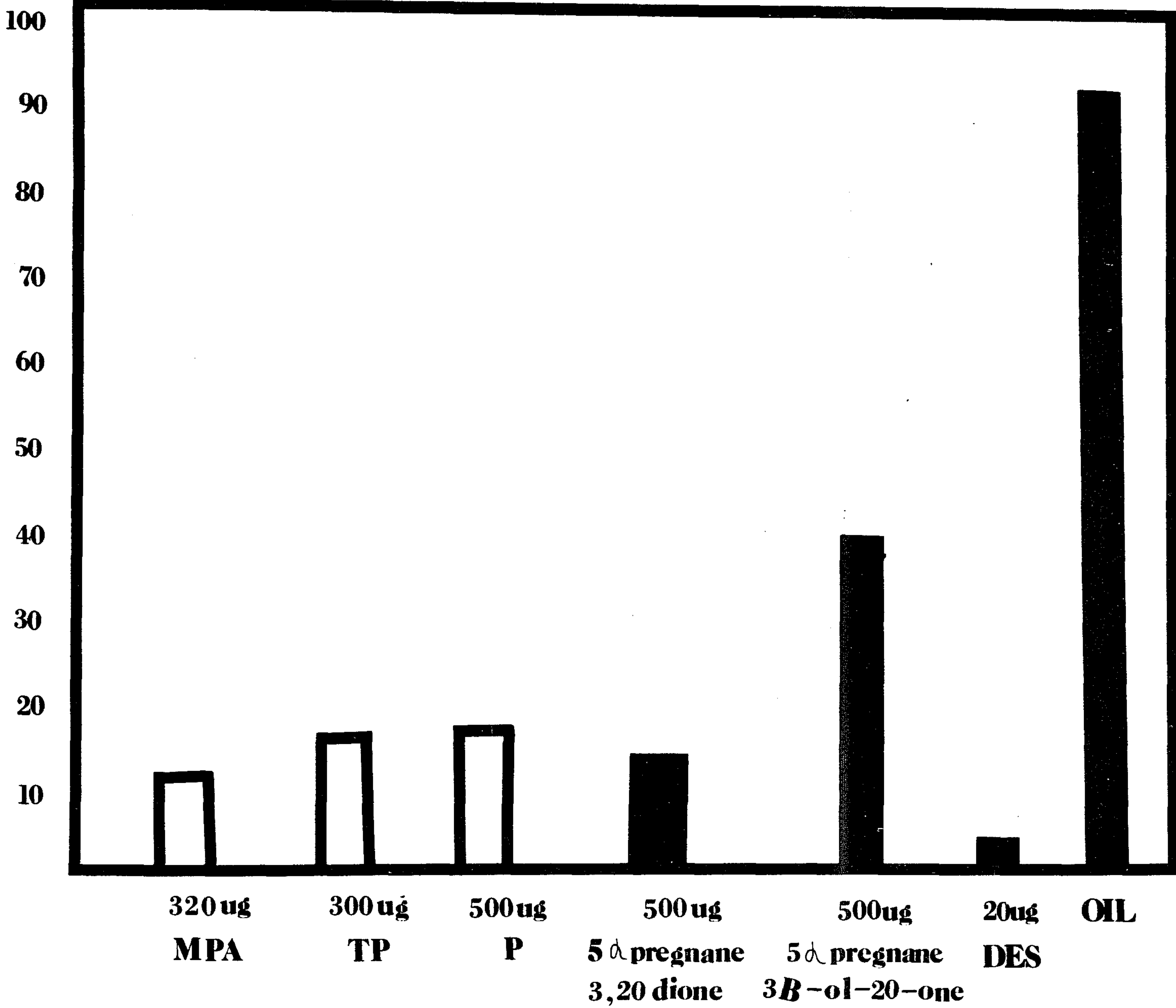
With regard to the processes of gonadotrophin and behavioral differentiation, the pattern changes. As shown in Figure 11, the two progestin metabolites did produce an anovulatory syndrome in adulthood. In all cases the smear was virtually always estrus and a state of constant vaginal cornification persisted into adulthood. All steroid groups differed from oil controls. Furthermore, the percentages of the various progestin groups were all approximately equal except for the 500 ug 5 α pregnane 3 β -ol-20-one group: MPA, 12%; TP, 15%; P, 16%; 5 α pregnane 3,20 dione, 12%; DES, 4%; and 5 α pregnane 3 β -ol-20-one, 40%. An analysis of variance with unequal sample sizes demonstrated a


% POSSIBLE CYCLES
100
90
80
70
60
50
40
30
20
10
320 ug
MPA
300 ug
TP
500 ug
P
500 ug
5α pregnane
3,20 dione
500ug
5α pregnane
3B-ol-20-one
20ug
DES
OIL

significant treatment effect ($F = 68.9$, $df = 8/58$, $p < .001$). None of the individual mean differences between any progestin groups reached an acceptable level of statistical significance.

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[Insert Figure 12 about here.]

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Likewise, all progestin neonatal treatments including the metabolite groups produced a strong refractoriness to exogenously administered EB + P. This can be detected in Figure 12, which depicts the lordotic quotients of the seven groups across four testing sessions. All steroids when administered within 24 hours of birth produced strong decrements compared to oil. Given that no significant change across the four trials for any of the groups was noted, a seven group one-factor analysis of variance was computed using the 4th trial. A significant treatment effect was noted ($F = 86.28$, $df = 6/58$, $p < .001$). Post hoc Tukey tests demonstrated that all groups were significantly depressed relative to controls ($p < .01$). Mean differences between MPA and 5α pregnane3,20 dione and P and 5 α pregnan 3,20 dione did reach an acceptable level of statistical significance ($p < .01$).

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[Insert Table 3 about here.]

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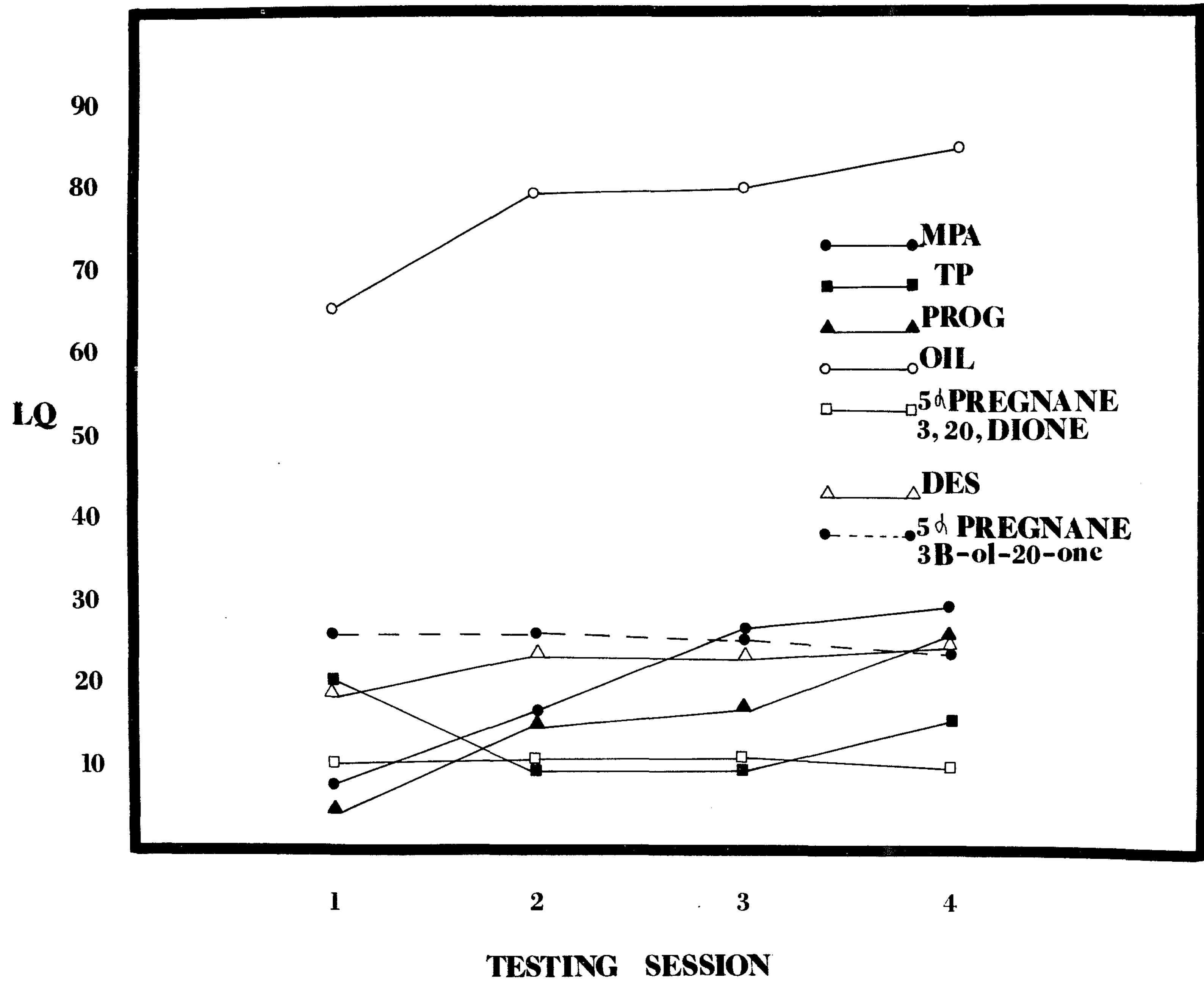

90
80
70
60
LQ
50
40
30
20
10
MPA
TP
PROG
OIL
5α PREGNANE
3, 20, DIONE
DES
5α PREGNANE
3B-ol-20-one
1
2
3
4
TESTING SESSION

Table 3

Masculine Behaviors in Genetic Females

		Concentration of Steroid					
Masculine Behavior	Oil	300 ug TP	320 ug MPA	500 ug P	500 ug 5α Pregnane 3,20 dione	500 ug 5α Pregnane 3 -ol-20-one	20 ug DES
Females							
N	10	11	9	10	8	8	9
% Mounting[a]	70	90	70	80	90	70	70
$\bar{X}$ Mount Freq.[b]	5	11	10	12	12	7	10
% Intromitting[a]	0	27	11	20	37.5	13	33
% Ejaculating[a]	0	0	0	0	0	0	0

[a]The percentage of total number of tests the animals within a group performed the behavior.

[b]Score represents the mean of four testing sessions.

Table 3 illustrates the mediocre levels of masculine behavior displayed by genetic females who had been exposed to one of various steroidal compounds on day 1 of life. One factor analysis of variance with unequal sample sizes was calculated. A significant treatment effect on mount frequency was noted ($F = 10.26$, $df = 6/58$, $p < .01$). Similarly, chi-square analysis of the percentage of trials in which subjects intromitted at least once was significant across the seven conditions ($X^2 = 15.98$, $df = 6$, $p < .05$). However, it should be pointed out that the absolute levels of intromission-like behavior were not impressive. For example, although oil females never displayed a proper intromission response, only in 11 out of 40 sessions (TP subjects), and in only 12 out of 32 sessions (5α pregnane 3,20 dione), did such a proper intromission response occur for experimental subjects. Furthermore, at no time did any female subject, experimental or control, ejaculate. Consistent with the defeminization data, 5α pregnane 3,20 dione produced a stronger neonatal effect than did 5α pregnane-3β-ol-20-one.

Discussion

In summary, early progestin or progestin metabolite administration produces a strong defeminization syndrome. This syndrome alters female type differentiation on anatomical, gonadotrophin, and behavioral parameters. This syndrome appears to be dose as well as time dependent. The assumed

complementary process of defeminization, that is masculinization, appeared to be enhanced relative to controls. However, the levels of masculine behavior in the various progestin groups did not approach or closely resemble the patterns characteristic of the genetic male. At this time it is difficult to speculate about this poor correlation between behavioral defeminization and behavioral masculinization. It is, of course, possible that more sensitive testing conditions or a more prolonged period of testosterone replacement therapy in adulthood could have minimized differences between progestin-treated females and normal genetic males. Secondly, it is possible that if progestin stimulation had begun earlier (for example, in prenatal life), a more complete masculinization syndrome could have developed. This possibility will be tested in Experiment 6. Lastly, it should be noted that one earlier mouse study has shown a similar nonparallelism between behavioral masculinization and behavioral defeminization (Edwards & Burge, 1971).

In addition, the results of Experiment 2 confirm and extend those of Experiment 1 by demonstrating that not only do progestins themselves disrupt the normal processes of female sex differentiation but progestin metabolites are as effective or in fact are more effective as disruptors with regard to two of the processes of differentiation. While testosterone propionate, progesterone, medroxyprogesterone acetate, and diethylstilbestrol were all effective in

initiating somatic virilization and clitoral hypertrophy on day 1 of life, two progestin metabolites, 5 α pregnane 3,20 dione and 5 α pregnane, 3 β -ol-20-one were both as ineffective as oil injections in inducing virilization. However, with regard to estrous cyclicity and responsiveness to exogenous estradiol and progesterone, neonatal treatment with 5 α pregnane 3,20 dione produced as great a defeminization syndrome as either TP, P, or MPA. 5 α pregnane 3 β -ol-20-one, however, was midway between oil and 5 α pregnane 3,20 dione in inducing defeminization. Thus, this is the first demonstration that progestins themselves, irrespective of their inherent androgenic potency, can defeminize a genetic female rodent. Similarly, this experiment raises the possibility that the actions of exogenously administered Progesterone may be due to its rapid metabolism to reduction products. If true, this case would provide another example of progestinic actions being influenced by the principal metabolites of Progesterone. Furthermore, the degree of masculinization in the metabolite groups paralleled those of the progestin groups but were much lower than levels previously reported for genetic males.

EXPERIMENT 3

The two preceding experiments have focused our attention on the role that progestins may exert in defeminizing the genetic female mouse. Recent evidence suggests, however, that progestins may function also as anti-androgenic compounds. Robson (1951) demonstrated that Progesterone will inhibit the action of exogenously administered testosterone propionate in orchidectomized male rats. Diamond and Young (1963) have investigated the masculinizing action of TP in both pregnant and nonpregnant guinea pigs and reported that the action of TP as a masculinizing agent was severely limited in the pregnant female. It was hypothesized that it assumed a "protective" function in the pregnant animal, which subsided within 3 days after parturition.

Progesterone may also act as an anti-androgenic agent on behavioral mechanisms. Erickson et al. (1967) have reported that Progesterone interferes with the elicitation of androgen-induced bowing-coo in ring doves. Progesterone has likewise been studied in relation to the early androgen syndrome in rats. Females treated with 1.25 mg of testosterone propionate at birth were anovulatory and sterile in adulthood. Females given the same amount of androgen followed by 50 ug of P were observed to be fertile in adulthood (Cagnoni et al., 1965). Kincl and Maqueo (1965)

similarly found that a single injection of P (3 mg) may function to block the effect of testosterone as a sterilizing agent in rat neonates.

This experiment will examine this possible anti-androgenic action of Progesterone in male mouse neonates. If Progesterone can exert such actions at this developmental period, then we may expect that a type of chemical castration can be induced in such animals. Furthermore, since medroxyprogesterone acetate has also been found to be anti-androgenic in rodents (Albin et al., 1973), the action of this compound will be studied as well.

Methods

Subjects. Male mouse neonates were offspring derived from litters described in Experiment 1.

Apparatus. All social behavioral testing was conducted in the home cages of the experimental subjects. Each polypropylene cage measured 13 x 8 x 5 inches.

Procedure. Litters of either C57BL/10J or C3H/HeJ pups were randomly assigned to one of ten conditions as follows: a) 320 ug MPA, b) 160 ug MPA, c) 80 ug MPA, d) 500 ug P, e) 250 ug P, f) 125 ug P, g) 50 ug P, h) oil. Each genetic male neonate was injected at the nape of the neck with one of the above concentrations within 24 hours after birth.

Progesterone and medroxyprogesterone acetate were injected in a sesame oil vehicle in a volume of .03 cc per injection. To prevent leakage, all injection sites were sealed with flexible colloidoin. Pups were weaned on day 28 and housed collectively in uni-sex groups with animals of the same age and treatment condition. Behavioral testing commenced on approximately day 75, at which time all subjects were singly housed. Testing in adulthood was divided into several components. One-half of the experimental males were first tested for male sexual vigor against lure estrus females. Males were tested twice weekly for two consecutive weeks at which time males were then tested for intra-specific aggression. Subjects were paired with submissive intruder C57/10J males of the same age. Aggression tests were conducted weekly for two consecutive weeks. The remaining other half of the subjects were tested in reverse order, that is, first they were exposed to the aggression interactions and then were tested for sexual vigor. Sexual vigor was assessed by pairing the subject with a behaviorally receptive estrus female. Latency to first mount, frequency of mounts, intromissions, and ejaculations were recorded on BRS counters and timers. Each session lasted 20 minutes following the first mount with pelvic thrusting by the male.

