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THE EFFECTS OF GONADOTROPIC HORMONES ON ADRENAL FUNCTION

IN THE MALE PSEUDOHERMAPHRODITE RAT

A DISSERTATION

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in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1968

THE EFFECTS OF GONADOTROPIC HORMONES ON ADRENAL FUNCTION

IN THE MALE PSEUDOHERMAPHRODITE RAT

APPROVED BY

DISSERTATION COMMITTEE

TO MY WIFE,

THE REASON FOR THIS ENDEAVOR

ACKNOWLEDGEMENT

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CHAPTER I

INTRODUCTION

The great variety of sexual characters and the pronounced sexual dimorphism found among many living forms have always intrigued the imagination of biologists. Indeed, much of the biological advancement in the area of reproductive physiology can be attributed to the peculiar, disparate or divergent sexual states existing in nature. These states have led to inquiries which have produced a greater understanding of reproductive systems and related phenomena. The present work was undertaken because an apparent hermaphroditic condition was found to exist in the laboratory rat which did not conform to current endocrinologic concepts.

The aim of this dissertation is to define and quantitate the factors involved in adrenal enlargement and hyperfunction of the adrenal androgen biosynthetic pathway in the male pseudohermaphrodite rat. It is hoped that the conclusions drawn will be more far-reaching than simply the elucidation of this anomaly.

Historical Review: the Endocrinology of Reproduction

Although the scope of this dissertation is centered on a small segment of reproductive physiology, the methods employed and conclusions drawn were dependent on concepts encompassing the entire field. It seems therefore quite appropriate to review, briefly, the development of basic concepts of reproductive physiology which have made this research possible.

The endocrinology of reproduction had its inception in the mid-1800's when Berthold (1849) demonstrated the existence of an internal secretion in the gonads by means of testis transplants in capons. However, the dependence of the internal secretion of the gonads upon the pituitary was not to be uncovered for another 50 years. In 1908, Paulesco first described gonadal atrophy following hypophysectomy. Two years later, Crow, Cushing and Homans (1910) reported amelioration of gonadal atrophy after hypophysectomy by pituitary implants. Regulation of gonadal function by pituitary hormones was firmly established as a result of the separate investigations of Smith (1927), Smith and Engle (1927), and of Aschheim and Zondek (1927) and Zondek (1927). During the 1920's, it was also learned that gonadal hormones modify hypophyseal secretion of gonadotropic hormones. A reciprocal relationship between the pituitary and gonads was first suggested by Engle in 1929. A correlation of the gonadotropic activity of the pituitary and the stage of the estrous cycle was made by Smith and Engle (1929) during this same period of time. They found that the guinea pig pituitary was less potent during estrus than during diestrus. Engle (1929) and Evans and Simpson (1929) reported that the gonadotropic hormones of the anterior lobe of gonadectomized animals increased in potency following castration. Engle suggested that the gonads affected the release of gonadotropins from the pituitary and thus in the absence of the gonads the gonadotropic hormones were stored. In 1927, Kallas reported that if a normal immature female rat was surgically united in parabiosis with an immature castrated rat, the normal twin came to precocious sexual maturity, indicating a marked increase in pituitary secretion following castration. Kallas was also able to prevent the increased gonadotropic activity of the castrate partner by injecting estrogen into the castrate animal. Shortly thereafter, Moore and Price (1932) reported that the deleterious effects of estrogens and androgens on the testes and ovaries did not appear if a gonadotropic extract were injected simultaneously with the gonadal hormones. From this experiment they concluded that the gonadal hormones did not have a harmful effect on the gonads, but inhibited the secretion of gonadotropic hormones by the pituitary with the result that the gonads became atrophic.

Evidence for the secretion of a follicle-stimulating hormone as a separate factor from the luteinizing hormone was first shown by Aschheim and Zondek in 1927 and by Zondek in 1930. Fractionation of the pituitary gonadotropic extracts into follicle-stimulating hormone and luteinizing hormone was first accomplished by Fevold, Hisaw, and Leonard in 1931. The fractionation of pituitary extracts into two separate and active constituents created a necessity for a definitive assay procedure for determining the completeness and potency of such fractions. This necessity ushered in the era of biological assays.

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an influence on the uterus through the medium of an internal secretion. Following the important work of Knauer and Halban, Adler, in 1912, prepared extracts from the ovary of sows which produced hypertrophy of the uterus. Stimulated by the pioneer work of Allen and Doisy in 1923 on the bioassay of the ovarian estrogenic substances and by the discovery of Aschheim and Zondek in 1927 of the presence of large amounts of estrogenic material in the urine of pregnant women, the stage was set for isolation and characterization of sex steroids as cyclopentanophenanthrene derivatives. The substance we now know as estrone was isolated from human pregnancy urine in a crystalline state simultaneously by Doisy, Veler and Thayer (1929), Butenandt (1929) and Dingemanse et al. (1930). In 1930, a second crystalline estrogen, estriol, was isolated by Marrian and by Doisy et al. After the sterol-bile acid formula had been proposed by Rosenheim and King in 1932, the likelihood that steroids were phenanthrene derivatives occurred to many investigators. This likelihood became a certainty when Butenandt, Weidlich and Thompson (1933) proved the estrogens to be phenanthrene derivatives. Complete proof of the structures of the natural estrogens was achieved largely as a result of the work of Cohen and his collaborators (1934; 1935). This work was facilitated by a new extraction procedure devised by Girard and Sandulesco (1936) for obtaining pure estrogens in high yield. Girard and his collaborators extracted large quantities of urine with an organic solvent and then used a compound (chloride of trimethylaminoacito-hydrazine) which reacts with ketones to render them soluble in water.

Perhaps the most remarkable achievement in all this early work on the estrogenic substances was the isolation of estradiol-17β from the liquor folliculi of sow ovaries by MacCorquodale, Thayer and Doisy in 1936. The amount of estrogen stored in the ovary is so small that 400 liters of liquor folliculi were processed in order to obtain the few milligrams of crystalline substance necessary for characterization.

The modern concepts of the hormonal action of the corpus luteum originated in part from experiments carried out by Fraenkel in 1903. He showed that removal of the corpus luteum in the first few days of pregnancy caused the embryos to disappear. Perhaps the first theory of function of the corpus luteum to receive support from later investigations was that it might exercise an inhibitory influence on ovulation. The theory of inhibition of estrus was tested experimentally by Loeb in 1911, who demonstrated that in guinea pigs removal of the corpora lutea accelerated the next ovulation. Bouin and Ancel in 1909 were the first to demonstrate that the corpora lutea were responsible for the progestational changes in the rabbit uterus. They showed that the removal of the corpora lutea by excision prevented formation of the progestational condition. Hence the classical observations of Fraenkel, Loeb, and Bouin and Ancel established the corpus luteum as an endocrine gland whose primary function was to prepare the uterus for implantation of the fertilized ovum.