Intraspecific aggression was observed by pairing each subject with an intruder for a 15-minute period commencing

after the first attack. Latency to first attack, total frequency of attacks, and cumulative time spent attacking were recorded.

After both types of behavior were assessed in each subject, subjects were bilaterally orchidectomized and allowed a five-day recovery period. Subjects were then tested for two additional sessions for both sexual vigor and agonistic behavior. All subjects were then subjected to testosterone propionate replacement therapy. Each subject was injected S.C. with 500 ug of TP dissolved in .1 cc sesame oil for 24 consecutive days. Subjects were tested for male sexual behavior on days 6, 12, 18, 24, and for aggression on days 3, 9, 15, 21.

Approximately seven days after the last injection and test, subjects were injected with an estrogen-progesterone regimen found to be successful in inducing sexual receptivity in female mice (10 ug EB and 500 ug P) 48 and 6 hours, respectively, before testing. Males were exposed to such a hormone regimen and tested biweekly for two consecutive weeks. During such tests males were exposed to sexually vigorous males of our breeding colony and a measure of female sexual receptivity, the lordosis quotient, was recorded.

Results

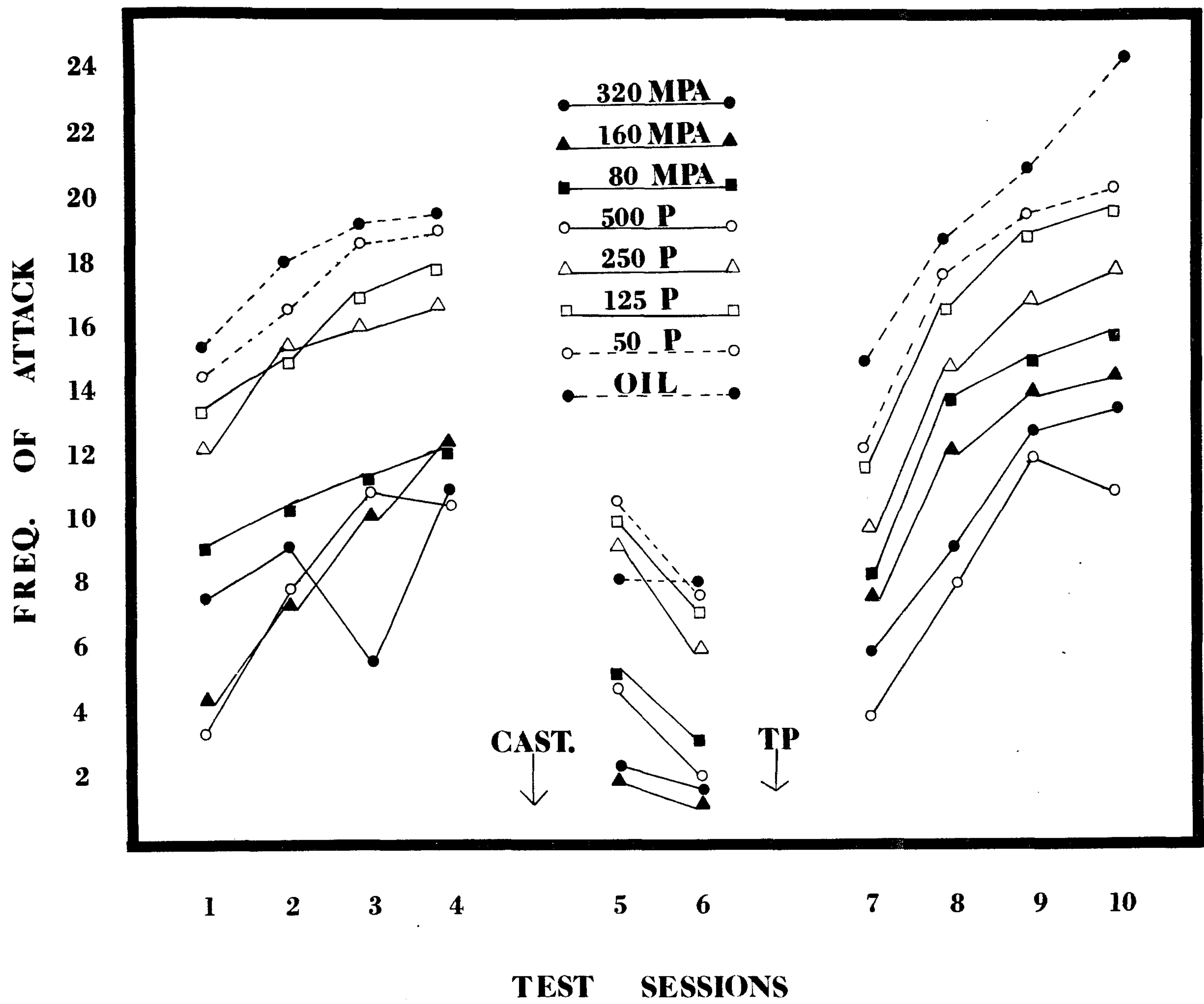
Aggression

Figure 13 describes the agonistic responses of genetic males treated neonatally with various steroid concentrations. The first four sessions were conducted prior to orchidectomy, sessions five and six were post-castration sessions while in sessions seven thru ten, subjects were tested during a prolonged period of Testosterone propionate replacement therapy. Pre-castration aggressiveness showed an over-all significant effect among the eight groups ($F = 9.32$, $df = 7/72$, $p < .01$) when fourth session scores were analyzed. Both MPA and P produced dose-dependent reductions in agonistic responding. Equivalent dosages of MPA produced significantly greater decrements than dosages P ($p < .01$).

Castration consistently produced sharp reductions in male aggressiveness across all treatment groups. The degree of decrement paralleled the aggressiveness observed prior to orchidectomy.

The responsiveness of males to adult androgenic stimulation was assessed during TP replacement therapy. A trend analysis was calculated according to Edwards (1972). A significant trial effect was noted ($F = 14.63$,

(Insert Figure 13 about here.)



FREQ. OF ATTACK
24
22
20
18
16
14
12
10
8
6
4
2
320 MPA
160 MPA
80 MPA
500 P
250 P
125 P
50 P
OIL
CAST.
TP
1
2
3
4
5
6
7
8
9
10
TEST SESSIONS

df= 3/72, $p<.01$) together with significant treatment effects ($F=12.95$, $df=3/72$, $p<.01$) and treatment by trial interaction ($F= 6.39$, $df= 7/72$, $p<.05$). Neonatal oil treated animals displayed the sharpest responsiveness to exogenous TP. Progestins showed a dose dependent responsiveness but on the fourth trial, all were significantly less responsive than oil injected subjects.

Male Sexual Behavior

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(Insert Table 4 about here.)

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Table 4 depicts the pre-castration sexual responses of genetic males exposed to one of several possible concentrations of progestins on day one of life. Typical measures of sexual arousal, percentage of subjects mounting and mean mount frequencies were not different among the eight treatment conditions. However, both the percentage of subjects intromitting in the four sessions and the percentage of subjects ejaculating did differ significantly among the groups ($X^2=49.57$, $df=7$, $p<.01$) and ($X^2=44.53$, $df=7$, $p<.01$). Furthermore, the effect appears to be highly dose-dependent in that the highest dosages of MPA and P produced the strongest deficits with the lower dosage groups being indistinguishable from

Table 4

Masculine Behavior in Genetic Males--Effects of Varying Concentrations of Progestins

Pre + Post-Castration Behavior	Concentration of Progestin							
	Oil	320 ug MPA	160 ug MPA	80 ug MPA	500 ug P	250 ug P	125 ug P	50 ug P
Masculine Behavior								
Males N	10	10	10	10	10	10	10	10
% Mounting[a]	100,3	90,25	90,30	100,20	90,10	80,30	80,30	90,20
$\overline{X}$ Mount Freq.[b]	12.6,5.1	13.2,6	9.5,5	11.6,6	13.21,7	12.19,6	13,6	12,15
% Total Intromitting[a]	95,20	62,15	75,20	87,15	50,20	90,20	90,15	95,20
% Total Ejaculating[a]	90,10	60,15	62,10	80,10	50,10	87,0	87,10	90,10

[a]First number refers to percentage of the total number of tests (from a possible 4) the animals within a group performed a behavior before castration; the second number refers to percentage of the total number of tests (from a possible 2) the animals within a group performed a behavior after castration.

[b]First score represents the mean of four pre-castration sessions; the second score represents the mean of two post-castration sessions.

controls.

Castration within ten days produced a dramatic reduction in sexual vigor and post castration levels did not differ as a function of neonatal treatment. Sexual vigor was also assessed when males were being exposed to a hormonal regimen of 500 ug Testosterone propionate.

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(Insert Table 5 about here.)

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As noted in pre-castration tests, typical measures of sexual arousal, for example, mount frequencies, do not differ across treatment conditions. However, the so called culminative aspects of sexual behavior did differ. Fourth trial percentages of intromissions (X^2=23.22, df=7, $p < .01$) and ejaculations (X^2= 19.26, df=7, $p < .01$) were significant. Similarly, a computation of the total possible occurrences of intromissions or ejaculations in the total of four trials, demonstrated that both intromission and ejaculation frequencies were not independent of neonatal treatment. (X^2= 62.2, df=7, $p < .001$) for intromission; (X^2=77.71, df=7, $p < .001$) nor for ejaculations.

Table 5

Masculine and Feminine Behavior in Genetic Males--
Progestin Effects

Masculine Behavior during TP Stimulation	Concentration of Progestin							
	Oil	320 ug MPA	160 ug MPA	80 ug MPA	500 ug P	250 ug P	125 ug P	50 ug P
Masculine Behavior								
Males N	10	10	10	10	10	10	10	10
% Total Mounting[a]	90	90	90	80	90	80	80	80
$\overline{X}$ Mount Freq.[d]	12.6	12.3	11.4	12.3	13.8	10.2	12.4	13.6
% Total Intromitting[a]	90	30	50	60	20	30	60	70
% Total Ejaculating[a]	80	10	30	40	10	40	60	70
Feminine Behavior								
RQ[a,b,d]	90	50	40	70	40	20	80	90
LQ[a,c,d]	10	50	30	10	60	20	20	10

[a]The percentage of the total number of tests the animals within a group performed the behavior.

[b]Number of active resistances/total freq. mounts x 100.

[c]Number of lordotic responses/total freq. mounts x 100.

[d]Frequency scores represent means for four sessions.

Feminine Behavior

Table 5 also depicts the mean lordotic quotients of castrated mice exposed to exogenous estradiol and progesterone. At least with the dosages of MPA and P sampled, over-all early progestin treatment did not significantly feminize the genetic male ($F=1.26$, $df=7/70$ $p < .10$). Yet a significant over-all effect would probably have been attained except for the high variability in several of the progestin groups. For example, in the high dosage MPA and P conditions, mean lordotic quotients were 50 and 60 respectively. It is of course also possible that a significant appearance of feminine sexual behaviors would have emerged if more than four testing sessions were employed.

Discussion

As in previous work reported from our laboratory, day one progestin treatment will result in strong deficits in sexual behavior among genetic males (Griffo et al., 1974). This was true in animals tested with testes in situ. Furthermore, plasma testosterone concentrations were depressed relative to controls. Therefore, it was plausible that the deficits in male sexual behavior were due to this depressed androgenic concentration. The present experiment, although confirming

the results of our earlier study with regard to pre-castration sexual vigor, demonstrated that progestin treated males were also refractory to exogenous adminstrations of high dosages of testosterone propionate after orchidectomy.

The mechanisms of such anti-androgenic action in the genetic male are at present obscure. It appears that several possibilities are tenable. One note of caution is in order before proceeding. The empirical support and background for each possibility is derived from work on the adult organism. Very limited biochemical data exists on the actions of progestins in the neonatal rodent.

The first possibility concerns the reported ability of progestins to compete for the limited capacity binding sites for Testosterone in the rat brain (Sar and Stumpf, 1973). Assuming that such action may exist in the neonatal mouse brain, we may hypothesize that neonatal progestins enter neural cells, block these androgenic binding sites and thus exert an irreversible demasculinization. This type of demasculinization would be similar to that typically produced by neonatal orchidectomy. Several issues concerning this hypothesis

require more extensive scrutiny. First, it is necessary to demonstrate such a binding competition effect in neonatal mice by progestins. Secondly, if we postulate that this is the necessary and sufficient condition for the anti-androgenic effects of progestin in the genetic male, then we must also postulate that this type of competitive inhibition in neural sites must be of long duration, extending with threshold strength at least to post-natal day four. Following from this, it would be plausible to suggest that the demasculinization syndrome induced by day one progestins could not be prevented nor reduced in magnitude by subsequent androgenic stimulation on day three of life since the progestins exogenously introduced on day one would still then be exerting competitive binding inhibition.

It is also possible that progestins alter masculine sex differentiation through a disruption of testicular-hypophyseal negative feedback. According to this hypothesis, progestins would interfere with the testicular secretion pattern of androgens whose absence could alter the processes of sex differentiation. If true, then introduction of exogenous testosterone on day three would restore this androgen deficiency and partially reverse demasculinization. Experiment 4 will examine this possibility.

EXPERIMENT 4

The preceding experiment has confirmed earlier work from our laboratory that progestins when administered neonatally may function as anti-androgenic compounds. This anti-androgenicity was true of both the natural compound, Progesterone, as well as the synthetic derivative, medroxyprogesterone acetate. These compounds, when administered neonatally, produced decrements in adult sexual vigor as well as agonistic behavior. Recall that Experiment 2 demonstrated that the behavioral action of progestins may have been due to their exclusive intraneural conversion via 5α reductase to two reduced products, 5α pregnane3,20 dione and 5α pregnane3B-ol-20-one. One key question concerns the possibility that the anti-androgenic effect of progestins, observed in Experiment 3, may have been due to that same metabolic conversion. In order hopefully to clarify this issue, male neonates were injected within 24 hours after birth with either progestins or progestin metabolites.

Method

<u>Subjects</u>. Male neonates, obtained from litters used in Experiment 2, were injected within 24 hours after birth with one of the following: (a) 320 ug MPA, (b) 500 ug P, (c) 500 ug 5α Pregnane3,20, dione, or (d) 500 ug

5 α Pregnan, 3B-ol-20-one, (e) 20 ug DES, (f) 300 ug TP, (g) oil, (h) 320 ug MPA-day 1 + 500 ug TP-day 2.

All steroids, dissolved in sesame seed oil, were injected in a volume of .03 cc per injection.

Apparatus. Same as used in Experiment 3.

Procedure. Behavioral testing procedures were identical to that conducted in Experiment 3 except that aggression tests were not conducted. In summary, subjects were first tested 4 times with testes in situ for sexual vigor. Orchidectomies and 2 post-operative additional tests for sexual vigor followed, and then all subjects were placed on a testosterone replacement therapy regimen and retested in four sessions. After a one-week period, males were injected with EB + P and assessed for female lordotic receptivity.

Results

Pre-castration behavior. Although mounting frequencies did not differ among the groups, the so-called culminative aspects of sexual behavior did. Percentage of intromitters as well as percentage of ejaculators was not independent of steroid structure: ($X^2 = 90.23$, df = 7, $p < .001$) and ($X^2 = 55.34$, df = 7, $p < .01$) for percentages of intromitters and ejaculators, respectively. Table 6 depicts this masculinization.

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[Insert Table 6 about here.]

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Table 6

Effect of Steroids on Masculine Behavior in Genetic Males

Pre, Post-Castration Behavior	Concentration of Steroid							
	Oil	300 ug TP	320 ug MPA	500 ug P	500 ug 5α Pregnane 3,20 dione	500 ug 5α Pregnane 3B-ol-20-one	20 ug DES	MPA Day 1 + TP Day 3
Masculine Behavior								
Males N	8	8	8	8	8	8	8	8
% Mounting[a]	90,40	90,30	100,30	90,20	100,30	100,40	70,30	90,30
$\bar{X}$ Mount. Freq.[b]	12,4	11,6	13,5	10,6	12,4	12,3	11,4	10,20
% Intromitting[a]	90,20	69,30	60,30	60,10	90,20	90,20	41,20	81,10
% Ejaculating[a]	90,10	60,20	50,30	50,10	90,10	90,10	31,10	81,10

[a]First number refers to percentage of the total number of tests (from a possible 4) the animals within a group performed a behavior before castration; the second number refers to percentage of the total number of tests (from a possible 2) the animals within a group performed a behavior after castration.

[b]First score represents the mean of 4 pre-castration sessions; second score represents the mean of two post-castration sessions.

After orchidectomy, all subjects in all groups displayed dramatic decreases in male sexual behavior from the 4th pre-operative trial to the 2nd post-operative trial. The difference score between these two trials was used as a raw score in a one-factor analysis of variance comparison. No significant treatment effects on these decreases was noted ($F = 0.29$, $df = 7/56$, $p > .50$).

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[Insert Table 7 about here.]

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<u>TP replacement and male sexual behavior</u>. Table 7 depicts the results of TP replacement therapy on male sexual behavior as a function of type of steroid administered neonatally. Consistent with the pre-castration data, mounting frequencies did not vary with neonatal treatment ($F = .25$, $df = 7/56$, $p > .50$). However, the culminative aspects of sexual behavior did. Since significant dramatic changes were not detected across the four trials, terminal levels of sexual vigor were employed by comparing each of the groups' performance on the 4th trial. A chi-square analysis yielded a significant effect using percentage of genetic males intromitting at least once in the test session ($X^2 = 25.22$, $df = 7$, $p < .01$). A similar effect was noted for percentage of ejaculators ($X^2 = 25.15$, $df = 7$, $p < .01$). There was almost a one-to-one correspondence between ability to intromit and ability to ejaculate. While

Table 7

Effect of Progestins on Masculine and Feminine Behavior in Genetic Males

	Concentration of Progestin							
Masculine Behavior during TP Stimulation	Oil	300 ug TP	320 ug MPA	500 ug P	5α Pregnane 3,20 dione	500 ug 5α Pregnane 3B-ol-20-one	20 ug DES	MPA-Day 1 TP-Day 3
Males N	8	8	8	8	8	8	8	8
% Total Mounting	100	80	90	90	100	100	63	90
$\bar{X}$ Mount Freq.	13	10	13	12	10	12	8	13
% Total Intromitting	90	60	20	30	90	90	40	80
% Total Ejaculating	90	40	20	20	80	70	20	70
Feminine Behavior during EB + P Stimulation								
LQ[b,c]	10	20	40	60	0	10	10	10

[a]The percentage of the total number of tests the animals within a group performed a behavior.

[b]Score represents the mean of four testing sessions.

[c]Number of lordotic responses/total freq. mounts x 100--means of four sessions.

progestins themselves produced these disruptions in sexual vigor, no such decrements were observed in progestin metabolite groups.

Female lordotic receptivity to genetic males. Table 7 likewise depicts the level of lordotic responding in genetic males exposed to various neonatal progestin treatments. As was true with other behavioral parameters, no important changes in responding transpired across the four testing sessions. Thus fourth trial scores were utilized as the basis for comparison. A significant treatment effect was noted ($F = 123.5$, $df = 7/56$, $p < .001$). While genetic males exposed to either of the two progestin metabolites were almost never observed to display high quality lordotic postures, males exposed to either MPA or P did exhibit significantly higher frequencies of lordotic responding than oil controls ($p < .01$). On the other hand, neither TP alone on day 1 or a combined treatment of MPA on day 1 and TP on day 3 nor DES alone on day 1 were capable of feminizing the genetic male. The two metabolite groups produced significantly less feminization than either TP, MPA, or P.

Discussion

The results of this experiment clearly demonstrate that while progestins themselves may act as possible anti-androgenic compounds, progestin metabolites known to possess strong affinity for neural tissue are not anti-androgenic

at least with respect to sexual behavioral processes.

By examining masculine sexual vigor subsequent to castration and TP replacement, it was shown that mounting frequencies and, therefore, arousal mechanisms were not altered by any steroid treatment. However, percentages of animals intromitting and ejaculating were diminished with several steroid treatments administered on day 1. While neonatally oil-treated animals always achieved intromission and ejaculated, MPA and P treated males did so on only 25% of the occasions. TP-treated males were higher at 37.5%. When MPA treatment on day 1 was combined with TP treatment on day 3, an almost complete restoration of masculine behavior was achieved. Only 12% of the DES males were able to achieve an intromission and ejaculation on the fourth testing trial.

The data, therefore, suggest that the anti-androgenic actions of progestins in the neonatal genetic male mouse are not due to a neural reduction of the parent compound to metabolites. Unfortunately, this experiment does not offer a greatly enhanced understanding of the possible mechanisms for the anti-androgenic effects of progestins.

Since both TP and DES treatments were unable to significantly feminize above control oil levels, it is likely that gonadotrophin negative feedback signal inhibition is not responsible for the heightened feminization in the progestin groups. However, the lack of demasculinization of

the combined day 1-MPA and day 3-TP groups suggests that a type of inhibition of or saturation of testosterone neural binding sites is responsible for the anti-androgenic effects. This interpretation is supported by the fact that Sar and Stumpf (1973b) have demonstrated that progesterone pretreatment will reduce the uptake and accumulation of ^{3}H testosterone in specific neural sites of the rat. This possibility will be assessed in Experiment 9.

EXPERIMENT 5

The four preceding experiments have explored the effects of neonatal progestins on anatomical, gonadotrophin, and behavioral differentiation in both genetic male and female mice. This experiment will explore the effects of neonatal progestin and androgen administration on mouse urinary cues. Recent research has indicated that male mice emit an androgen dependent urinary cue which serves to trigger aggression in trained male mouse fighters (Lee, 1976). Previous work from our laboratory has demonstrated that neonatal androgenization (500 ug per genetic female) alters their adult urinary output so as to provide an effective aggression-promoting cue to the trained fighter (Lee & Griffo, 1973). Vandenberg and co-workers have likewise found that neonatal androgenization modifies the urine of genetic female mice. Such urine was found to induce the previously reported puberty acceleration phenomenon. In this experiment, the Lee and Griffo (1973) data will be replicated and a possible mechanism for the effect will be presented. As the first two experiments have demonstrated, neonatal progestin or androgen treatment disrupted the estrus cycles, often resulting in either a state of constant vaginal cornification (constant E) or a state of constant diestrus. In this experiment, the urine from subjects in these two conditions

will be compared.

Subjects

Pups were born to male and female C57BL/10J progenitors purchased from the Jackson Memorial Laboratories. Pups were injected with one of the following steroid concentrations within 24 hours after birth: a) 300 ug TP, b) 320 ug MPA, c) 500 ug P, d) oil. A random assignment determined the particular steroid condition for each subject. Pups were weaned on day 28 and housed collectively with animals of the same sex and treatment condition. On day 50, cyclicity tests were initiated and continued until a stable pattern emerged (generally 20 days). Females exhibiting either a constant estrus or a constant diestrus pattern were chosen and daily urine samples were taken at 10 a.m. Urine from subjects of a particular condition was pooled and stored in a freezer at -20° C for approximately 7 days.

Aggression-Testing Paradigm

SJL/J male mice, purchased from the Jackson Memorial Laboratory at six weeks, were trained as fighters at nine weeks of age. Training followed the procedures described by Scott (1966). Fighters were randomly assigned to one of nine conditions as follows: 1) MPA-estrus, 2) MPA-diestrus, 3) TP-estrus, 4) TP-diestrus, 5) P-estrus, 6) P-diestrus, 7) oil at estrus, 8) oil at diestrus, 9) ovariectomy. One-half of the fighters were first paired against a castrate

male C57/10J colony mouse coated with .30 cc of distilled water. The remaining fighters were paired against a castrate C57 male coated with .30 cc of urine of their respective treatment condition as described above. In the second test session, two days after the first interaction, the pairings were reversed. During these sessions latency to first attack, total frequency of attacks, and cumulative attack time were recorded on BRS counters and timers.

Results

As described in Experiments 1 and 2, early progestin administration resulted in dramatic disruptions of the estrus cycle. In certain cases, a constant estrus pattern was found; in others, a constant diestrus pattern was displayed. The reactions of fighters exposed to urine-coated castrates were compared to their reaction toward distilled H_2O-coated castrates. In each case a difference score as well as a score representing the percentage of control H_2O reaction was computed. These difference scores were then used as the dependent measure in a one-factor nine group analysis of variance. Such an analysis yielded an over-all treatment effect ($F = 15.22$, $df = 8/54$, $p < .001$). Tukey *a posteriori* tests demonstrated ($p < .01$) that when either MPA, TP, or P neonatal treatments produced constantly estrus females, their urine was more aggression-promoting than the urine from females treated identically except for the fact

that they were in constant diestrus. The urine from control females, who were injected on day 1 with oil, was no more aggression-promoting when collected during estrus than during diestrus. Furthermore, the three progestin estrus conditions were all as equally aggression promoting.

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[Insert Figure 14 about here.]

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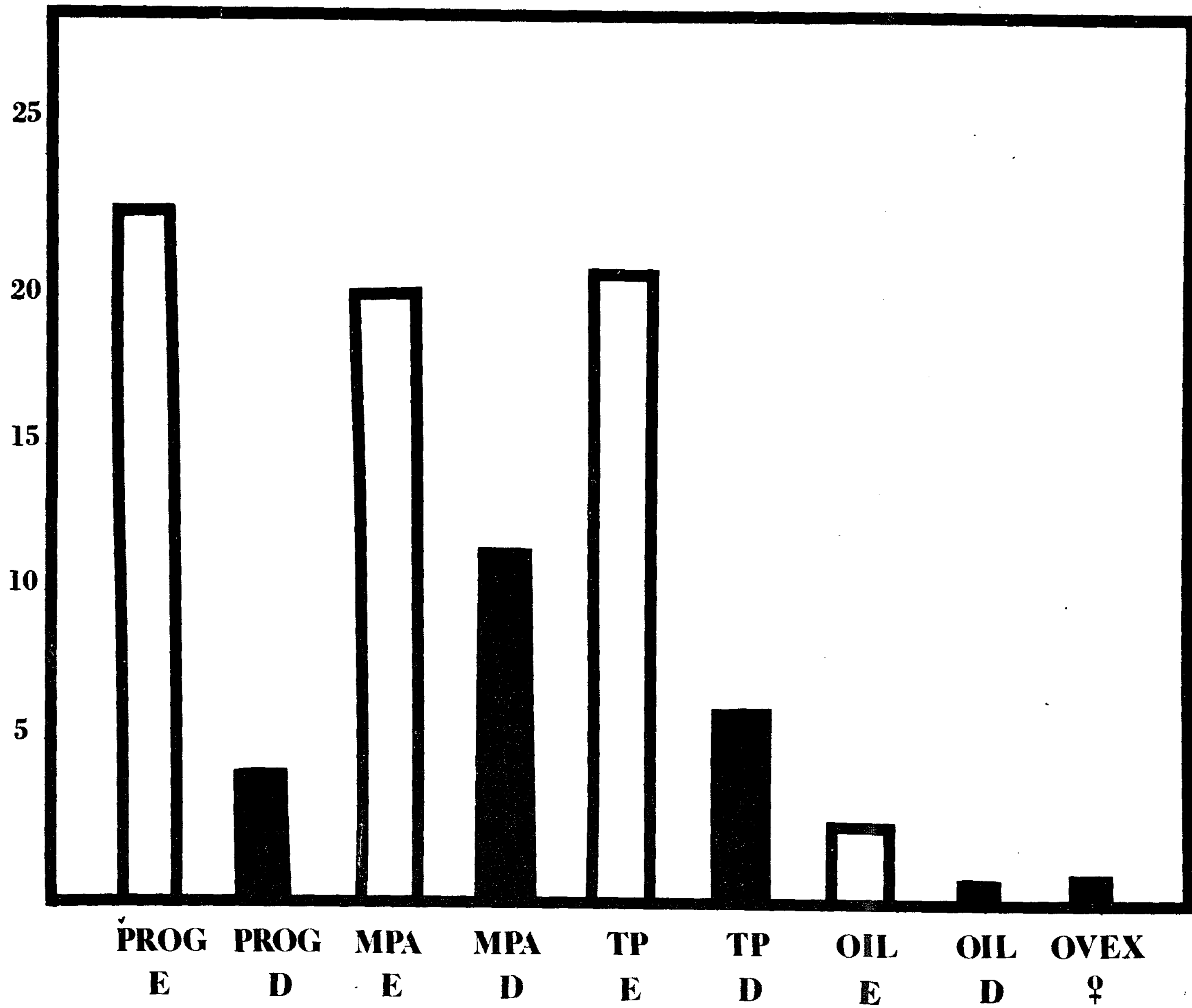
Discussion

Previous studies have reported that neonatal androgenization of genetic female mice alters some as yet unidentified component or components of their urine. Lee and Griffo (1973) have shown that the urine of the androgenized female is capable of promoting intense aggressive displays. Androgenized females with intact ovaries were themselves perceived as suitable targets for aggression by male mouse fighters. Dominic (1969) reported that the urine of early androgenized females was able to induce the pregnancy block phenomenon. Vandenberg (personal communication) has found that the urine of early androgenized females was able to accelerate the processes of puberty in female mice. This acceleration effect was as strong as that produced by adult intact male urine.

All of the above studies failed to establish a mechanism for these effects. If we examine the reproductive physiology of such androgenized females, we notice that


FREQ. ATTACK
DIFF. FROM H_2O CONTROLS
25
20
15
10
5
PROG E
PROG D
MPA E
MPA D
TP E
TP D
OIL E
OIL D
OVEX ♀

estrogen secretion from the ovary is at a steady-state level yet progesterone is not secreted. Furthermore, in animals that exhibit a pattern of constant vaginal cornification (continual estrus smear), we may assume that estrogen levels are higher than in animals exhibiting a constant diestrus pattern (Turner & Bagnara, 1971).

The results of Experiment 5 demonstrate that this differential estrogen secretion pattern may be a crucial variable in determining the effectiveness of the urine of androgenized females. When any of the three steroids (MPA, TP, P) produced an anovulatory persistent vaginal cornification syndrome, a concomitant urinary modification ensued. This modification resulted in an aggression-promoting property in the urine. However, when a state of persistent diestrus was induced, levels of aggression promotion did not differ from controls, nor did they differ from levels induced by urine derived from normal ovariectomized females.

We may argue that it is just a property of estrus urine in general which is responsible for such an effect. However, data from this experiment would directly contradict such a proposition. The urine of neonatally oil-treated females was no more aggression-promoting at estrus than at diestrus.

Therefore, the data suggest that some urinary component associated with a constant secretion of estrogen by the ovary of the androgenized female is responsible for the heightened

levels of aggression promotion. It is also possible that some other estrogen sensitive peripheral target organ like the clitoral gland may be the source of these aggression promoting cues. The exact chemical structure determination remains a task for future researchers. Furthermore, these data may lead one to hypothesize that the other pheromonally related androgenization effects may share a similar mechanism.

EXPERIMENT 6

The results of the first five experiments have demonstrated that neonatal progestin administration will disrupt the processes of sex differentiation in mice of both sexes. Anatomical, gonadotrophin and behavioral indices as well as urinary cue output were altered. However, with regard to masculine behavior in genetic females, neonatal progestin does not appear to completely reverse sexually dimorphic behavior. For example, it was found in Experiments 1 and 2 that ejaculatory tendencies were not increased in progestin-treated females despite the fact that somatic virilization was extensive. In fact, none of the females, experimental or control, were ever observed to ejaculate. The purpose of this experiment is to assess the role that prenatal administrations of progestins will have on the processes of sex differentiation. It is possible, for example, that the development of masculine behavior potential depends upon prenatal hormonal stimulation.

This view is supported by the fact that Ward (1969) demonstrated that prenatal administrations of testosterone propionate from days 13-19 of pregnancy masculinizes and defeminizes female rat fetuses. On the other hand, Whalen, Peck, and Lo Piccolo (1966) administered 17 alpha 19 Nortestosterone for five days during the last quarter of

gestation and reported genital virilization but normal female sexual behavior.

Method

Subjects. C57BL/10J subjects were purchased at 8 weeks of age and mated under laboratory conditions. The day of conception was noted by examination of the vaginal smear.