Clear-cut evidence for the presence in extracts of the corpus luteum of a principle active in producing progestational changes in the uterus was obtained by Corner (1929) and by Allen (1930). Six years later the isolation of this active principle as a pure crystalline substance was reported simultaneously by Butenandt and Westphal (1934), Allen and Wintersteiner (1934), Slotta, Ruschig and Fels (1934) and by

Hartman and Wettstein (1934). The complete structure of progesterone was elucidated by Butenandt and Westphal (1934) and independently by Fernholz (1934). Pregnanediol, which later was to assume a position of some importance in the progesterone story, had originally been isolated from human pregnancy urine by Marrian in 1929. Shortly thereafter Butenandt (1930) established the correct molecular formula. The possible biochemical significance of pregnanediol, although quite obscure at the time of its first isolation, became apparent as soon as the molecular formula and chemical nature of progesterone were established. Final proof of the metabolic relationship between pregnanediol, the excretory metabolite of progesterone, and progesterone was obtained by Venning and Browne in 1937. They were able to show that during the menstrual cycle, pregnanediol was excreted in the urine only during the luteal phase of the cycle.

The foundation was laid for the extraction and characterization of the androgens with the discovery by McGee, Juhn and Domm (1928) that a lipid concentrate from the testes of bulls produced a growth response in the combs of capons. The first pure crystalline androgen was obtained not from the testis, however, but from urine. In 1928, Loewe *et al.* reported the presence of an androgenic substance in human male urine, and six years later Butenandt and Tscherning (1934a; 1934b) and Ruzicka *et al.* (1934; 1935) were successful in isolating an active saturated hydroxyketone, androsterone, from this source. A second crystalline androgen, dehydroepiandrosterone, was isolated from human urine by Butenandt and Dannerbaum in 1934. Finally, in 1935 the testicular androgen was isolated as a pure crystalline substance by David, Dingemanse and Laqueur and named testosterone. Almost immediately afterward, testosterone was obtained by a series of transformations, using dehydroepiandrosterone as a starting material, by Butenandt and Gunter (1935) and by Ruzicka and Wettstein (1935).

In 1934, Reichstein isolated a crystalline substance from the adrenal cortex, adrenosterone, which was subsequently shown to be a C_{19} compound possessing androgenic potency. This fact, together with the fact that certain of the C_{21} compounds could be degraded to androgenic compounds led Reichstein to suggest that the adrenocortical substance might be similar in structure to the sex steroids. Research on the urinary androgens and the other related neutral 17-ketosteroids was greatly facilitated by the well-known colorimetric method which was first suggested by Zimmermann in 1935. Many modifications in the Zimmermann method have since been made. Nevertheless, it seems clear that the chief credit for first developing a reliable and accurate method for assaying urinary 17-ketosteroids must be given to Zimmermann.

Male Pseudohermaphroditism: Description of Genotype and Phenotype in the Rat and Man

Male pseudohermaphroditism is a sexual condition characterized by a disparity between the genotype and phenotype of a male individual. A defect involving responsiveness of somatic tissues to androgens is the primary lesion which produces this anomaly (Morris and Mahesh, 1963). Genetic study of this anomaly in the rat began with the discovery by Stanley and Gumbreck (1964) of a single litter of F_3 King x Holtzman hybrid rats. This litter was born of parents of normal phenotype and contained 1 normal male, 5 females and 2 pseudohermaphrodites. From this single litter a colony has been established. If only the litters containing all three types are considered, a recent sample of 210 litters comprising a total of 1364 animals contains 620 females, 372 males and 372 male pseudohermaphrodites. Inspection of the above figures shows that the defect is found in half of the males in litters in which it occurs. This is the classical ratio produced by a sex-linked gene.

When dams that had produced pseudohermaphrodites were outbred to males of an unrelated line, the ratio of normal males to male pseudohermaphrodites was still 1:1. Of such matings there were 26 litters containing 160 animals: 71 females, 44 males and 45 male pseudohermaphrodites. When sires of pseudohermaphrodites were outbred to females of an unrelated line there were no pseudohermaphrodites produced. In 19 such litters, there was a total of 152 animals: 85 normal females and 67 males with no pseudohermaphrodites. Thus, these data show that only the female transmits the defect. Since male offspring obtain their single X chromosome only from their mothers and since there are the two classes of males, normal and pseudohermaphrodites in equal numbers, the gene for this trait must be carried on the X chromosome. According to Castle and King (1940) there are now five well established linkage groups for the rat and none of the known genes thus far mapped occurs in association with the gene for pseudohermaphroditism. Its sporadic occurrence in widely separated general populations such as that of the mouse, pig, goat, rat and man would seem to indicate that a normal gene must mutate with some regularity to product it.

Since pseudohermaphrodites are all sterile and cannot therefore transmit the gene for the defect, it cannot be said with certainty that this gene is either a dominant or a recessive one. There seems to be no logical alternative over that of a single sex-linked recessive. Evidence now at hand proving that these animals are genetic males consists of the following facts:

1) The nuclei of liver cells of pseudohermaphrodites are chromatin negative as in normal male littermates, while Barr bodies are clearly observed in nuclei of liver cells of female littermates (Allison, Stanley and Gumbreck, 1964).
2) The karyotype of the pseudohermaphrodite is the same as that of normal males, both showing in particular a single X and Y chromosome (Allison, Stanley and Gumbreck, 1964).
3) The sex ratios among offspring indicate that these animals are genetic males (Stanley and Gumbreck, 1964).

In affected animals the entire reproductive tract, with the exception of small bilateral testes, is missing. These testes usually lie in the perineal region and since there is no scrotum they can only descend through the inguinal ring to a position just outside the body cavity. The possibility of high temperature damage to these gonads exists and may account partially for the failure of spermatogenesis to occur. Occasionally one or both of these testes will remain inside the body cavity.

Nipples are formed along the milk line in the same number and position as in the female. They become visible at an early age and although the amount of growth does not approach that of the female they can be easily seen throughout the life of the animal. A vaginal opening is patent but small in about 50 per cent of the affected animals, being only 1 to 2 millimeters in depth and ending in a blind cul de sac. The other half of the affected animals exhibits only a scar which is completely closed but visible in what would be the vaginal position in a female, indicating late closure of the embryonal raphe (Stanley and Gumbreck, 1964). Affected animals resemble females in early life and the difference in external genitalia and nipple line are not readily apparent at birth. Even at 21 days of age, it is often difficult to identify a pseudohermaphrodite with certainty (APPENDIX I, Page 129). Body size and weight of the adult animals are intermediate between those of normal males and females. The average weight of 15 normal females of the strain is 239 grams, that of 15 males 415 grams, while that of 15 male pseudohermaphrodites is 318 grams. Significant difference in the wet weights of some of the endocrine glands is also seen. The pituitary and adrenal glands of the pseudohermaphrodites are significantly larger than those of normal males and females. The kidneys are also significantly smaller in size and weight than those of either normal males or females on the basis of body weight. The kidney weight/body weight ratio for normal males is .0067, for normal females .0068 and for the pseudohermaphrodites .0062. APPENDIX II (Page 135) lists the comparative organ weights for males, females and pseudohermaphrodites.