Apparatus. Identical to that described in Experiment 3.

Procedure. Pregnant females were randomly assigned to one of 9 treatment conditions as follows: (a) 320 ug MPA-prenatal, (b) 300 ug P-prenatal, (c) oil-prenatal, (d) 320 ug MPA-neonatal, (e) 300 ug P-neonatal, (f) oil-neonatal, (g) 320 ug MPA-pre + neonatal, (h) 300 ug P-pre + neonatal, (i) oil-pre + neonatal. Depending upon the condition as described above, pregnant females were either injected intramuscularly on days 14 to 17 of gestation (conditions a, b, c) or were injected on days 14 to 17 of gestation and their female offspring injected on day 1 of neonatal life (conditions g, h, i) or their female pups alone were injected on day 1 of neonatal life (conditions d, e, f).

The sequence of anatomical, gonadotrophic and behavioral assessments were identical with those employed in Experiment 1 with virilization noted, estrous cyclicity analyzed over a 30-day period, ovariectomies performed, lordotic responding noted after EB + P stimulation, and lastly

masculine behaviors recorded after a period of TP therapy.

Results

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[Insert Table 8 about here.]

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Anatomical differentiation. Table 8 depicts the percentage of virilized females as a function of time of exposure to one of two progestins. Prenatal administrations of either MPA or P were sufficient to virilize 100% and 86% of the respective genetic females. Virilization was extensive with clitoral hypertrophy being the major feature since scrotal folds were not observed. Internal reproductive structures were also extensively masculinized. Seminal vesicle sproutings were observed in each subject but testicular differentiation did not develop. Neonatal treatments alone altered the external genitalia, but the percentages of animals displaying virilization was slightly smaller. However, internal reproductive structures were indistinguishable from oil females. An over-all chi-square analysis revealed no significant independence between time or type of steroid administration and the presence or absence of virilization ($X^2 = 37.83$, df = 8, $p < .01$).

Gonadotrophin assessments. As in Experiment 1, estrous cyclicity was used to indirectly infer the gonadotrophin secretion pattern. Table 8 also depicts the percentage of

Table 8

Effects of Prenatal Progestins in Genetic Females

Feminine Characteristics	Days of Treatment								
	MPA Neo-natal	P Neo-natal	Oil Neo-natal	MPA Pre-natal	P Pre-natal	Oil Pre-natal	MPA Pre + Post	P Pre + Post	Oil Pre + Post
N	7	7	7	7	7	7	7	7	7
% Virilization	85.71	71.43	0	100	85.71	0	71.42	71.42	0
% Possible Cycles	11.57	9.29	82.86	80	85.71	85.71	9.29	9.12	82.86
4th Trial, LQ	40	30	80	60	60	85	45	30	85
Masculine Behavior									
Mount Freq.	5.88	3.38	6.88	8.88	9.75	4.63	8.63	10.88	3.13
% Intromissions (% out of (4 Interactions)	14.29	7.14	10.71	71.43	67.86	3.57	85.71	68.75	7.14
% Ejaculations (% out of 4 interactions)	3.13	6.25	0	56.25	56.25	0	71.88	62.50	0

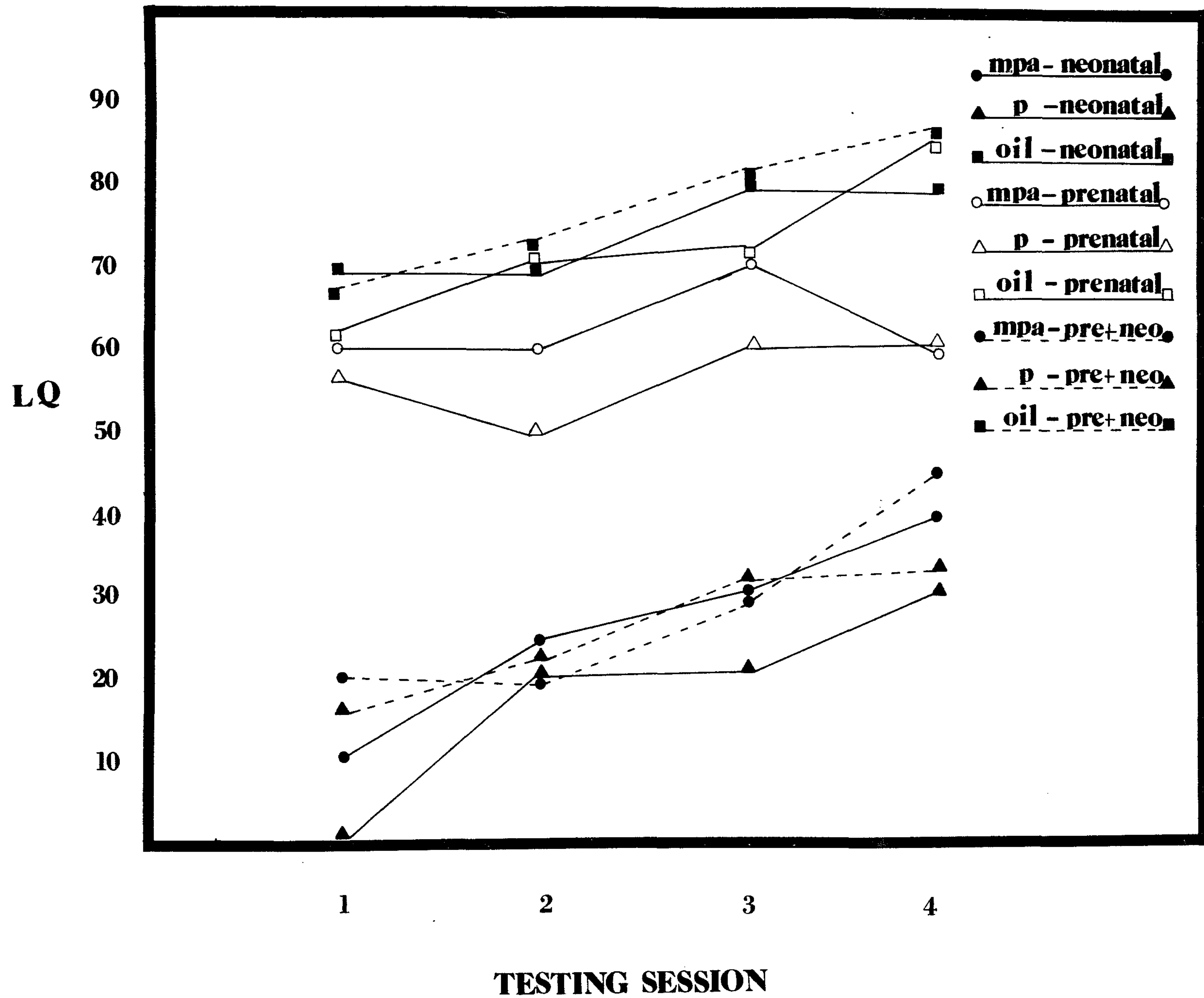
Scores represent means.

possible cycles in a 30-day period as a function of type of progestin and time of exposure. A significant effect was obtained using a one-factor analysis of variance ($F = 75.42$, $df = 8/54$, $p < .001$). Tukey comparisons revealed that prenatal administrations were not capable of altering or disrupting estrous cyclicity, yet both progestins when administered neonatally caused very significant disruptions. Neonatal and prenatal treatments together did not produce any greater degree of disruption than neonatal treatment alone.

The type of estrous cyclicity disruption was similar to that observed in Experiments 1 and 2. The majority of animals in the neonatal and neonatal + prenatal group exhibited patterns of constant vaginal cornification. Uterine hypertrophy was also markedly increased.

[Insert Figure 15 about here.]

<u>Female behavioral differentiation</u>. Behavioral responsiveness to exogenous estradiol and progesterone was assessed on four weekly sessions. Although performance levels in all groups did increase over the 4 trials, such increases did not reach an acceptable level of statistical significance. Therefore, fourth trial LQ's were chosen as the dependent measure in a one-factor analysis of variance. A significant treatment effect was noted ($F = 21.16$, $df = 8/48$, $p < .01$). Individual Tukey comparisons demonstrated that oil neonatally was less disruptive of lordotic receptivity than either neonatal MPA or P or prenatal + neonatal MPA or P.



mpa - neonatal
p - neonatal
oil - neonatal
mpa - prenatal
p - prenatal
oil - prenatal
mpa - pre+neo
p - pre+neo
oil - pre+neo
LQ
90
80
70
60
50
40
30
20
10
1
2
3
4
TESTING SESSION

Neonatal progestin treatments of either MPA or P were more disruptive of female sex behavior than any oil treatment ($p < .01$). Furthermore, neonatal progestin treatments of either MPA or P were more defeminizing than prenatal treatments ($p < .05$). Yet prenatal progestin therapy did produce intermediate levels of behavioral defeminization since both types of prenatal progestin treatment were more disruptive of lordotic receptivity as compared to any of the three oil treatments ($p < .05$). Combined prenatal and neonatal progestin treatments did not result in a greater degree of behavioral defeminization than did neonatal treatment alone.

Masculine behavioral differentiation. Masculine type behaviors were assessed in biweekly testing sessions when each subject was being primed with the male sex hormone, testosterone propionate. As in the preceding segment on lordotic receptivity, no significant trial by trial increase was noted either in percentage of mounters, mount frequency, percentage of intromitters, or percentage of ejaculators. This was largely due to the extensive subject variability in performance over the four trials. Therefore, 4th trial mount frequencies and percentage of 4 possible intromissions and percentage of 4 possible ejaculations were used as dependent measures. All three measures were statistically analyzed by means of an equal sample analysis of variance. Mount frequencies were significantly different

across the nine treatment groups ($F = 24.3$, $df = 8/63$, $p < .01$). Individual Tukey comparisons were made. Prenatal progestin treatment of either type significantly increased 4th trial mount frequencies as compared to any of the three oil groups or either of the two neonatal progestin groups ($p < .01$). Similarly combined prenatal and neonatal progestin treatments were equally effective in masculinizing the genetic female as prenatal treatments alone were. Neonatal progestins in fact were as ineffective as masculinizers as were any of the three oil treatments.

The so-called culminative aspects of male sexual behavior also followed a similar pattern. Percentage of intromissions in four possible sessions was assessed for each subject. An over-all treatment effect was noted across the nine conditions ($F = 34.52$, $df = 8/63$, $p < .01$). Neonatal treatment alone did not increase the percentage of possible intromitters in the four testing sessions compared to oil treatment, yet prenatal progestin treatment dramatically raised the probability of intromission in the genetic female ($p < .01$). Furthermore, combined prenatal and neonatal treatment did not produce an additive effect.

Percentage of each subject displaying a complete ejaculatory response in the four possible trials was computed. A significant treatment effect was noted ($F = 4.70$, $df = 8/63$, $p < .01$). Furthermore, Tukey comparisons yielded the same pattern as that presented for percentage of possible

intromitters. Neonatal treatment alone produced essentially no ejaculatory responses in genetic females, yet 56% of prenatally progestin-treated females did ejaculate. Prenatal plus neonatal treatment did not produce an additive effect.

Discussion

Several conclusions have emerged from the results of the previous experiment. While prenatal progestin treatment did not produce a substantially greater degree of virilization than respective day 1 treatments, internal reproductive structures of the prenatal groups were dramatically masculinized. In such females, seminal vesicle sproutings and male-like preprutial glands were noted. This masculinization pattern was sufficient to permit complete ejaculatory responses and seminal fluid discharge in the genetic female. Although seminal fluid could be detected, no sperm could be detected because such animals lacked testicular structures. No doubt this anatomical virilization of internal reproductive structures parallels the behavioral masculinization of ejaculatory potentials in prenatal groups but not neonatal groups. Highly correlated with this enhanced ejaculatory capacity is the increase in percentage of possible intromissions as a function of progestin prenatal treatment. In fact, mount frequencies followed a similar pattern.

These high levels of masculine behaviors in genetic females treated prenatally with progestins must be compared

with the opposing defeminization produced by either MPA or P. It appears that defeminization was not absolutely accomplished during the prenatal period but, rather, extended into neonatal life. This type of temporal sensitivity was very similar to that observed for gonadotrophin differentiation yet markedly different from that of the so-called masculinization process.

Mechanisms responsible for these differential temporal sensitivities are obscure at present. It is, of course, possible that these particular types of patterns are only representative of the particular testing and dosage conditions employed in this experiment. A remote possibility is that a placental barrier exists which restricts the ability of these progestins to reach the fetus or, more importantly, its neural tissue. Of course, such an interpretation must assume that behavioral masculinization in the prenatal progestin groups requires a significantly smaller steroid threshold concentration than does behavioral or gonadotrophin defeminization. The next experiment is designed to extend our knowledge of progestins by estimating their respective levels in fetus and mother after an intravenous maternal injection of tritiated progesterone.

EXPERIMENT 7

The results of the previous experiment have focused on several interesting aspects of this early progestin syndrome. Defeminization of gonadotrophin and behavioral levels was maximally induced by early neonatal exposure to progestins. Prenatal exposure to progestins induced intermediate levels of behavioral defeminization with no evidence of cyclicity disruption. Furthermore, the degree of behavioral and gonadotrophin disruption was similar for neonatal versus pre- and postnatal groups. The pattern of somatic virilization, however, was unique. Prenatal exposure to progestins induced the greatest degree of peripheral virilization with combined pre- + neonatal exposure producing the greatest qualitative degree of clitoral hypertrophy in the genetic female.

Several interpretations have been advanced for this differential sensitivity with developmental stages. The purpose of this experiment is to assess the validity of one of these interpretations. The lack of behavioral and gonadotrophin defeminization in prenatal groups could have been due to the relative inability of progestins to cross the placental barrier. The fact that peripheral virilization was induced by prenatal progestins appears to contradict this possibility. However, it is of course possible

that prenatal progestins could have crossed the placental barrier reaching and thereby altering the external genitalia of the fetus but were prevented from entering fetal neural tissue. This interpretation is consistent with the absence of gonadotrophin and behavioral defeminization reported in Experiment 6.

In this experiment, the levels of tritiated progesterone will be monitored in fetal tissues after maternal intravenous injections. The experiment has been designed to examine two questions. First, do high levels of exogenous progesterone label cross the placental barrier? If so, does the steroid enter fetal neural tissues with high affinity?

Method

Subjects. Female C57BL/10J mice were mated under laboratory conditions. Pregnancies were noted and recorded by daily vaginal smear determinations or the presence of a copulatory plug.

Procedure. Steroid preparation and administration: Between days 17-19 of gestation female pregnant mice were intravenously injected with 12.5 uCi [1,2 - ^{3}H] progesterone (specific activity 48 Ci/mM) purchased from New England Nuclear Corporation. The steroid was prepared for injection by boiling off the benzene and ethanol solvents and redissolving the residue in 20% ethanol. Thin-layer

chromatography revealed that at least 90% of the radioactivity was associated with a single peak whose R_F corresponded to the parent progesterone. Nuclear Chicago Salubilizer (NCS) was purchased from Amersham/Searle.

Isolation of samples: Each subject was sacrificed either 5, 15, 30, 45, 60, 75, 120, 240, or 480 minutes after injection by cervical dislocation. The ventral muscle wall was immediately opened; fetuses were then dissected from the uterine wall. A maternal blood sample was taken using a heparinized syringe. Fetal torsos were separated from fetal brains and fetal blood was pooled. Maternal neural tissues were also randomly sampled.

Uptake analysis in vivo: All samples were placed in preweighed scintillation vials and weighed. NCS was pipetted into each vial in the proportion of six times the wet weight of the tissue or plasma sample. Vials were transferred to a 37° C incubator and samples were digested for a period of 10 days. After tissue digestion, 15 ml scintillation solution (15 ml Bray's solution, Robinson & Karavolas, 1973) was added, the vials were recapped, shaken and returned to the 37° incubator until after the cessation of chemofluorescence (approximately 10 days). All samples were counted in a Packard Tri-Carb liquid scintillator counter. The counter was set to read the full tritium range and to accumulate sufficient counts to achieve a 95% confidence interval $\pm$ 3% of the mean. All results are

reported as counts per min/mg wet weight (cpm/mg) or as the ratio of cpm/mg of two samples. Each data point represents the mean of three separate determinations.

Results

Accumulation of ^{3}H progesterone was observed across the placental membranes after intravenous maternal injections. Levels of plasma fetal radioactivity were negligible at five and fifteen minutes after the single i.v. injection but dramatically rose 345X by 30 minutes. Similarly, maternal plasma levels rose significantly by 30 minutes. At all time intervals, maternal plasma levels of radioactivity were higher than fetal plasma levels. Between 75 and 120 minutes, plasma radioactivity abruptly dropped in both mother and fetuses. Levels of radioactivity were negligible at 4 hours and 8 hours post-injection in plasma samples.

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[Insert Figure 16 about here.]

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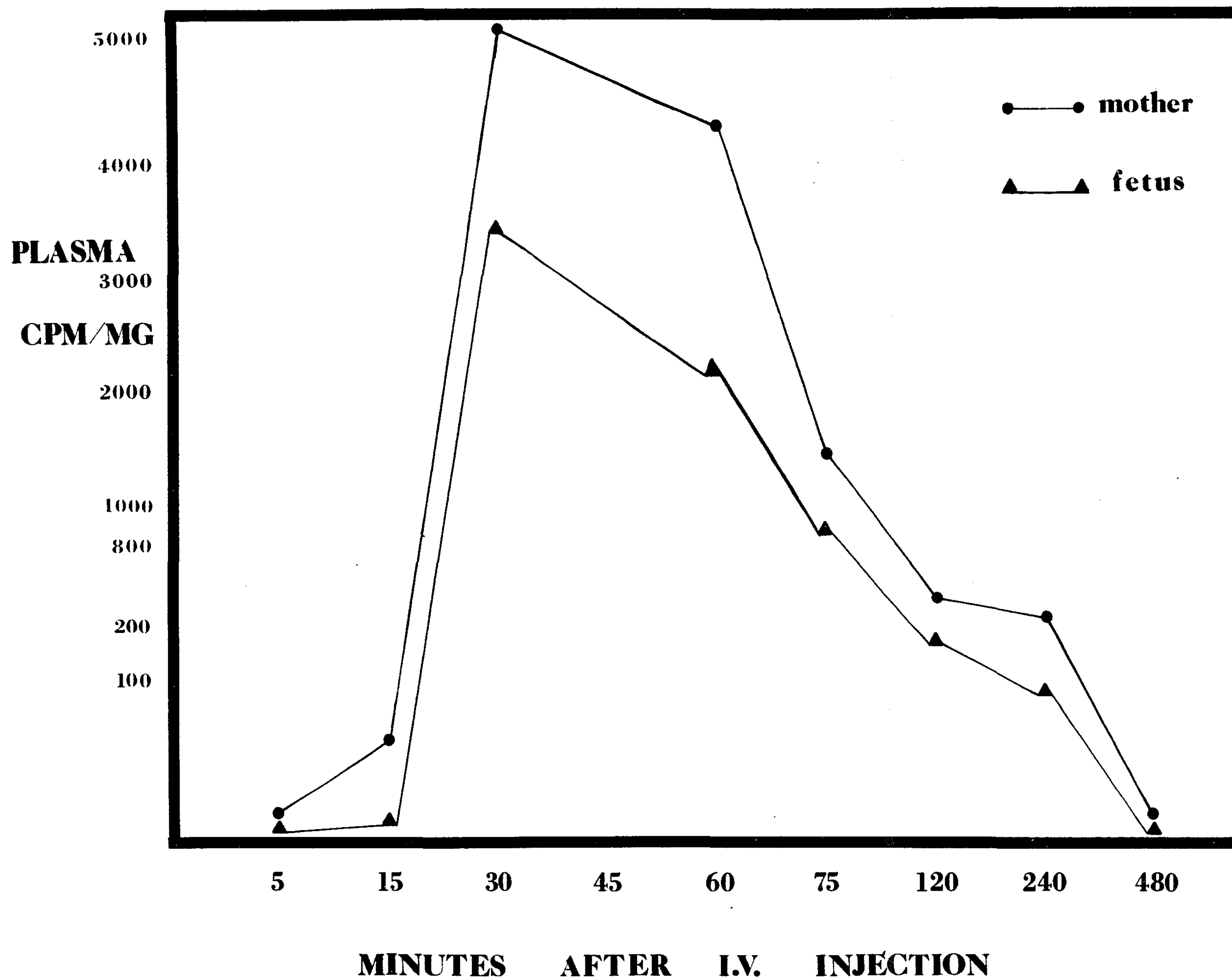
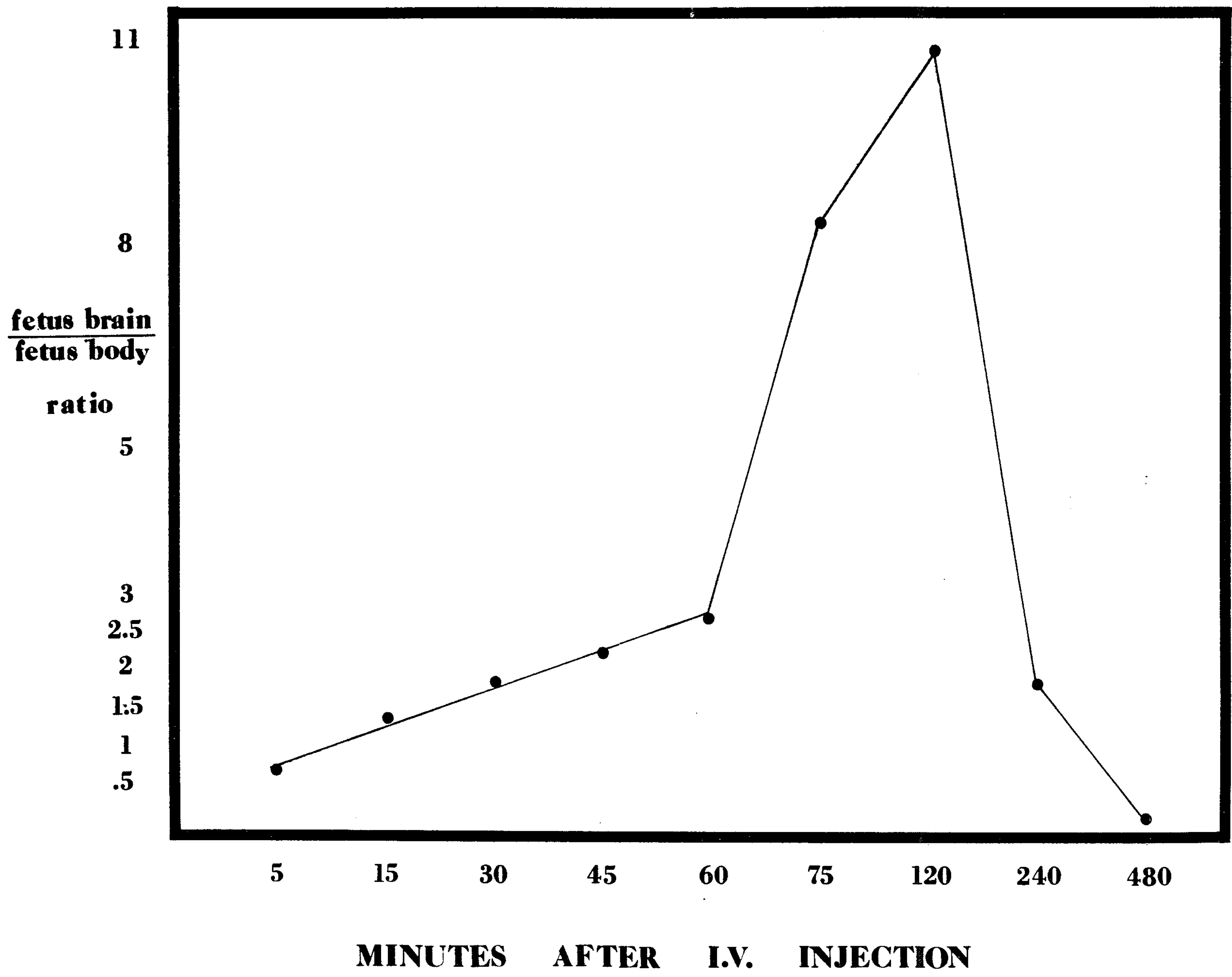
Comparisons between levels of radioactivity in gross neural areas of the fetus and combined peripheral areas were also calculated for each time interval. Brain to torso ratios were computed and analyzed by a one-way analysis of variance with unequal sample sizes. A significant time effect was noted ($F = 16.06$, $df = 9/46$, $p < .01$). Tukey post-hoc comparisons demonstrated that brain to body ratios


5000
4000
3000
2000
1000
800
200
100
PLASMA
CPM/MG
mother
fetus
5
15
30
45
60
75
120
240
480
MINUTES AFTER I.V. INJECTION


11
8
fetus brain
fetus body
ratio
5
3
2.5
2
1.5
1
.5
5
15
30
45
60
75
120
240
480
MINUTES AFTER I.V. INJECTION

were greatest at the 120-minute interval and then declined thereafter. Significant decreases in the ratio became apparent from interval 120 minutes until radioactivity levels became negligible by 480 minutes post-injection ($p < .01$).

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[Insert Figure 17 about here.]

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Several other interesting insights emerged. Levels of radioactivity in maternal neural tissues were extremely high. For example, in our laboratory we have observed hypothalamic/cortex ratios of ^{3}H Progesterone to average approximately 1.35 in normal C57 ovex BL/10J mice. Yet ratios in the pregnant animal ranged from 9 to 11 despite the fact that endogenous ovarian steroids are elevated during pregnancy and would therefore be expected to saturate the limited capacity neural binding sites. This same type of proposed saturation was observed in the placenta. Levels of radioactive progesterone were minimal in every one of the placentas sampled irrespective of time interval.

Discussion

The results complement those of other studies aimed at tracing the course of hormones or drugs across placental membranes. Backstrom, Hansson, and Ullberg (1965), for example, demonstrated the passage of C^{14}- DDT across the

placental barrier of mice. Bengtsson and Ullberg (1963) compared the autoradiographic patterns of diethystibestrol and natural estrogens. They did this in both the intact and pregnant female mouse. High levels of label were detected in adrenal and neural regions, yet diethylstilbestrol only partially was able to transverse the membranes of the placenta. In the present experiment, progesterone was found to pass the barrier with the greatest plasma levels of radioactivity being noted 30 minutes after the intravenous injection. Yet at all time intervals, maternal plasma levels were higher although not significantly due to large variability.

The data of Experiment 6 must now be viewed in light of the present findings. Progesterone was readily detected in fetal whole brains. Whole brain/torso ratios were as high as 11, 120 minutes after the single i.v. injection. This finding would thus contradict the hypothesis that the inability of prenatal progestins to defeminize the genetic female results from a neural barrier to progestins in the fetal organism. Limited evidence also suggests that fetal hypothalamic sites are particularly sensitive to progestins. In a limited sample of fetuses (N = 3), neural tissue was dissected into separate cortex, cerebellar, and hypothalamic regions. In all three subjects, hypothalamic/cortex ratios exceeded two and hypothalamic/fetal plasma ratios exceeded unity.

Unfortunately, this study does not offer us a complete understanding of the transplacental passage of steroids. Additional studies are necessary in order to probe several other key questions. For example, does the placenta alter its permeability characteristics with advancing gestational age? Also, will the placenta alter these permeability characteristics if confronted with exogenously introduced steroid over-load? These are important questions for they may have particular clinical relevance. Lastly, an important drawback of the present study concerned the failure to characterize the nature of the radioactivity in fetal plasma or neural tissue. For example, given the results of Experiment 2, it is possible that the major tritiated product or products were pregnane derivatives of ^{3}H Progesterone.

EXPERIMENT 8

Within the last several years, a great deal of evidence has been gathered illustrating the regulatory nature of steroidal hormones. It is known for example, that estrogens will interact at the molecular level with uterine receptors subsequently inducing a hormone specific protein (Hamilton, 1968).

As noted in the literature review, neural tissues have also been implicated as receptor sites for the three key gonadal hormones of estrogen, progesterone and testosterone. With regard to estradiol neural retention patterns, both biochemical tracer counting techniques as well as light microscopy autoradiography, have demonstrated that certain regions of the female rat and bovine brain selectively concentrate and retain estradiol: the hypothalamus and pre-optic area (Eisenfeld and Axelrod, 1965; Kato and Villee, 1967b; McEwen and Pfaff, 1970; Pfaff, 1968); the septum and amygdala (Stumpf, 1970; Pfaff, 1968; Zigmond and McEwen, 1970). More elaborate biochemical techniques have shown that these highly saturable neural binding sites are, like those of the uterus, found in cell nuclei (Zigmond and McEwen, 1970) and soluable (cytosol)

fraction (Eisenfeld, 1970) and are stereospecific for 17β-estradiol.

Given the fact that female rodents, exposed to perinatal steroids, are often refractory to exogenous adminstrations of ovarian hormones, it has occurred to several researchers that this refractoriness may be due to altered steroid binding capacities in the defeminized female. Although the data is often not unanimous, several independent laboratories have observed that treatment of neonatal female rats with testosterone propionate shortly after birth decreases the uptake and subsequent binding of estradiol in the hypothalamus and uterus in sixty day old females (Flerko et al., 1969; McEwen and Pfaff, 1970; Tuohimaa and Johansson, 1971; Vertes and King, 1971).

However, the situation is complicated by the fact that female behavioral receptivity is also dependent upon progesterone. The neural retention characteristics of tritiated progesterone have also been studied. Recently, tissue uptake studies have shown that after injection of ovariectomized rats, guinea pigs or hamsters with tritiated progesterone, a high concentration is found in the brain (Leavitt, 1972; Wade and

Feder, 1972; Whalen and Luttge, 1971). In the guinea pig, the major component of the radioactivity was unmetabolized progesterone with extensive metabolism being noted in rat and hamster brains. Midbrain areas retained the greatest degree of radioactivity with hypothalamic areas second (Luttge and Wallis, 1973; Wade, Feder and Harding, 1973). In mice, Luttge, Chronister and Hall (1973) demonstrated a similar midbrain affinity for progesterone in the mouse brain.

Paradoxically, although researchers agree that neonatally sterilized females are particularly distinguishable from normals in their responsiveness to progesterone, to my knowledge no study has explored the neural affinity for progesterone in such sterilized genetic females. One purpose of the present experiment is to assess this neural affinity for progesterone.

In both males and females, the pattern of testosterone uptake is less well defined. In cell fractionization studies, McEwen et. al. (1972) reported that specific testosterone uptake may exist in the preoptic hypothalamus. Pfaff (1968) likewise found some evidence for ^3H nuclear accumulation in preoptic, central and posterior hypothalamic regions and the anterior pituitary.

Neonatal anti-androgenic treatment will reduce the concentration of labeled testosterone in hypothalamic areas (Tuohimaa & Niemi, 1972). Uptake and retention of ^{3}H testosterone in both sterilized and control females were examined.

Method

Subjects. Genetic female C5710/J pups, born in this laboratory served as subjects.

Procedure. Within 24 hours after birth, genetic female pups were injected in the usual manner with either oil or 320 ug MPA. Each litter was randomly assigned to one of the two groups. Pups were weaned at age 28 days, housed collectively with animals of the same sex and treatment condition until day 50, at which time all subjects were singly housed in polypropylene cages. Vaginal smears were then taken daily for a period of 30 days. MPA subjects that displayed a pattern of constant vaginal cornification were chosen and oil subjects that displayed a five-day cyclicity pattern were chosen for brain uptake studies. All were then bilaterally ovariectomized. Constant estrus animals were chosen since they are more commonly produced given the dosages of neonatal steroids and it was assumed that the majority of subjects in former uptake studies (McEwen & Pfaff, 1970) were constant estrus females.

Steroid preparation and concentration: The following radioactive steroids were purchased from the New

England Corporation: (1, 2 - ^{3}H)testosterone (specific activity 45 Ci/mM); (6, 7 - ^{3}H)estradiol (specific activity 45 Ci/mM); (1 , 2 , - ^{3}H) progesterone (specific activity 48 Ci/mM). The steroid was prepared and assessed for purity as described in Experiment 7.

For the in vivo uptake studies, 8uCi of steroid was dissolved in .2cc of 20% ethanolic solution and injected I.V.. For the in vitro procedure each tissue was incubated with 1.33×10^{-10}M of tracer.