Sterility in the pseudohermaphrodite is rather obviously reflected in the histological picture of the testes. In most of the seminiferous tubules, the germinal epithelium consists of a thin layer, one or two cells in thickness. The cell types consist of spermatogonia, Sertoli cells and usually some large primary spermatocytes (APPENDIX I, Page 130). Secondary spermatocytes, spermatids and spermatozoa are never observed. In contrast to the germinal line, there is a marked hyperplasia of the interstitial cells of Leydig (APPENDIX I, Page 131). In a large proportion of the older pseudohermaphrodites, one and occasionally both of the testes develop Sertoli cell tumors. Some of these become quite large weighing up to 10 grams. The testicular neoplasms vary considerably in structure from area to area. Some sections reveal a peripheral rim of seminiferous tubules which are lined by Sertoli cells showing moderate to marked hyperplasia. This testicular tissue then merges into neoplastic tissue. The neoplasm varies from areas in which there is a tubular arrangement associated with an overgrowth of Sertoli cells, while in other areas there are islands of cells which are generally polygonal in shape and appear to be finely vacuolated. Many areas, however, are characterized by spindle-shaped cells which have a whorled appearance. In these areas there is no resemblence to parent tissue. No crystalloids of Reinke or lipochrome pigment are ever encountered (APPENDIX I, Page 132-133).

The time and site of action of the gene which produces pseudohermaphroditism have now been established. By removal of embryos at different stages of development from pregnant mothers that had produced one or more litters containing pseudohermaphrodites, Allison (1966) found that the reproductive tract began to degenerate by day 17 of the pregnancy and by day 19 all vestiges of it had completely disappeared, with only the testes remaining. The anlage for the ductal and accessory systems were present and appeared to be normal until day 17 in utero. Regression appeared to be under way at day 17 and was complete by day 19 in utero.

In order to understand the significance of this anomaly, reference must be made to the work of Jost (1958). Using the rabbit as an experimental model, Jost castrated embryos of pregnant females before day 19 in utero. He found that castration did not have any adverse effect on the subsequent development of the female reproductive tract of castrated female embryos. In marked contrast to the females, castrated males developed a complete female reproductive tract. This classical experiment demonstrated the necessity of the presence of the testes for the masculinization of the male reproductive anlage. To go one step further, Jost transplanted unilaterally a testis into the vicinity of the developing reproductive anlage of female embryos. On the operated side of the females, the Müllerian anlagen (female counterpart) were inhibited and the Wolffian (male counterpart) derivative developed. On the intact contralateral side, the Müllerian anlagen developed as expected.

The results of the work of Jost offer a functional explanation for the absence of the reproductive tract in the male pseudohermaphrodite. The Wolffian or male counterpart of the reproductive tract does not develop because pseudohermaphrodites are insensitive to androgens. Consequently, the pseudohermaphrodite cannot induce formation of a male reproductive system. Furthermore, the Müllerian or female counterpart of the reproductive tract does not develop because the testes inhibit development of the female anlage. Hence, the net result is a complete absence of the reproductive tract of either male or female origin.

In 1953, Morris suggested the term "testicular feminization" for an hereditary syndrome occurring in man, characterized by individuals with testes but otherwise female phenotype. These individuals have complete female urogenital sinus derivatives, lack development of both Müllerian and Wolffian duct systems and exhibit female secondary sex characteristics. The syndrome is hereditary with transmission through the maternal line. The carriers are usually normal females, but decreased axillary hair or pubic hair has been noted in otherwise normal mothers.

Jacobs et al. (1959) and Puck, Robinson and Tijo (1960) have found the sex chromosome complement in cases of this syndrome to be XY. Others have confirmed this finding (Bergada et al., 1962; Allexander and Ferguson-Smith, 1961). Grumbach and Barr (1958) have pointed out that the ratio of female to male to affected male approximates 2:1:1 leading to the conclusion that this syndrome is either a sex-linked recessive trait or a sex-limited autosomal dominant one.

In man, the term "testicular feminization" has been inappropriately applied to cases exhibiting clitoral enlargement. In the "complete" form of the syndrome the clitoris is normal or small. Undoubtedly, there may be some with minimal clitoral enlargement who present a closely related anomaly, especially when associated with female secondary sex characteristics. There may be a similar hereditary etiology, but secondary sex characteristics even with slight enlargement of the clitoris are unpredictable. They may resemble the complete form of feminization and in such instances probably have a similar etiology, they may be essentially males, they may be intermediate with sexual hair and breast development, or they may have no pubic hair and no breast development (Morris, 1953). While both syndromes are hereditary, the complete feminization syndrome and the syndrome with clitoral enlargement do not occur in the same family tree (Morris and Mahesh, 1963). Because the secondary sexual characteristics and the genital abnormalities vary, cases with clitoral enlargement should be considered as separate. Any attempt at comparison between pseudohermaphroditism in man and the rat is extremely hazardous, unless the complete form in the human is well established.

The histological picture of the testes in human pseudohermaphrodites closely resembles that occurring in the rat. There is a predominance of tubules containing mature Sertoli cells (Morris and Mahesh, 1963; David et al., 1963; Kase and Morris,1965). The tubules appear to be lined principally by germ cells in association with the Sertoli cells. The occurrence of large aggregates of Leydig cells bears an amazing resemblance to Leydig cell hyperplasia found in male pseudohermaphrodite rats. The incidence of neoplasia in these gonads is difficult to ascertain, since the majority of cases reported in the literature have only reached their late teens.

Male Pseudohermaphroditism: Endocrine Aspects in the Rat and Man

The similarity of male pseudohermaphroditism in the rat to that in man is striking in several aspects, one of the most interesting being the lack of response of somatic and sexual tissue to administered androgens. Notable in the rat is the lack of effect of testosterone on the kidney, as well as the urinary papilla which shows no stimulation by growth or even hyperemia (Stanley, Gumbreck and Easley, 1966). The positive growth effect of testosterone on the kidney of the normal rat has been well documented (Korenchevsky and Ross, 1940). The presence of high urinary 17-ketosteroid levels in human pseudohermaphrodites with absence of body hair suggested to Wilkins (1957) that the disorder might be due to a peripheral lack of responsiveness to androgens. This hypothesis was supported by his observation that large doses of methyl testosterone caused no growth of sexual hair, enlargement of the clitoris or change in voice. These studies have not established whether the defect is due to an end organ refractoriness to androgen, to the formation of an androgen inhibitor or to peripheral conversion of androgens to estrogens.

It has been amply demonstrated that testosterone is an anabolic agent capable of bringing about a positive nitrogen balance (Brown and Samuels, 1956; Kenyon et al., 1940; Knowlton et al., 1942; Landau et al., 1950). When food intake is held constant, urinary nitrogen decreases. In addition, there is a similar fall in urinary phosphorus. The finding that patients with the complete form of testicular feminization fail to show this anabolic response when treated with large doses of testosterone supports the hypothesis of somatic tissue refractoriness and indicates that the defect involves the anabolic as well as the virilizing effects of the hormone (French et al., 1966). The urinary excretion of citrate has been shown by Shorr, et al. (1948) to decrease after treatment with testosterone. This effect of testosterone appears to be the result of an increased renal tubular reabsorption of citrate. The lack of this response in human pseudohermaphrodites makes it appear that the somatic tissue defect in the complete form of so-called testicular feminization encompasses all effects of the hormone (French et al., 1966).