In vivo analyses: Both neonatally oil and MPA treated females were each randomly assigned to one of three in vivo conditions: ^{3}H Progesterone, ^{3}H estradiol, ^{3}H testosterone. In all cases, 8uCi of tritiated steroid was administered I.V. (tail vein) and each animal sacrificed by cervical dislocation 120 minutes thereafter. The ventral body wall was opened, a sample of blood was taken using a heparinized syringe and neural tissues were dissected and immediately weighed. The following samples were taken with the aid of a dissecting microscope:

a) Adenohypophysis

b) Hypothalamic Block extending from the mammillary bodies to the rostral edge of the optic chiasm, to the medial border of the cerebral peduncles laterally and to the anterior commissure dorsally.

c) Cortex

d) Midbrain

e) Cerebellum

As controls, muscle samples were also taken. All samples were processed in an identical manner as that described for the uptake analysis in vivo of Experiment 7, with the exception that each cpm value was converted to a dpm value since counter efficiencies were estimated at 49%.

In vitro analyses: Subjects from both neonatal groups were sacrificed by cervical dislocation and decapitated. The brain was removed from the skull case, dissected with the aid of a dissecting microscope and tissues were immediately placed on glassine weighing paper to yield tissue samples identical to those described above for the in vivo analysis. All tissue samples were transferred to 25 ml Erlenmeyer flasks together with 2 ml of Krebs-Ringer-Phosphate buffer (pH 7.4) containing 10mM glucose and 1% bovine serum albumin. Flasks were capped and tissues were incubated for twenty minutes at 37 $^{\circ}$C in a shaking incubator. After this period, either ^{3}H Testosterone, ^{3}H estradiol or ^{3}H Progesterone were added depending upon a random assignment of subject to condition. All flasks were

recapped, returned to the incubator for an additional 60 minutes. Tissues were repeatedly washed with .154M NaCl and transferred along with 1 ml of distilled water to individual scintillation vials. The water was removed by suction aspiration and cotton tipped swabs. One ml of NCS tissue solubilizer was pipetted into each vial. All vials were tightly capped and returned to the 37°C incubator for a period fo five days. Following the digestion of all samples, 10 ml of toluene-based fluor were added and all vials were returned to the incubator for an additional five day period. The fluor consisted of PPO (2,5-diphenyloxazole) and POPOP (1,4-bis-(4-methyl-5-phenyloxazolyl)-benzene) dissolved in analytical grade toluene at concentrations of 5.0 and 0.05 g/l, respectively. Counting techniques and efficiency were identical to that utilized in Experiment 7.

Results

In vivo analyses

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(Insert Table 9 about here.)

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Table 9 depicts the uptake of tritiated gonadal

Table 9

In Vivo Analysis of Gonadal Steroid Uptake in Various Neural Regions

Neonatal Treatment	^{3}H Steroid	Hypothalamic Block[a]	Pituitary[a]	Cortex[a]	Plasma/ Hypothal.	Hypothal./ Cortex	Pituitary/ Cortex
MPA Female	T	169.69	100.23	95.33	1.33	1.78	1.05
	EB	181.35	215.69	120.10	2.01	1.51	1.79**
	P	135.36	258.43	123.06	.95	1.06***	2.09*
Oil Female	T	122.83	100.25	89.01	1.42	1.38	1.12
	EB	263.50	280.58	115.53	3.20	2.28	2.43**
	P	204.89	666.76	113.01	1.93	1.84**	5.90*

[a]Values represent average dpm/mg obtained from 4 animals.

*Values differ from one another ($p < .01$).

**Values differ from one another ($p < .05$).

***Values differ from one another ($p < .05$).

steroids in various neural tissues from animals of two neonatal treatment groups. Although MPA treated subjects did exhibit a tendency to retain more ^3H testosterone than did oil controls, the differences did not reach an acceptable level of statistical significance when compared by independent t tests. Significant differences also were not apparent in estradiol retention between the two groups in hypothalamic samples, although MPA treated subjects were deficient in hypophyseal estradiol uptake ($t=7.46$, $df=6$, $p<.01$). However, the major differences existed between the two groups with regard to progesterone accumulation. In both hypothalamic and pituitary regions, ^3H progesterone levels were significantly reduced in neonatal MPA subjects. Furthermore, while mean hypothalamic/cortex ratios and pituitary/cortex ratios were 2.10 and 5.90 respectively for oil treated subjects, similar ratios in MPA subjects were both less than unity. A comparison of these two groups yielded statistically significant effects ($t=51.67$, $df=7$, $p<.001$) for both hypothalamic/cortex ratios as well as pituitary/cortex ratios ($t=5.63$, $df=7$, $p<.01$).

<u>In vitro analyses</u>

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[Insert Table 10 about here.]

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Table 10

<u>In Vitro</u> Analysis of Gonadal Steroid Uptake in Various Neural Regions

Neonatal Treatment	Tritiated Steroid	Hypothalamic Block[a]	Pituitary[a]	Cortex[a]	Hypothalamic/ Cortex	Pituitary/ Cortex
MPA Female	T	304.27	229.60	186.67	1.63	1.23
	EB	213.90	293.89	173.90	1.23	1.69**
	P	67.88	116.37	193.95	0.35*	0.60***
Oil Female	T	202.93	209.75	170.53	1.19	1.23
	EB	385.74	366.68	146.67	2.63	2.50**
	P	211.54	459.90	183.96	1.15*	2.50
Untreated Male	T	483.19	307.82	186.56	2.59	1.65
	EB	474.82	396.95	189.93	2.50	2.09
	P	206.12	418.88	221.63	0.93	1.89

[a]Values represent dpm/mg. Each value is the mean of 4 samples.

*Differences between each value ($p < .05$).

**Differences between each value ($p < .05$).

***Differences between each value ($p < .01$).

Table 10 illustrates the in vitro accumulation of various tritiated steroids in genetic females treated neonatally with MPA or oil and also the accumulation in untreated genetic males.

The in vivo analysis demonstrated that the greatest distinction in patterns of steroid uptake concerns the differences in the two groups with respect to ^{3}H progesterone accumulation. To explore this finding a bit more completely, an in vitro analysis was initiated. A similar pattern was observed. Neither ^{3}H estradiol nor ^{3}H testosterone uptake was different in hypothalamic areas of the two groups but ^{3}H progesterone levels were. Pituitary/cortex ratios were significant ($t=81.52$, $df=7$, $p < .01$).

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(Insert Figure 18 about here.)

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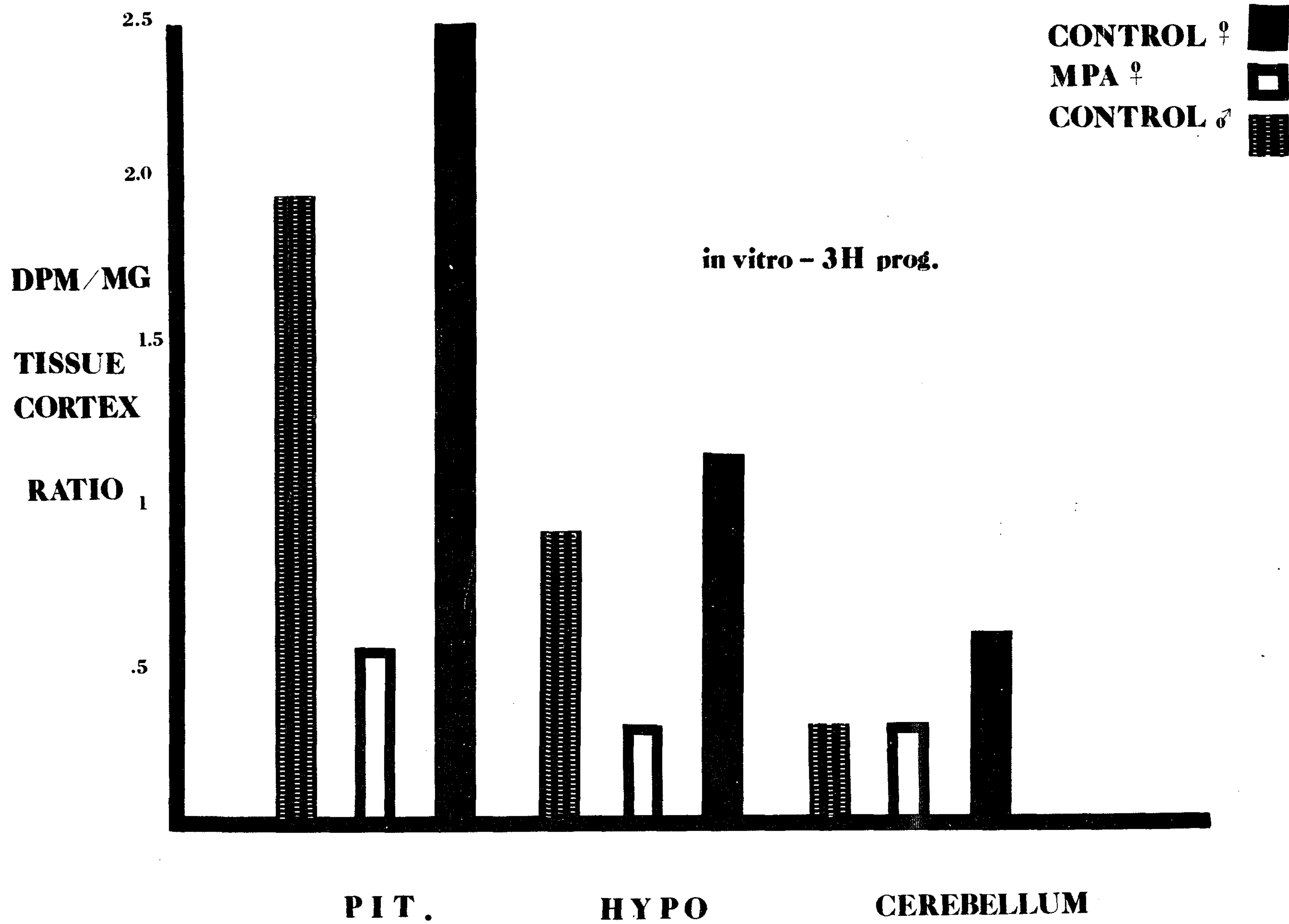
Hypothalamic/cortex ratios approached unity in the control female subjects but were less than unity in MPA treated females (.32). An independent t test was calculated and a significant difference in hypothalamic/cortex ratios was observed between MPA and oil treated subject in their ability to accumulate ^{3}H progesterone ($t=10.58$, $df=7$, $p < .01$). Pituitary/cortex ratios likewise were significant;


CONTROL ♀
MPA ♀
CONTROL ♂
in vitro – 3H prog.
DPM/MG
TISSUE
CORTEX
RATIO
2.5
2.0
1.5
1
.5
PIT.
HYPO
CEREBELLUM

MPA subjects displayed a sharply reduced ability to concentrate progesterone from the blood ($t=36.36$, $df=7$, $p<.01$). Interestingly enough, uptake of ^{3}H progesterone in control males was actually higher than in the de-feminized females. These differences due to small sample sizes in the male group, were not statistically reliable.

Discussion

The purpose of this study was to test the hypothesis that the behavioral and gonadotrophin disruptions observed in adult females treated neonatally with medroxyprogesterone acetate may be correlated with a deficiency in the ability of neural tissue to concentrate the appropriate gonadal hormones from the blood. Although the different groups of females were not distinguishable in their hypothalamic affinity for estradiol, pituitary responsiveness was greater in oil treated females than in MPA females. This was true in both *in vivo* and *in vitro* analyses.

It appears, however, that the major distinguishing characteristics between the two groups in both *in vivo* and *in vitro* conditions is the diminished accumulation of ^{3}H progesterone in both hypothalamic as well as

pituitary sites. It therefore seems plausible that the refractoriness to ovarian hormones may be due to this inability of MPA females to concentrate progesterone from the blood. However, it is very important to recognize that normal genetic males did accumulate ^3H progesterone in levels intermediate between normal control females and neonatally MPA treated females. This suggests that the reduced accumulation and uptake of tritiated progesterone in sterilized females is not a concomitant of a masculinization process per se but rather reflects a process correlated with defeminization. Whether this reduced neural sensitivity to progesterone is a cause of or an effect from gonadotrophin and behavioral defeminization remains a question for future research studies.

Several weaknesses of the present study should be assessed in future research endeavors. First, no effort was made in identifying the final radioactive product in neural tissue. Since 5 α- dihydrotestosterone and 5 α dihydroprogesterone are major metabolites of testosterone and progesterone in neural tissue respectively; this omission may be significant and possibly responsible for the lack of reliable statistical differences in testosterone accumulation among the three groups.

Second, the NCS technique of plasma solubilization did not appear to be ideally suited to our laboratory for high individual subject variability obscured tissue/plasma differences. However, it should be noted that absolute plasma radioactivity did not differ as a function of neonatal treatment. So it cannot be argued that differential ability of label to enter blood or differential binding of tritium label in blood is responsible for the diminished hypothalamic/cortex ratios in the MPA females. However, a resolution of this problem could be achieved if a solvent extraction procedure were used in conjunction with the NCS tissue solubilization technique.

EXPERIMENT 9

We have discussed the multiple effects of neonatal progestins in inbred mice. It was shown that progestins may exert a synandrogenic action in genetic females while at the same time exerting anti-androgenic like actions in the genetic male. The purpose of the present experiment is to briefly explore possible sites in the neonatal mouse brain which may be implicated in the production of these effects. Two particular questions will be examined. First, does ^{3}H Progesterone display a specific accumulation and uptake pattern in the neonatal mouse brain? Similar studies have analyzed this same question with respect to ^{3}H Testosterone but with conflicts in the literature. Diamond and Dale (1967) reported selective retention of testosterone by the neonatal hypothalmus relative to uptake by the cerebellum and the liver. However, Alvarez and Ramirez (1970) could not confirm this selective retention.

The second question of interest concerns the validity of one hypothesis advanced for the anti-androgenic effects of progestins expressed in the genetic male. Progesterone has been shown to compete for binding sites in Testosterone sensitive neural regions. In fact, Sar and Stumpf (1973) have shown that high dosages of either Progesterone

or Cyproterone acetate can reduce the uptake of Testosterone in hypothalamic and pituitary areas of male rats. Similar ideas on peripheral sites of Testosterone action were presented by Tveter and Aakvaag (1969). In this experiment, in vitro uptake of ^{3}H Testosterone will be examined in neonatal mice pre-treated with MPA in order to assess whether such a neural competition effect exists in the neonatal mouse brain.

Method

Subjects. Subjects for both part A and B were C57BL/10J mice born and bred in the laboratory.

Procedure. Part A: Pups were taken from their mothers, within 24 hours after birth and injected with 8 uCi of 1 , 2 ^{3}H) Progesterone (Specific Activity 48 Ci/mM). The radioactive steroid was prepared and assessed for purity as described in Experiment 7. Pups were injected subcutaneously at the nape of the neck with a .05 cc. 20% ethanolic solution. Pups were sacrificed either 30, 60, 90, or 120 minutes after s.c. injection.

Pups were sacrificed by decapitation and a blood sample was taken via a heparinized syringe. The brain was removed from the skull case, frozen at -20°C and then dissected with the aid of a dissecting microscope.

Anatomical landmarks were identical to those of Experiment 8. All remaining procedures including the NCS tissue digestions were identical to those employed in the *in vivo* procedures of Experiments 7 and 8.

Part B: In this experiment, pups were removed from their mothers randomly assigned to one of two conditions: 1) 300 ug MPA pretreatment + ^3H Testosterone incubation, 2) ^3H Testosterone incubation alone. Tissues were processed for an *in vitro* analysis as described in Experiment 8.

Results and Discussion

Part A - *In Vivo* Analysis

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(Insert Figure 19 about here.)

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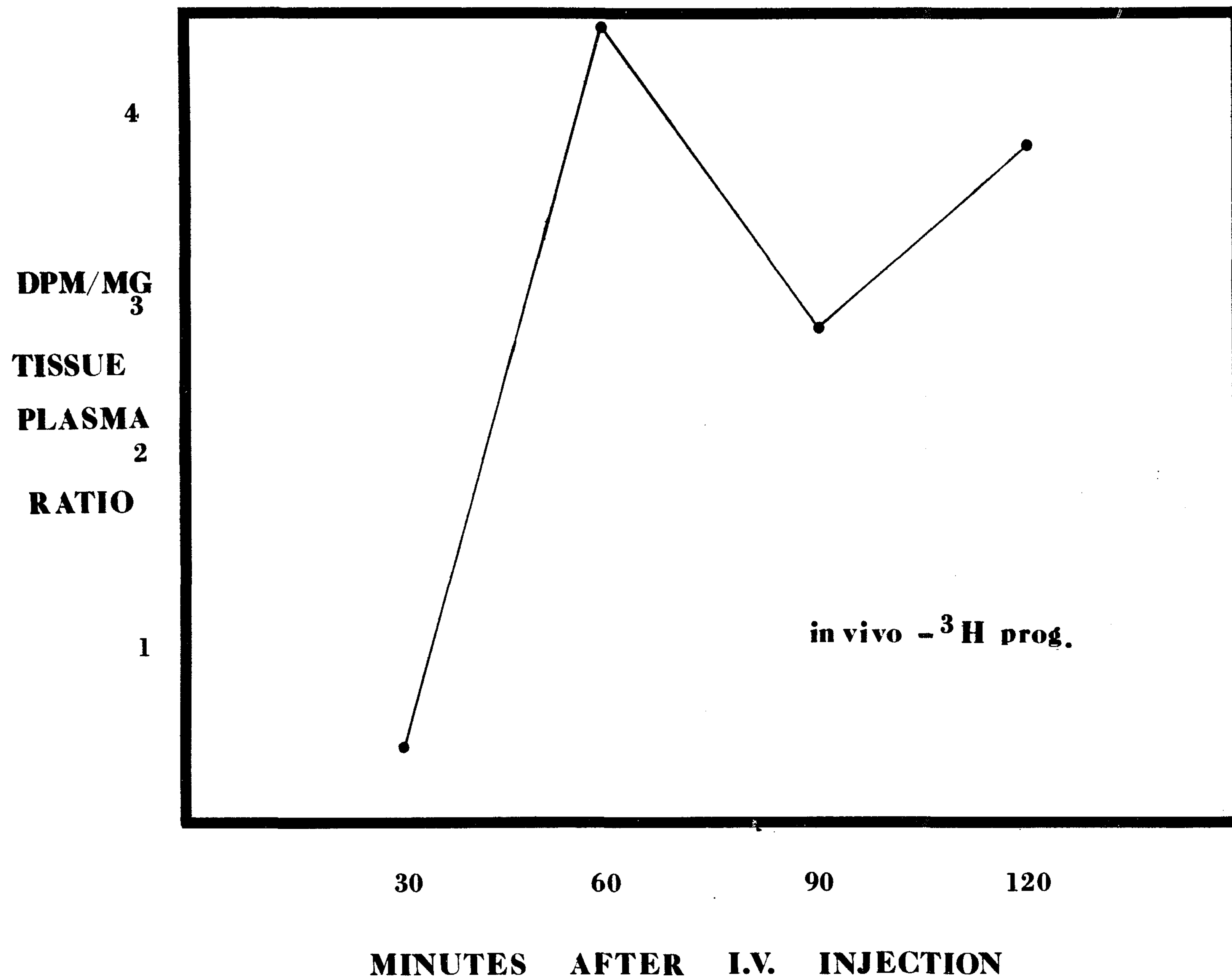
Figure 19 depicts the *in vivo* uptake of ^3H Progesterone after four injection - sacrifice intervals. By examining tissue/plasma ratios, you observe a high hypothalamic uptake of Progesterone 60 minutes after injection, which drops to 2.9 by 90 minutes but rises again to four by two hours post-injection. Although this pattern is a bit strange, the data is consistent with the idea that the progestins can be accumulated in neonatal neural tissue from plasma.


4
DPM/MG
3
TISSUE
PLASMA
2
RATIO
1
in vivo - ^{3}H prog.
30
60
90
120
MINUTES AFTER I.V. INJECTION

Part B - In Vitro Analysis

- - - - - - - - - - - - - - - - - -

(Insert Table 11 about here.)

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Table 11 depicts in vitro uptake and accumulation of ^{3}H Testosterone in mouse neonates half of whose tissues were pre-exposed to 300 ug unlabelled MPA. An independent t test between saturated versus unsaturated tissue groups demonstrated a significant reduction in hypothalamic/cortex ratios in tissues pre-exposed to MPA ($t=6.39$, $df=7$, $p=.01$). This reduction was caused by a reduction in hypothalamic accumulation of ^{3}H Testosterone.

Although incomplete, the two parts of this experiment are consistent with the hypothesis that progestins alter the processes of sex differentiation by changing sensitivities in hypothalamic areas and that the neonatal hypothalamus is sensitive to progestins on day one of life (Part A). Part B supports the idea that the anti-androgenic actions of progestins may be due to their ability to compete for the limited capacity neural receptor sites for Testosterone in the neonatal mouse brain. This data, therefore, is consistent with the idea that the demasculinization syndrome induced by progestins is associated with competitive receptor site inhibition.

Table 11

In vitro uptake of ^{3}H Testosterone inhibition by MPA

In vitro uptake

UNSATURATED	dpm/mg	cortex tissue
hypothalamus	227.6*	1.687*
cortex	143.41	-
muscle	10.20	.071
SATURATED	**dpm/mg**	**cortex tissue**
hypothalamus	105.6*	.88*
cortex	119.93	-
muscle	21.53	.17

*significantly different from each other ($p < .05$)

General Discussion

Experiments 1 through 9 have analyzed the processes of sex differentiation in mice exposed to progestins perinatally. The original purpose of this dissertation was to provide a broader empirical understanding of the possible pathological actions of progestins during development.

Perinatal progestins in the genetic female. In one group of studies, female mice that had been injected with either of two progestins, MPA or P, were defeminized in adulthood. This defeminization syndrome was apparent with regard to the three proposed processes of sex differentiation, namely, anatomical, gonadotrophin, and behavioral. Defeminization was inferred if the dependent measure score in progestin treated neonates was significantly depressed relative to oil-treated neonates. In these studies, comparative differences between intact males and neonatally treated females were not assessed. Therefore, absolute levels of either masculinization, feminization, demasculinization, or defeminization could not be ascertained.

On the anatomical level, either MPA or P produced dose-dependent somatic virilization chiefly characterized by clitoral hypertrophy and decreased anal-genital distances. The display of clitoral hypertrophy was apparent at weaning and continued throughout the life-span of the organism. Somatic virilization was equally extensive in each group of genetic females treated with TP, P, or MPA. Furthermore,

when subjects were exposed to androgenic stimulation during masculine behavior tests, clitoral hypertrophy became more pronounced.

These data on the virilizing actions of perinatal progestins are consistent with data on the Sprague-Dawley rat (Revesz, 1963; Whalen, Peck, & Lo Piccolo, 1966). Furthermore, in human beings, Wilkins (1960) reported a pseudohermaphroditic syndrome in the female offspring of mothers who received a synthetic progestogen, Norlutin, during pregnancy.

Since it is widely known that progestins may serve as intermediates in the metabolic conversion of pregnenolone to testosterone (Turner & Bagnara, 1971), it had always been assumed that progestins virilize due to their inherent androgenicity. The results of Experiment 1 are consistent with this idea since a given dose of progestin produced a slightly smaller percentage of virilized females than an equal dose of testosterone propionate.

In Experiment 2, genetic females were exposed to one of two progestin metabolites, 5 α pregnane, 3, 20, dione and 5α pregnane 3β -ol-20-one. These two compounds, in contrast to the parent progestins of MPA and P, did not alter the normal processes of female anatomical differentiation relative to oil treated females.

Both Experiments 1 and 2 have also examined gonadotrophin differentiation. The type of differentiation

process induced by progestins was assessed in genetic females by a 30 day examination of the estrous cycle. Using two different criteria of cyclicity, % of possible cycles in a thirty day period and % of animals cycling, Experiment 1 demonstrated that neonatal progestins alter and disrupt estrous cyclicity in both a dose-dependent and a time-dependent fashion. Furthermore, Experiment 2 extended this finding by suggesting that the principle 5α reduced neural metabolite of Progesterone, 5α pregnane 3, 20, dione, is as potent in seriously disrupting estrous cyclicity. In fact, the patterns of cyclicity disruption observed in TP, MPA, P and 5α pregnane 3, 20, dione treated animals were nearly identical. There were distinct individual differences in the expression of the cyclicity disruption. The most common manifestation was a constant vaginal cornification, in some cases extending throughout the entire 30 day observation period.

Interestingly enough, females were differentially sensitive to the two progestins at distinct developmental ages. At all ages, natural Progesterone produced a significantly greater degree of disruption at day 10 than MPA at day 10. By day 20, neonates were no longer sensitive to gonadotrophin disruption by either MPA or P.

Comparing the two processes of differentiation in the genetic female, certain similarities as well as differences emerge. MPA and P both alter relative to oil controls anatomical as well as gonadotrophic differentiation. The two processes are unique in the sense that while anatomical differentiation is complete by day 10 and maximally sensitive to MPA at that time; gonadotrophin differentiation is still disruptable by both MPA and P at neonatal day 10. Progesterone is more effective at this time, however.

The third class of differentiation processes was likewise altered and modified in genetic females by exposure to progestins. Females exposed to either MPA or P on day 1 were quite resistant and unresponsive to the advances of the stud male despite the exogenous introduction of estorgen and progesterone. This refractoriness to female sex hormones was both dose-dependent as well as time dependent. Even at day 10, 320 ug of MPA was capable of altering the potential for lordotic receptivity. Progesterone on day 10, however, was as ineffective as oil in defeminizing the genetic females. This differential temporal sensitivity is a common feature in both gonadotrophin and female behavior differentiation.

Experiment 2 examined the behavioral alterations produced by various gonadal steroids and steroid derivatives.

Female receptivity was dramatically depressed in TP, MPA, P, DES and 5α pregnane 3, 20, dione treated females, even after four sessions of EB+P priming. In these groups, lordotic quotients on the fourth session ranged from 9 to 31 while the oil group averaged 88.3. Qualitative observation indicated that the lack of female receptivity was not a mere passive process nor one caused by a general inactivity. Rather, females were intensely resistant and aggressive toward the male. Wright and Fitzpatrick (1975) reported that neonatal MPA will produce similar resistance behavior in the adult female Sprague-Dawley rat. This disruption of normal female behavioral differentiation was produced even at dosages incapable of producing somatic virilization. Like the changes produced in normal gonadotrophin differentiation by progestin metabolites, behavioral development was also modified by these compounds.

Therefore the results of Experiment 2 are consistent with the idea that progestin metabolites may play a key role as the major steroid in the expression of progestogenic action. This idea is certainly not a new one for reports have indicated that 5α pregnane 3, 20-dione exerts progesterone-like effects on ovulation (Sanyal & Todd,]972; Sridharan et al.,]974), on female receptivity (Wade & Feder,

1973; Czaja et al., 1974) and on luteinizing hormone releasing hormone induced LH release from rat pituitaries in vitro (Schally et al., 1973). Experiment 2 demonstrates that progestin induced defeminization may indeed result via metabolic conversion. Prior studies, both experimental and clinical have assumed that perinatal progestins exert their actions via metabolic conversion to potent androgens (Wilkins, 1960; Whalen, Peck & Lo Piccolo, 1966). It is possible that such a conclusion, although tenable and likely for an explanation of anatomical defeminization, is not readily convincing with respect to gonadotrophin or behavioral defeminization. 5α pregnane 3, 20-dione, although unconvertible to androgens (Karavolas et al., 1976), is independently capable of producing cyclicity disruptions and refractoriness to female sex hormones. Based on the results of Experiments 1 and 2, we can propose that neonatal progestins act at two loci in the genetic female mouse. In fact, the particular mechanism of action at these sites are unique. The original studies assumed since they observed extensive clitoral hypertrophy that progestins act via androgenic conversion. However, they mistakenly assumed that the processes of sex differentiation were homogenous. Experiments 1 and 2 demonstrate that the processes of

differentiation are probably distinct in that each is uniquely sensitive to different progestins at specific developmental stages. However, while virilization can be produced by MPA or P on day 1 and to a lesser extent on day 4, large doses of progestin metabolites were unable to virilize even when administered within 24 hours after birth.

The fact that the 5 α reduced metabolities relative to oil controls were disruptors of female sex and gonadotrophin differentiation suggests that neural tissue is capable of reducing progestins to 5α pregnane derivatives and in fact this reduction is a primary step in the defeminization syndrome. It is possible that the reduction process is the normal metabolic step in neural tissue and therefore can trigger the developmental events of defeminization. Conversely, androgens are the usual product of progesterone metabolism in peripheral tissues. This model of dual progestin action at different sites is consistent with various studies which have shown the reduction of ^{3}H Progesterone in neural tissue (Cheng & Karavolas, 1973; Robinson & Karavolas, 1973; Karavolas et al., 1976), but not in peripheral target organs like the uterus (Wiest, 1972). This model has explanatory value for the differential results of Experiments 1 and 2 if this predominant intraneural conversion of ^{3}H Progesterone to pregnane derivatives is observed in the neonatal mouse as it has been in the adult rat.

Although a defeminization syndrome was observed, the results of Experiments 1 and 2 did not provide a great deal of support for a corollary masculinization process induced by progestins. There was a slight enhancement in mount frequency and percentage of subjects mounting in the highest dosage conditions, but actually qualitatively the differences were not very impressive. With respect to intromissions and ejaculations, no reliable differences were observed between progestin and oil groups. In fact, despite extensive peripheral masculinization, progestin treated females never were able to consummate the masculine behavior act with an ejaculatory reflex.

Experiment 6 was conducted to assess whether this lack of complete behavioral masculinization was due to an inappropriate choice of temporal parameters in Experiments 1 and 2. For example, it is possible that the neural systems for masculinization develop pre-natally rather than post-natally while the systems for female development are sensitive during the neonatal period. Experiment 6 supports this idea. While pre-natal progestin stimulation was capable of producing extensive virilization and masculinization of the internal reproductive tracts, no decrements in estrous cyclicity were noted. In fact, such females were indistinguishable from controls.

With regard to defeminization, sensitivities were greater during days 1 to 4 after birth although during the prenatal period sensitivity probably is initiated. In fact, pre and postnatal exposure to progestins is as capable of reducing responsiveness to adult female sex hormones as is postnatal exposure alone.

Masculinization appeared to follow a uniquely different pattern. Prenatal progestin treatments of either type significantly increased 4th trial mount frequencies as compared to any of the three oil groups or either of the two neonatal progestin groups. Likewise, combined prenatal and neonatal progestin treatments were equally effective in masculinizing the genetic female as prenatal treatments alone were.

It again must be emphasized that the absence of comparisons between normal intact genetic males and the various progestin treated females precludes any interpretation which would suggest that an absolute masculinization and defeminization was obtained by perinatal progestin exposure. The employed experimental design does not permit one to conclude, for example, that progestin treated females are as "masculine" or as "feminine" as the genetic male. Additional group comparisons are, therefore, necessary before a meaningful statement concerning the absolute degree of masculinization and defeminization can be made.

Several other issues emerge as we discuss the effects of perinatal progestins in the genetic female. As we view social-behavioral interactions in animals, the complexity of the situation immediately appears. Let us consider the female sex behavior testing procedure. An adult female that had been treated with EB+P is presented to a sexually vigorous stud male. A mount to lordosis quotient is computed. Although the male stud is aroused and does vigorously mount the female, females that had been exposed neonatally to progestins are unresponsive to the advances of the male. Yet it was observed in Experiment 5 that the urine of progestin treated females exhibiting a continual pattern of vaginal cornification was aggression promoting. In fact, whether a genetic female was injected with either progestinic or androgenic compounds was immaterial, what was important was the presence of a constant estrus smear. Urine from sterilized females exhibiting a constant pattern of diestrus was no more aggression promoting than water. Taken together, data from experiments 1, 2 and 5 suggest that the endrogenous metabolic pathways may be altered in the sterilized genetic female which are thus responsible for the heightened aggression promoting cues of their urine.

However, when such females are exogenously administered

estrogen and progesterone, no such aggression promoting cue exists, since stud males did not attack the females themselves in sex behavior tests.

One last point of clarification, we tested the reactions of trained male mouse fighters to castrates coated with urine of the particular condition rather than test the fighters against the neonatally treated females themselves. This procedure was employed due to the high aggressiveness of female mice treated neonatally with sterilizing steroids (Bronson & Desjardins, 1968). It was assumed that if the female subject attacked the fighter, an understanding of the aggression promoting qualities of urine would have been obscured. In the earlier 1973 study (Lee & Griffo, 1973), neonatally sterilized females, who had been treated with 500 ug TP on day 1 themselves were highly aggression promoting targets for BALB male mouse fighters.