Endocrine studies performed on patients with this syndrome have in general shown normal or elevated levels of urinary 17-ketosteroids, female levels of urinary estrogens, cornification of the vaginal mucosa and elevated gonadotropins. With castration there has been reported a fall in 17-ketosteroids and estrogens, decrease in vaginal cornification and a rise in gonadotropins indicating at least in part a gonadal hormone source. Stimulated by studies such as those summarized above, Stanley and co-workers (1967) reported steroid determinations of the plasma of male pseudohermaphrodite rats. The titers of the three estrogens, estrone, estriol and estradiol-17β in the pseudohermaphrodite rats were approximately double those of their normal male controls. Dehydroepiandrosterone was about 6 times greater in plasma of pseudohermaphrodites than in that of normal male controls, while androsterone values were equal in both. Adrenosterone and etiocholanolone were between 2 and 3 times as great in pseudohermaphrodites as in the controls. APPENDIX II (Page 136) summarizes the above results. These values indicate some similarity between this syndrome in the rat and male pseudohermaphroditism with feminizing testes in man. David _et al_. (1965) have also reported a marked increase of urinary dehydroepiandrosterone in human pseudohermaphrodites. Normally the testes secrete little or no dehydroepiandrosterone, the adrenal being the chief contributor to this neutral 17-ketosteroid. _In vitro_ studies of human pseudohermaphrodite testes demonstrate two biosynthetic pathways to testosterone, one from 5Δ pregnenolone via 17-hydroxypregnenolone and dehydroepiandrosterone and a second via progesterone and 17-hydroxyprogesterone (David _et al_., 1965; Green, Miller and Hamwi, 1963). The fact that urinary dehydroepiandrosterone of testicular origin is negligible in normal men and is present in significant quantity in rats and patients with this syndrome supports the view that the preponderance of the former pathway is an unusual finding that may be of significance in the etiology of this syndrome.

In human pseudohermaphrodites, measurement of urinary steroids before and after castration have shown that the testes make a significant contribution to the urinary 17-ketosteroids and estrogens (Ikkos, Tillinger and Westman, 1959; Salassa _et al_., 1961; Morris, 1953). The ability of testis slices to synthesize estradiol-17β from labeled progesterone and testosterone is consistent with the higher concentration of estrogen in testicular venous blood (French et al., 1965; Griffiths, Grant and Whyte, 1963). French et al., (1965) reported that the concentration of estradiol-17β in testicular venous blood of human pseudohermaphrodites was about 25 times greater than in peripheral blood and peripheral blood values were in the normal female range. Urinary 17-ketosteroid and 17-hydroxycorticosteroid excretion of human pseudohermaphrodites were within the normal limits for both males and females (David et al., 1963; French et al., 1965; Griffiths, Grant and Whyte, 1963; Ikkos, Tillinger and Westman, 1959; Molinoff and Armstrong, 1962; Morris and Mahesh, 1963; Salassa et al.,1961). The significant drop in urinary 17-ketosteroids following gonadectomy indicates that the testes contribute approximately one-fourth the initial control level (French et al., 1965). The high blood levels of testosterone support the view that lack of virilization in these patients is not due to a deficient production of testosterone. The results obtained from progesterone-4-^{14}C incubation studies serve to indicate that the testes are capable of producing substantial amounts of testosterone and that the enzymes of the well established pathway involving 17-hydroxyprogesterone and androstenedione are present (David et al., 1965; French et al., 1965).

Development of the features of testicular feminization in the presence of a substantial production of testosterone as well as estradiol 17β might result from one of the following mechanisms:

1) Formation of an androgen inhibitor (Neher et al., 1965)
2) Peripheral conversion of androgens to estrogens (French et al. 1965).

3) Other disorders of androgen metabolism (David _et al_., 1965; Rivarola _et al_., 1967).

4) An end organ refractoriness to androgens (Morris and Mahesh, 1963).

Recent studies have demonstrated that estrogens can inhibit the effects of androgens (Hoskins and Koch, 1959; Lauritzen, 1964). The inhibition is seldom complete, however, and can be only accomplished by doses which greatly exceed physiological levels. If there was a defect in catabolism of estradiol-17β that blocked its conversion to less active compounds, then a large quantity would be available to inhibit the effects of testosterone. Although plasma estradiol-17β is elevated in pseudohermaphrodites, the proportion of plasma estrogen to testosterone is still less than that in females, who develop pubic and axillary hair in response to androgens. Hence, it seems unlikely that lack of virilization can be explained on the basis of androgen inhibition by estrogen. Furthermore, an androgen inhibitor would necessarily have to originate outside the testes, since patients with feminizing testes do not respond to treatment with testosterone even after removal of the gonads. A large peripheral conversion of testosterone to estrogen can be excluded both by failure to recover labeled estrogen in the urine following administration of ^{14}C labeled testosterone and by the absence of increase in either plasma or urinary estrogens during the course of treatment with testosterone (French _et al_., 1965). Another possibility which can be ruled out is a block in the conversion of dehydroepiandrosterone to androstenedione, involving the enzyme 3β-hydroxysteroid dehydrogenase. This possibility seemed probable since dehydroepiandrosterone is elevated in the pseudohermaphrodite. However, the finding of testosterone levels in the human pseudohermaphrodite in the normal male range excludes this possibility (Gwinup *et al.*, 1966; Deshpande *et al.*, 1965; Horton, Shinsako and Forsham, 1965; Kase and Morris, 1965; Pion *et al.*, 1965; Rosner *et al.*, 1965; Sharma, Dorfman and Southren, 1965; Simmer, Pion and Dignam, 1965; Southren *et al.*, 1965.

A final possibility which must be considered is an alteration of the target site, making the peripheral organs refractory to the effects of testosterone. This mechanism is suggested by the observation that testosterone blood levels are equivalent to those of normal males, and the observation that these patients fail to virilize when given massive doses of testosterone (Morris and Mahesh, 1963).

Working Hypothesis: Supporting Evidence for Methodological Approach

The information given in the previous sections has laid the groundwork for the formulation of a working hypothesis originally set forth before the work undertaken in this dissertation was begun. The working hypothesis is as follows:

> The cause of adrenal enlargement and hyperfunction of the adrenal androgen biosynthetic pathway in the male pseudohermaphrodite rat is due to a tropic stimulation of the adrenal cortex by an elevated titer of pituitary gonadotropic hormones.

It is to be noted that the above postulate excludes the pituitary hormone, adrenocorticotropin (ACTH), as the tropic factor responsible for producing the adrenal enlargement in the male pseudohermaphrodite rat. The rationale for the working hypothesis is based on a number of observations previously noted. Paramount among these observations are the enlarged pituitary and adrenal glands of the male pseudohermaphrodite rat. Hence, the objective of this dissertation is directed towards elucidating the cause or causes of enlargement of these organs. Implicit in this objective is a thorough study of the endocrine status of the pituitary and adrenals in these animals.

The enlarged adrenal glands were construed to result from an increased metabolic activity of the adrenal cortex. According to a widely accepted view, the pituitary hormone, adrenocorticotropin (ACTH), is the sole pituitary factor which can mediate such an increased metabolic activity of the adrenal cortex. A disparity in the above observation and this accepted view soon became apparent when it was noted that the pseudohermaphrodite rats seemed to be as healthy as their littermate siblings. Adrenal hypertrophy and hyperplasia are generally associated with pathology of the Cushingoid type, due to an increased output of glucocorticoids which results in protein catabolism, gluconeogenesis and gastrointestinal pathology. In other words, if adrenal enlargement was due to an increased secretion of ACTH from the pituitary gland, one would expect to find an increased titer of glucocorticoids and possibly some concomitant pathology. Since these animals were asymptomatic of Cushingoid pathology, doubt was cast on the idea that adrenal enlargement was mediated via ACTH.

An additional finding which led to the formulation of the working hypothesis was the demonstration of a markedly elevated titer of androgens in the pseudohermaphrodite rats (Stanley, Gumbreck and Easley, 1967). Again, if adrenal enlargement was related to an increased adrenal output of androgens, current concepts would suggest mediation by ACTH. This being true, there would then have to be a concomitant increase in the glucocorticoids at least of the magnitude as that seen in the sex steroids. Such an increase would surely lead to a pathological state.

The last finding which led to the establishment of the working hypothesis was the demonstration that the pseudohermaphrodite pituitary had an FSH content of only 20 per cent of that of normal male controls (Easley, 1966). In association with the low pituitary FSH content, the interstitial cells (Leydig cells) of the pseudohermaphrodite testes showed some hyperplasia. This finding indicated an increased output of gonadotropins, resulting in an increased stimulation of the interstitial cells.

The working hypothesis seemed to be consistent with the findings of adrenal and pituitary enlargement, absence of Cushingoid pathology, elevated androgen titer and indications of an increased gonadotropin output. However, before accepting the working hypothesis, an experimental design was constructed to test the validity of the hypothesis. Included in the design are the following four conditions:

1) Gonadotropin titers must be greater than that of male and female controls.
2) 17-ketosteroid production from the adrenal glands must be greater than that of male and female controls.
3) Corticosterone production must be in the normal range of male or female control values.
4) Supression of gonadotropin output must depress adrenal 17-ketosteroid production without affecting the corticosterone values.

The rationale for these conditions is based upon the concomitant effects one would expect to find from an elevated gonadotropin titer, if indeed the gonadotropins are capable of acting as a tropic factor on the adrenal cortex. The gonadotropins (primarily LH) are known to induce steroidogenesis of androgens in the testes. Since the biosynthetic pathway in the adrenal glands is the same as that occurring in the gonads for the sex steroids, it does not seem unreasonable that gonadotropins could stimulate the production of androgens in the adrenal glands. On the other hand, stimulation of the adrenal corticosteroid pathway by the gonadotropins is not expected. Hence, corticosterone values should be within the normal range of control animals. Finally, suppression of gonadotropin output should decrease the adrenal production of androgens without affecting the corticosteroid values, if the gonadotropins are the tropic factors involved. Failure to confirm any of the stated conditions would negate the validity of the hypothesis.

Because the basic postulate of this dissertation differs to some extent from the current concepts of adrenal function, these concepts are reviewed here. A view that came into wide acceptance during the 1940's held that the pituitary hormone, adrenocorticotropin (ACTH), was the only pituitary factor involved in maintenance of adrenal cortical function (Sayers and Sayers, 1948). Implied in this view was the assumption that there exist only one hormonal secretory zone, the zona fasciculata or middle cortical zone. This postulate supported the cell migration theory, which accordingly, assigned a germinative role to the zona glomerulosa (outer zone), secretory role to the zona fasciculata (middle zone) and considered the zona reticularis (inner zone) an area of cell decay (Deane and Greep, 1946; Jones, 1948). In more recent times, facts have accumulated which challenge this scheme. Deane and Greep were the early protagonists who favored a theory suggesting that the zona glomerulosa secretes a hormone of the deoxycorticosterone type concerned primarily with electrolyte metabolism, while that of the zona fasciculata produced steroids dealing with carbohydrate and protein metabolism. The zonal theory of adrenal function, as it is known today, was based on the observation that after hypophysectomy the zona fasciculata atrophied. On the other hand, the zona glomerulosa was not altered markedly after hypophysectomy (Dean and Greep, 1946). Tying together morphology and function, the zona glomerulosa exists independently in animals with adequate salt intake, while the zona fasciculata degenerates in hypophysectomized animals. Hence, it is a logical conclusion that the zona glomerulosa is connected with salt metabolism, involving the deoxycorticosterone type of steroids, while the zona fasciculata is the site of production of the glucocorticoid type of steroids concerned with carbohydrate and protein metabolism. Furthermore, according to the zonal theory, the functions of the zona fasciculata are directly under the control of ACTH while the zona glomerulosa is largely independent of pituitary control.

Greep and Deane (1947) studied the effects of the salt retaining steroid, deoxycorticosterone acetate, on the adrenal cortex in an attempt to define the function of the zona glomerulosa. They found that this steroid produced a zona glomerulosa lacking in sudanophils, Schiff-positive substances, and in birefringent and autofluorescent materials. These changes were in accord with the idea that the zona glomerulosa was no longer secreting a hormone for which deoxycorticosterone acetate would serve as a substitute and that they were due to atrophy of disuse. Going one step further, Deane and Greep (1948) altered the ratio of sodium and potassium by giving diets deficient in sodium or potassium. Sodium depletion produced a zona glomerulosa much thicker than normal, while potassium depletion produced a zone reduced in thickness. Likewise, administration of potassium chloride caused hypertrophy of the zona glomerulosa. These changes were interpreted to mean that depression of the sodium-potassium ratio caused increased secretion by the cells of the zona glomerulosa. Conversely, elevation of the sodium-potassium ratio produced changes in the zona glomerulosa indicative of inactivity. Further support was added to the zonal theory of adrenal function when it was demonstrated that ACTH administration did not cause salt retention in the rat (Ingle, Li and Evans, 1946; Ingle et al., 1947; Bergner and Deane, 1948). Also, on the basis of histochemical changes, the zona fasciculata showed evidence of increased activity after ACTH administration while the zona glomerulosa remained unchanged (Bergner and Deane, 1948).

The concept of adrenal cortical physiology as it has been developed above indicated two secretory adrenal zones and two types of hormones. An extension of this theory will be considered which involves the very basis of this dissertation. Androgens are produced by the adrenal cortex and the zona reticularis has been designated to be the site of secretion (Reifenstein et al., 1945; Albright, 1947). Implicit in this extension is the possibility that some other pituitary hormone besides ACTH has a direct influence on the adrenal cortex. In this regard, the mouse adrenal is of special interest because it possesses a characteristic juxtamedullary zone which shows variations with age and sex and which has been thought to be the androgenic layer (Howard-Miller, 1927; Grallman, 1936; Waring, 1935).