Experiments 8 and 9 have attempted to examine several of the biochemical correlates of this defeminization syndrome. The *in vivo* study of Experiment 9 demonstrated that Progesterone does reach and in fact is selectively accumulated in hypothalamic areas of the day one mouse. More elaborate studies (Lieberburg & McEwen, 1975) have shown that ^{3}H estradiol is accumulated in a limbic-hypothalamic complex. Experiment 9 however, should be extended by examining the saturability of this neural system together with the stereospecificity of analysis of the accumulation of many different steroids. Furthermore, given the results of Experiment 2, the data on the *in vivo* uptake of ^{3}H Progesterone is incomplete since no effort was made to characterize nor identify the final tritiated product after I.V. injection. This remains an important question for an understanding of progestin action neonatally.

Experiment 8 attempted to approach the question of the mechanism of defeminization by an examination of the *in vivo* and *in vitro* binding of sex steroids in adulthood. It was hypothesized that possibly the refractoriness and unresponsiveness to exogenous female sex hormones was due to an actual insensitivity

in uptake mechanisms to estradiol or progesterone. Despite numerous studies, the relative ability of normal females, androgenized females and male rats to accumulate and retain estradiol in brain and peripheral tissues is not clear. Several reports indicate that the hypothalamus and pituitary of male or androgenized female rats accumulate less estradiol than the same tissues of normal females (Flerko et al., 1969; McGuire and Lisk, 1969, Vertes and King, 1971). Several other studies noted a diminished uptake in only hypothalamic areas (Anderson and Greenwald, 1969; Clark et al., 1972; McEwen and Pfaff, 1970; Maurer and Wooley, 1971; Tuohimaa and Johansson, 1971). While still a third group of researchers could not detect a difference in uptake in either the hypothalamus, or pituitary of androgenized female or male rats as tissues were compared with those of females (Eisenfeld and Axelrod, 1966; Green et al., 1969; Luttge & Whalen, 1970).

Experiment 8, although attempting to extend this knowledge to the mouse was actually aimed at providing a different perspective. The data supports the idea that ^{3}H estradiol uptake is reduced in pituitary sites of the acyclic sterilized mouse but hypothalamic uptake patterns

did not differ significantly from control females nor from untreated males. A similar lack of significance was noted for ^3H testosterone uptake.

However, a striking gap in earlier work in this area concerned the ability of neural hypothalamic and hypophyseal sites to accumulate progesterone from the blood. This gap was apparent despite the fact that progesterone is as important in inducing female sexual receptivity. Experiment 8 demonstrates that neonatal MPA treatment, producing a pattern of constant vaginal cornification was strongly associated with an unresponsiveness of hypothalamic and pituitary sites to accumulate and retain ^3H progesterone. Hypothalamic/cortex ratios as well as pituitary/cortex ratios were significantly different in MPA treated females as compared to oil females. However, males did not differ from control females.

The data are consistent with the idea that neonatal treatment with sterilizing steroids, which produces a persistent estrus syndrome reduces the ability of the female mouse hypothalamus and pituitary to retain progesterone. However, given the ability of the male brain to retain progesterone suggests that this

unresponsiveness to progesterone is a concomitant of an acyclicity syndrome and thus reflects a defeminization and not necessarily a masculinization.

Thus, a hypothesis can be advanced to explain the behavioral defeminization. A basic neural insensitivity to progesterone would exist in acyclic females exposed to neonatal sterilizing gonadal hormones. The data are consistent with a very recent report which asserts that cold progesterone pretreatment will increase hot estradiol retention in normal but not in neonatally estrogen or androgen-sterilized female rats. This conclusion was reached in both in vivo and in vitro studies (Lisk & Reuter, 1976).

The idea that the behavioral defeminization observed in Experiments 1 and 2 is due to an insensitivity to progesterone is also supported by behavioral studies themselves. Two independent studies suggested that neonatal androgen stimulation might differentiate males from females by suppressing responsiveness to progesterone but not to estrogen (Clemens et al., 1969; Davidson & Levine, 1969).

Before accepting this model as valid, it is important to explore the phenomenon in more detail.

For example, the study was designed only to examine the uptake of sex steroids in genetic neonatally treated females who displayed a constant estrus pattern. It is unclear whether a neural modification caused the acyclicity and the diminished uptake of ^{3}H Progesterone or that acyclicity per se results in a diminished uptake of ^{3}H Progesterone. Possibly, we can find evidence to suggest that any acyclicity syndrome, for example, one caused by a 24 hour light cycle, will alter progesterone uptake.

Secondly, since normal males and normal females are indistinguishable in ^{3}H Progesterone uptake, it may be that the lack of any progesterone secretions in the sterilized persistent estrus female is the crucial variable in contrast to the viewpoint that a permanent neural substrate change is indelibly imprinted by neonatal steroids. Thirdly, in order to insure the replicability of this phenomenon, an *in vivo* method of solvent extraction should be employed. This procedure would be able to establish more reliable and less variable levels of plasma radioactivity.

Perinatal progestins in the genetic male. Experiments 3 and 4 have demonstrated that in the genetic male both male agonistic and sexual behavior is depressed in subjects exposed to neonatal progestins. These decrements in social behaviors were true prior to orchidectomy but become more prominent during TP replacement therapy. In a sense, early progestin treatment induced a type of neonatal pharmacological

castration since the data of Experiments 3 and 4 are similar to that reported previously in rats (Grady, et. al., 1965).

Experiment 3 noted that lordotic receptivity was not significantly enhanced in progestin treated males, however in Experiment 4 , the highest dosages of MPA and P did enhance receptivity. For obscure reasons, subject to subject variability masked these effects in Experiment 3.

Experiments 1 and 2 illustrate that while neonatal progestin treatment results in diminished aggressive and sexual behavior before castration, such treatment also strongly inhibits responsiveness to exogenous testosterone after castration. Griffo et al. (1974) have previously reported that neonatally progesterone treated male mice, when adult, secrete a significantly reduced amount of testosterone into the peripheral circulation. This sub-normal level of testosterone may be responsible for the depressed pre-castration scores. Furthermore, we may hypothesize that some neural substrate modification also ensued since later TP therapy was not capable of initiating either sexual or agonistic behavior.

The mechanisms for these neonatal anti-androgenic effects of progestins are unclear at present. The results of Experiment 4 clearly show that the proposed anti-androgenic effects of progestins in the genetic male are not due to 5 reduction to pregnane metabolites as was the case for the defeminization syndrome in the genetic female. From the results of the <u>in vitro</u> analysis in Experiment 9, we can tenatively propose that the neonatal anti-androgenic effects of MPA and P are associated with the fact that MPA can competitively inhibit the retention of testosterone in the neonatal mouse brain. However, the results of Experiment 4 question whether this progestational competitive ability is a necessary and sufficient condition for this anti-androgenicity. Males, who received both MPA on day 1 and TP on day 3 were indistinguishable from oil controls in responsiveness to androgenic stimulation in adulthood. It appears that progestins, although capable of competitively binding with testosterone in the neonatal brain, probably also inhibit the secretion of endogenous testosterone from the testes. This idea is supported by the fact that if neonatal MPA demasculinized the genetic male only by competing for

active "androgenic retention sites" then this demasculinization syndrome would only be moderately intense. According to this viewpoint, MPA competition would be possible over the first several hours or days of neonatal life. At the end of that competition period, testosterone would still be capable of "masculinizing" neural tissue and therefore, only limited demasculinization would ensue due to the lack of androgenic priming in the first hours or days after birth. Results of Experiments 3 and 4 with regard to either aggressive or male sexual behaviors contradicts this idea. The demasculinization syndrome was quite intense and in fact remained so despite exogenous administration of large dosages of TP. We can therefore predict that progestins are anti-androgenic in the neonatal male mouse possibly due to both a competive retention effect as well as a testosterone secretion or production inhibition. While the former property may be a necessary condition for progestin anti-androgenicity, it is not a sufficient one. Conversely, the direct effect on testosterone secretion rate may be both a necessary and sufficient condition for the demasculinization of the genetic male observed in

Experiments 3 and 4.

The literature review has focused attention on two models of sex differentiation. One view is that masculinity and femininity are really opposite ends of the same continuum. In other words, the experimental animal that displays a high probability and frequency of lordotic receptivity is considered feminine. Conversely, the subject that displays mounting, but not lordosis, will be termed masculine. This is the so-called linear model as discussed by Whalen (1974). An alternate model, the orthogonal model, postulates that hormones can defeminize without masculinizing and masculinize without defeminizing. In a sense, the data from these experiments lend a preliminary degree of support to the orthogonal model. In Experiments 1, 2, and 6, it was shown that progestins as compared to oil will defeminize primarily during the neonatal stage of development while masculinizing during prenatal stages of development. Furthermore, progestin metabolites were capable of dramatically defeminizing but were completely impotent in masculinizing, at least during neonatal life when compared to oil controls.

With regard to the demasculinization of genetic males, high dosages in Experiment 4 did lead to both

demasculinization as well as feminization. However, the dosages required to feminize were in excess of those required to demasculinize. Another example providing support for Whalen's orthogonal model is the behavior of the DES treated males. 20 ug DES administered within 24 hours after birth in Experiment 4, dramatically demasculinized genetic males but completely failed to feminize. For example, mean mount frequencies, percentage total of interaction with an intromission or ejaculation were 63, 40 and 20 respectively while lordotic quotients only averaged ten. In summary, both Experiments 1 and 2 in genetic females and Experiments 3 and 4 in genetic males provide limited support for the orthogonal model.

Several other studies are consistent with this orthogonal model. Whalen and Edwards (1967) gonadectomized male and female rats within 12 hours of birth. Subgroups of animals were administered TP simultaneously. After the appropriate hormonal priming in adulthood, masculine and feminine behavior were assessed. Mount frequencies were not modified by the presence or absence of TP but lordotic receptivity was drastically depressed in animals exposed to androgenic stimulation.

Since the presence of TP after birth did not facilitate masculine-like mounting behavior but did inhibit feminine receptivity, the authors concluded that postnatal androgen stimulation defeminized, but did not masculinize. Masculinity and femininity need not be looked at as opposite limits of discrete continuum but as "orthogonal or independent dimensions of the organism's sexuality" (Whalen, 1974). Another supportive study for this orthogonal model has been published in dogs. Beach et al. (1972) found that androgen treatment shortly after birth dramatically reduced the potential of the bitch to display feminine behavior. However, masculine behaviors were not observed. These data appear quite similar to those found in Experiments 1 and 2 of this dissertation.

However, it must be stressed that the support which these data provide for the orthogonal model is limited since, as mentioned previously, absolute reference points for "masculinization" and "feminization" were not provided. Unfortunately even studies which claim to provide direct support for a model of orthogonality (Whalen, 1974) have not included these absolute reference points.

Physiological integration. It is difficult to precisely define the physiological significance of these progestational effects, particularly since progesterone levels in neonatal or fetal mice are unknown to my knowledge. It can be argued that these studies, like all others in this field of

behavioral endocrinology have employed pharmacological dosages of steroids rather than physiological ones. Of course, a clinical interest of the effects of these compounds could have justified this type of methodology. Very little information has been gathered prior to this dissertation on the effects of progestins and their possible physiological significance for differentiation, at least in the mouse. We are required to utilize information from a primate species, the rhesus monkey, to establish such physiological function. In the rhesus monkey, Goy and Resko (1972) have demonstrated that although cyclic gonadotrophin secretion is normal, masculinization both anatomically and behaviorally did occur with androgenization.

Concentrations of progesterone display an interesting sex difference in this species. Larger amounts of progesterone are found in the circulatory system of the fetus than of the mother (Hagemenas & Kittinger, 1972) and in the fetal circulation of the female fetal rhesus than of the male (Hagemenas & Kittinger, 1972; MacDonald et al., 1973). All studies likewise demonstrated that more progesterone is concentrated in the umbilical vein than in the umbilical artery. Even more significant are the testosterone/progesterone ratios (T/P). In the male such a ratio was $.289 \pm .092$, while in the female rhesus such a ratio determination averaged $.023 \pm .009$. The respective concentrations of androgen and progesterone were relatively low.

Opposite values were noted in the female. Resko (1974) has suggested that it may be the ratio of androgen (testosterone) to antiandrogen (progesterone) that affects development.

A type of physiological model can be proposed which would attempt to explain the differential effects of progestins observed in the present series of experiments. The relatively high levels of progesterone in the female fetus would antagonize the action of testosterone found in the female. The normally low levels found in the genetic male would permit testicular androgens to "masculinize" the neural site or sites responsible for the tonic release of gonadotrophins and behavioral processes. A downward shift in this ratio would produce an anti-androgenic effect as observed in Experiments 3 and 4. In the female, circulating high levels of progesterone would effectively antagonize the low levels of testosterone composed of secretions from fetal ovary and adrenal sources. It can be argued that if such secretions were not negated by the relatively high levels of progesterone, then varying degrees of masculinization would ensue. However, if the T/P ratio is dramatically either reduced or increased, then a disruption of feminine gonadotrophin and behavioral differentiation would develop. However, this model (Resko, 1974) must be slightly modified, given the results of Experiments 1, 2, and 6.

If T/P levels become too low, then progesterone will likewise lead to a disruption of female gonadotrophin and behavioral development. Such was the case in Experiments 1 and 2. In fact, rodent studies have suggested that combined TP + P neonatal treatment, rather than additively masculinizing the female fetus, actually prevented steroid induced sterility (Cagnoni et al., 1965; Kincl and Maqueo, 1965).

Theoretical and clinical issues. This series of studies have examined and analyzed the three processes of sex differentiation in mice. Progestins were found to alter the respective differentiation systems of males and females. The data also raise theoretical questions concerning the physiological mechanism for normal masculine differentiation.

Many researchers have been concerned with the actual physiological conditions which foster male development. The key question concerns the actual hormone that triggers or indelibly imprints masculinization in both the normal untreated genetic male or the neonatally steroid stimulated genetic female. Biochemical and behavioral studies have supported a theory which postulates that aromatization of testosterone to estradiol is the crucial and rate-limiting step for masculine differentiation. The fact that the central nervous system is capable of aromatizing androstenedione to estrone (Naftolin et al., 1972) and that the administration of estrogen to newborn female rats will

function like testosterone to inhibit the adult display of lordotic responsiveness (Whalen & Nadler, 1963) makes this theory credible. In fact, advanced biochemical techniques have lent support to this aromatization hypothesis (Lieberburg & McEwen, 1975).

The results of this dissertation raise questions concerning an aromatization hypothesis which asserts that steroids _must_ be aromatized to estrogens to trigger or "organize" the processes of male sex differentiation. Although the results of Experiment 1 with the parent progestational compounds can be interpreted to support this hypothesis, the results of Experiment 2 are inconsistent with such a theory. Progestin metabolites, incapable of being aromatized to estrogens in neural tissue, nevertheless defeminize both gonadotrophin and behavioral processes. Although aromatization may be a sufficient condition for male sex differentiation and defeminization, it is not a necessary one.

Although an understanding of progestin action clinically was not a primary purpose of this research, nevertheless certain clinical applications may be drawn. Progestins have often been prescribed as therapeutic agents during pregnancy (Wilkins, 1960; Yalom et al., 1973). Yet the safety and utility of such compounds have never been fully ascertained and in fact warnings to the contrary have been ignored. For example, in 1958, Wilkins et al. published a

paper in the Journal of Clinical Endocrinology entitled "Masculinization of the female fetus associated with administration of oral and intramuscular progestins during gestation," in which they described the extensive peripheral virilization (clitoral hypertrophy and labia-scrotal fusion) resulting from gestational therapy with progestins. Two pages prior to the account of this research, a drug manufacturer ran a half page advertisement on the benefits of Norlutin, a synthetic progestin. Clearly, at that time an integration between laboratory experimentation and clinical practice was not as yet achieved.

Current medical practice (Goodman & Gillman, 1975) still suggests that non-androgenic progestins be administered in threatened or abortion-prone pregnancies. The results of Experiment 2 may now require a re-examination of this practice. Progestin metabolites, which were non-androgenic and non-virilizing anatomically, nevertheless were still defeminizing. Thus the absence of anatomical pathology should not necessarily be taken as a sign that a steroid is harmless.

On a more positive note, Experiments 3 and 4 have e extended the idea that progestins are anti-androgenic. This anti-androgenicity of progestational compounds may therefore allow their application in clinical cases of prostatic carcinoma (Hansson & Tveter, 1971) and precocious puberty (Schoen, 1966) and sexual behavior pathologies (Money, 1970).

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