Mice possessing a juxtamedullary x-zone, fall into three classes:

male mice before puberty, prepuberally castrated male mice and female mice. Jones (1948a; 1948b) reported that when mice in these categories were hypophysectomized, the x-zone disappeared. However, ACTH administration to hypophysectomized mice caused the zona fasciculata to assume the appearance of a normal adrenal, while the x-zone was unaffected. In addition, Jones found that administration of a pituitary gonadotropin extract, principally LH in nature, maintained the x-zone but did not affect the zona fasciculata. To what extent pituitary LH influences the adrenals of other species is not known, but it is interesting to note that in the ground squirrel the adrenal cortex hypertrophies during the breeding season and this can be duplicated in the non-breeding period by giving pituitary gonadotropins (Moore _et al_., 1934; Zalesky, 1934; Zalesky, Overholser and Gomez., 1941). It is of particular interest that the hypertrophy appears only in the outer part of the zona reticularis.

CHAPTER II

MATERIALS AND METHODS

Management of Experimental Animals

Male pseudohermaphrodite rats were obtained from a colony of King x Holtzman hybrids established in 1959 by Stanley and Gumbreck. Large numbers of these animals were produced by breeding females who carried the gene for pseudohermaphroditism. Since the anomaly was transmitted as a sex-linked recessive gene, statistically one-half of the males in any sibship were affected. Experimental animals were housed in the animal quarters at the University of Oklahoma Medical Center. Litters were weaned at 30 days of age and placed on Purina laboratory chow consisting of 23% protein, 4.5% fat, 6% fiber and 9% ash. Unless otherwise specified, animals employed in experiments were between 5 and 6 months of age. Male and female control rats were littermates of male pseudohermaphrodites. When females were employed as control animals, only virgins were selected. Experimental and control animals were comparable in age and within group variation in body weight was held to a minimum by selecting animals within a 25 gram weight range.

Urine for steroid determinations was collected by placing experimental and control rats in metabolic cages equipped for this purpose. Urine was collected twice daily at 12 hour intervals, the volume measured in a graduate cylinder and pooled into 24 hour specimens. Several drops of hydrochloric acid were placed in the collection bottles to decrease bacterial growth, and pooled 24 hour collections were stored in a freezing unit. Metabolic cages were cleaned thoroughly each day and resupplied with water and laboratory chow.

Assay of the Luteinizing Hormone

The principle utilized in the assay of the luteinizing hormone (LH) depends on the ability of LH to specifically deplete the ovary of ascorbic acid. The degree of depletion is proportional to the dose of LH employed. A sample containing an unknown amount of LH was compared at several dosage levels with an NIH-LH-S_{11} reference standard. From the regression lines so obtained, the potency of the unknown was computed in terms of the reference standard.

Preparation of Assay Animals

Animals used for the bioassay of LH were females of the Holtzman strain. They were shipped from Madison, Wisconsin at 21 days of age and at 25 days of age they were prepared for bioassay with a single subcutaneous injection of pregnant mare serum (PMS, 50 I.U.). The PMS injection was followed 60 hours later by a single subcutaneous injection of human chorionic gonadotropin (HCG, 25 I.U.). As a result of this treatment the animals became pseudopregnant. They were used for bioassay during the period from 6 to 8 full days following the administration of HCG. At the time of use the ovaries were heavily luteinized and weighed consistently more than 100 milligrams each. Ovaries weighing less than 70 milligrams were discarded, since they sometimes yielded erratic results.

Bioassay Procedure

At the time of assay the animals were lightly anesthetized in an ether jar. The material to be tested was dissolved in 0.25 ml of saline and then injected intravenously via a dorsal tail vein, using a 27 gauge, half-inch needle. Application of ethanol (70%) to the tail helped to visualize the vein. All injections of pituitary extracts were made uniformly over a period of 10 to 15 seconds. All injections of plasma were made uniformly over a period of 75 seconds. Four hours after the intravenous injection, each animal was sacrificed by cervical fracture and the left ovary was dissected free and removed. The ovary was rapidly freed of surrounding fat on a moistened filter paper and briefly rolled on dry filter paper to remove adherent blood and moisture. The ovary was then quickly weighed on a Roller-Smith torsion balance to the nearest 0.2 mg and transferred to a ground-glass homogenizer where it was vigorously homogenized. Ten milliliters of 2.5% metaphosphoric acid was added to the homogenate, thoroughly mixed with the homogenate, and filtered through Whatman #40 filter paper. The filtration process removed all remnants of cell membranes. The clear metaphosphoric acid filtrates were then analyzed for ascorbic acid by the method of Mindlin and Butler (1938), as modified by Parlow (1961).

Each assay included 2 doses of a reference preparation, NIH-LH-S_{11}, in addition to the same number of doses of the unknown (pituitary extract or plasma) and a control group which received only 0.9% saline. Ten assay animals were used for each dosage level. Consequently, a usual assay employed a total of 50 animals. A total of 12 assays were carried out on pituitary extracts and plasma obtained from male, female and male pseudohermaphrodite rats ranging from 5 to 6 months of age. Plasma was separated from blood obtained by aortic puncture. For the pituitary assays, the pituitary powder was homogenized in 0.9% saline and allowed to extract for 4 hours at 4°C. All doses of the pituitary extract were administered in 0.25 ml of 0.9% saline.

Estimates of potency of the pituitary extracts and plasma were expressed in terms of NIH-LH-S_{11} reference standard. Values for the slope and standard deviation of the dose-response curves were calculated according to the method of Finney (1964). For purposes of statistical treatment, individual sample results were recorded as milligrams of ascorbic acid per 100 milligrams of ovarian weight and then converted to per cent depletion of ovarian ascorbic acid.

Chemical Determination of Ascorbic Acid

Ascorbic acid by virtue of its redox properties has the ability to reduce certain colored dyes to the colorless form. According to the method of Mindlin and Butler (1938), a solution of ascorbic acid was added to a buffered solution of the dye, 2, 6-dichlorophenol indophenol, and the degree of decoloration, which was proportional to the concentration of ascorbic acid, was observed by means of a spectrophotometer.

Reagents and Standards

1. Metaphosphoric acid solution

a. 5% solution: one liter of distilled water was added to 50 gm metaphosphoric acid (A.R. grade) and allowed to dissolve by standing in the cold. The metaphosphoric acid solution was stored at refrigerator temperature and prepared fresh every two weeks.

b. 2.5% solution: prepared fresh daily by dilution of a 5% solution with an equal volume of distilled water.

2. Ascorbic acid standards

a. Stock solution, 1.0 mg/ml: 100 mg of U.S.P. Reference Standard Ascorbic Acid were dissolved and made to 100 ml volume with 2.5% metaphosphoric acid. The stock solution was stored in a freezer.

b. Concentrated standard solution, 0.10 mg/ml: the stock solution was diluted 1:10. A sufficiently large quantity of concentrated standard solution was prepared to be used for the entire series of assays and was stored in a freezer in a number of small vials. Each vial was ample for the preparation of dilute standard solutions for one series of analyses.

c. Dilute standard solutions

	Concentration	Concentrated Standard Solution	2.5 per cent metaphosphoric acid used for dilution
	μg	ml	ml
1.	2.0	0.5	25.0
2.	4.0	1.0	25.0
3.	6.0	1.5	25.0
4.	8.0	2.0	25.0

3. 2, 6-dichlorophenol indophenol solution: 20.0 mg of 2, 6-dichlorophenol indophenol were dissolved in 100 ml distilled water at 85° to 95°C, and then diluted to 500 ml with distilled water. The solution was stored in the cold and was prepared fresh every two weeks.

4. Sodium acetate solution: 22.65 gm of sodium acetate were dissolved in 500 ml distilled water and the pH was adjusted to 7.0 by the addition of 6% acetic acid (v/v). It was stored in the cold and made fresh every week.

Analytical Procedure

The following description is based on the use of a Beckman DB-G spectrophotometer: 5 ml of indophenol-acetate solution were pipetted into each of a series of quartz colorimeter tubes. Distilled water was added to a second tube. Reading at 515 mμ in the DB-G, the distilled water blank was placed in the split beam and adjusted to read 100% transmittance. A process blank was prepared by adding 5 ml of metaphosphoric acid to 5 ml of indophenol-acetate solution. The time of addition was noted, the metaphosphoric acid added rapidly, and the tube was immediately inserted into the colorimeter so that the galvanometer reading was observed not less than 20 seconds, and not more than 45 seconds after addition of the acid solution. Ascorbic acid dilute standard solutions containing 2.0, 4.0, 6.0, and 8.0 μg/ml, respectively, and filtered ovarian gland extracts were treated in the same manner as the blank.

The galvanometer readings of the standards and blank plotted against ascorbic acid concentration on semi-logarithmic paper resulted in a straight-line curve, and from this calibration curve the ascorbic acid concentrations of the ovarian gland extracts were calculated. The total amount of ascorbic acid in the glands was obtained by multiplying the value taken from the standard curve by the total volume of metaphosphoric acid solution in the homogenate.

Assay of Plasma Follicle-Stimulating Hormone

The technique of parabiosis was employed for the assay of plasma FSH. In this method a female assay animal is surgically united to an experimental animal (male pseudohermaphrodite rat). This procedure leads to an anastomosis of the two circulatory systems thus producing an exchange of blood. Consequently, any FSH not metabolized by the experimental animal will cross over to the assay animal via the united circulation and stimulate ovarian enlargement. Hence, the endpoint of the assay is the increase in ovarian weight of the female parabiont. This method indicates only an elevated titer of FSH, since normal titers are readily metabolized by the experimental animal.

Assay Procedure

Animals used for bioassay of plasma FSH were female littermates of male pseudohermaphrodite rats. Thus, the probability of an immune response between experimental and assay animals was markedly reduced. At the time of surgical union, both experimental and assay rats were 30 to 33 days of age and weighed 80 to 90 grams. The animals were anesthetized in an ether jar and the hair was clipped over the dorsal and lateral body regions. After scrubbing the exposed areas with zephirin an oblique skin incision was made in the animals to be united extending from the gluteal region anteriorly to a midline point at the base of the skull. Likewise, an incision was made in the abdominal musculature, latissimus dorsi and trapezius muscles following the course of the skin incision. Next, the scapuli of the two animals were united with several silk sutures followed by surgical union of corresponding edges of the muscle incisions with absorbable surgical sutures. Edges of the skin incision were united with noncorrosive nickel-silver wound clips. The angulation of the skin incision resulted in an oblique angle between the heads of the parabiotics and prevented any biting that might otherwise have occurred.

The animals remained in surgical union for 15 days at which time they were sacrificed. The ovaries of the female assay parabiont were dissected free and weighed on a Roller-Smith torsion balance. The per cent increase in ovarian weight of the assay animals from male pseudohermaphrodite x female pairs were compared with those of male x female pairs. Also, pseudohermaphrodites were castrated and united to female assay animals in order to determine if the pituitary under this condition would increase the secretory output of FSH.

Determination of 17-Deoxycorticosteroids

The determination of the total 17-deoxycorticosteroids (unsubstituted at C_{17}) included the 21 hydroxypregnan-20-ones and pregnane-20: 21-diols. The chemical procedure was carried out according to the method of Exley and co-workers (1961). The principle involved in this determination is based on the oxidation of the $C_{20:21}$ constituent of the 17-deoxycorticoid molecule to a C_{20} aldehyde. The Angeli-Rimini colorimetric reaction is then used for the detection of the aldehyde. Only aldehydes lacking substituents interacting with the aldehyde group, such as an hydroxyl group at position C_{17}, will give the colorimetric reaction. The aldehydes are treated with benzenesulphohydroxamic acid in a weakly alkaline solution. The hydroxamic acids formed are then detected in the reaction mixture by conversion into their purple ferric complexes. Since in the process of oxidizing the $C_{20:21}$ constituent to an aldehyde, the $C_{20:21}$ ketols are oxidized to etiocholanic acids, the total steroid extract is first subjected to a reduction process. This process reduces the $C_{20:21}$ ketols to $C_{20:21}$ glycols which are then oxidized to C_{20} aldehydes.

Reagents and Standards

1) Ethylene dichloride; spectroanalyzed

2) Tertiary butanol; A.S.C.

3) Methylene dichloride; spectroanalyzed

4) Ethyl acetate; spectroanalyzed

5) Petroleum ether; boiling point 36° to 45°C

6) Anhydrous sodium sulphate; granular

7) Sodium bismuthate; A.S.C.

8) Hydrochloric acid; 5N solution: 100 ml of 12 N HCL were diluted to 5N by the addition of 140 ml of distilled water.

9) Sodium hydroxide; 3N solution: 500 ml of distilled water were added to 60 gm of sodium hydroxide pellets and allowed to dissolve by standing in a cold water bath.

10) Perchloric acid; 2.5 N solution: 100 ml of 7N perchloric acid were diluted to 2.5 N by the addition of 180 ml of distilled water.

11) Glacial acetic acid; 40% v/v: 40 ml of concentrated acetic acid were diluted to a 40% solution by the addition of 60 ml of distilled water.

12) Alkaline aqueous tertiary butanol solution: 0.125 N sodium hydroxide and 50% aqueous tertiary butanol were mixed in equal volumes.

13) Potassium borohydride; 20% w/v: an aqueous solution of potassium borohydride was made fresh each time it was used by dissolving 0.5 gm of the reagent in 2.5 ml of distilled water.

washed successively with water (10 ml), 3N-NaOH (10 ml; shaken for 5 minutes) and water (2 x 10 ml). The washed extract was filtered through a column of anhydrous sodium sulphate and a 30 ml portion evaporated to dryness.

Conversion of 17-Deoxycorticosteroids to Aldehydes

The sample containing the residue of the ethylene dichloride extract was dissolved in tertiary butanol (0.2 ml) and treated with a freshly prepared aqueous solution of potassium borohydride (0.1 ml; 20%, w/v), overnight at room temperature. This procedure reduced the $C_{20:21}$ ketol constituent. On the following morning aqueous acetic acid (3 ml; 40%, v/v) and sodium bismuthate (0.5 gm) were added, the mixture shaken for 10 minutes and then centrifuged at 4,000 r.p.m. in a Universal refrigerated centrifuge. This procedure disrupted the $C_{20:21}$ glycol bond which resulted in the formation of a C_{20} aldehyde. A portion of the supernatant (2.5 ml) was treated with aqueous sodium metabisulphite (0.05 ml; 20%, w/v) and with hydrochloric acid (1 ml; 5N) and then shaken with methylene dichloride (3 ml) for 15 minutes. The aqueous phase was removed and the extract (lower phase) washed successively with water (1 ml), 3 N-NaOH (1 ml; shaken for 5 minutes) and water (2 x 1 ml). The washed extract was then filtered through a column of anhydrous sodium sulphate and a portion (2 ml) evaporated to dryness, care being taken not to overheat the residue.

Determination of C_{20} Aldehydes

The sample containing the aldehyde was dissolved in tertiary butanol (0.2 ml), treated with a freshly prepared solution of benezene-sulphohydroxamic acid (0.1 ml; 0.125 N) in aqueous tertiary butanol

(50%, v/v). After 30 minutes at room temperature, water (0.5 ml) was added and after a further 15 minute period the mixture was shaken with ethyl acetate (2 ml) for 15 minutes and then centrifuged at 4,000 r.p.m. for 5 minutes. A portion of the top layer (2 ml) was carefully removed and shaken for 5 minutes with a solution (0.1 ml) of ferric nitrate (0.5 N) in perchloric acid (2.5N). Petroleum ether was added (2 ml) and the mixture shaken again. It was then centrifuged at 4,000 r.p.m., the organic phase carefully removed and the aqueous phase washed with a mixture of ethyl acetate and petroleum ether (2 ml; 1:2, v/v). The extinction of the aqueous phase was determined at 440 mμ, 510 mμ and 580 mμ against a process blank obtained by submitting tertiary butanol (0.2 ml) alone to the treatment. Urinary blanks were also determined to account for colored impurities. This involved treating the ethyl acetate extract with perchloric acid only.

The transmittance readings at the various wavelengths were carried out in a Zeiss spectrophotometer, employing the microcell unit. A calibration curve was constructed from aldehydes prepared from a pure corticosterone reference standard. Eight graded concentrations of the reference standard were employed in each determination. The galvanometer readings were plotted on semi-logarithmic paper against the various concentrations of the reference standard. From the calibration curve so constructed, the value of the unknown was calculated.

Determination of Total Neutral 17-Ketosteroids

The majority of colorimetric methods for the determination of the total neutral 17-ketosteroids depend for their final determination on the reaction originally described by Zimmermann (1935). This reaction relies on the fact that steroids containing the group $-COCH_2-$ will give a purple color when treated with m-dinitrobenzene in the presence of alkali. The literature contains information on a large number of methods for the estimation of total urinary 17-ketosteroids in which the final determination is made colorimetrically using the Zimmermann (1935) reaction. These methods have been reviewed by Loraine (1958) and Dorfman (1962). The method employed in the present work was a modification of the procedure devised by Drekter _et al_., (1952) for the determination of total neutral urinary 17-ketosteroids. The modification involved the substitution of carbon tetrachloride for ethylene dichloride because experience with the latter indicated that excessive contaminating chromogens were extracted and alcoholic potassium hydroxide was substituted for aqueous potassium hydroxide. Furthermore, filtration through anhydrous sodium sulphate was added as an additional step. Otherwise, the procedure was carried out as originally described by Drekter and co-workers, but employed larger volumes of urine and solvent.

Reagents and standards

1) Hydrochloric acid; A.C.S.
2) Carbon tetrachloride; spectroanalyzed.
3) Absolute ethanol; U.S.I.
4) Anhydrous sodium sulphate.
5) Sodium hydroxide; 10% solution: 500 ml of distilled water were added to 50 gm of sodium hydroxide pellets and allowed to dissolve by standing in a cold water bath.
6) Potassium hydroxide; 2.5 M alcoholic solution: The alcoholic KOH solution was made fresh each time it was used by grinding

7.0 gm KOH in 25 ml of 100% ethanol, filtering the supernatant fluid to remove insoluable carbonate and adding another 25 ml portion of alcohol to the residue which was ground again and filtered. This grinding was done with a glass rod in a 50 ml graduate cylinder set in ice water.

7) M-dinitrobenzene; 2% solution: m-dinitrobenzene solution was made by placing 1 gm of the reagent in a 50 ml volumetric flask and filling to the mark with 100% ethanol. The flask was warmed slightly to facilitate dissolution.

8) Dehydroepiandrosterone reference standard; 50-500 μg/ml: a graded series of concentrations of this steroid were made by dissolving 50 mg of dehydroepiandrosterone in 100 ml of 100% ethanol and diluting to the appropriate concentration with additional 100% ethanol.

Acid Hydrolysis and Steroid Extraction

A pooled 24 hour urine specimen (250 ml) was placed in a round-bottom flask (500 ml capacity) and to this were added 25 ml of concentrated hydrochloric acid. A reflux condenser equipped with a water jacket was attached to the flask which was then heated on a hot plate for 10 minutes. This procedure removed the glucuronate and sulphate moities conjugated to the 17-ketosteroids and rendered them soluble in an organic medium. The flask containing the hydrolyzed urine was rapidly cooled and 80 ml of carbon tetrachloride added and permitted to reflux in a boiling water bath for 20 minutes. The flask was again cooled and the aqueous layer removed in a separatory funnel. The organic extract was filtered through Whatman #40 filter paper in a Buchner funnel with suction and washed twice with 20 ml of water. The carbon tetrachloride extract was shaken with solid sodium hydroxide pellets followed by a wash with 10% aqueous sodium hydroxide (2 x 20 ml) and again subjected to a water wash (2 x 20 ml). This procedure removed interfering chromogens and the acidic and phenolic fractions. The washed extract was filtered through a column of anhydrous sodium sulphate and evaporated to a residue.

Analytical Procedure

For the colorimetric determination a series of 50 ml test tubes were prepared as follows:

1) Reference standard solutions: from a solution containing 500 μg/ml of dehydroepiandrosterone (U.S.P.), a graded series of concentrations ranging from 50 to 500 μg were prepared by diluting with an appropriate amount of 100% ethanol. To each test tube in the series was pipetted 1 ml of the appropriate concentration.

2) Unknown urine extract: 1 ml of the urine extract.

3) Unknown urine extract blank: 1 ml of urine extract plus 1 ml of 100% ethanol.

4) Process blank: 1 ml of 100% ethanol.

One milliliter of 2.5 M KOH in 100% ethanol was pipetted into every tube, and to each tube, except the one containing the unknown urine extract blank, 1 ml of 2% m-dinitrobenzene in 100% ethanol was added. The tubes were set in a dark place at about 25°C and left for 45 minutes. They were occasionally shaken to facilitate the color development. After color development, 25 ml of 70% ethanol was added to each tube and the contents mixed by inversion. The extinction of the reference standards and unknown was determined at 440 mμ, 510 mμ and 580 mμ against the process blank. A calibration curve was constructed from a pure dehydroepiandrosterone reference standard. Six graded concentrations of the reference standard were used to construct the calibration curve in each determination. The galvanometer readings were plotted on semi-logarithmic paper against the various concentrations of the reference standard. From the calibration curve, the value of the unknown was calculated.

Method of Histological Preparation

Histological sections were prepared to investigate the possibility of a correlation between the high titer of gonadotropins, the high adrenal androgen output and the zonation of the adrenal gland, and to define histologically the region of cortical hyperplasia.

Reagents and Stains

1) Absolute ethanol; U.S.I.
2) Xylol; AP
3) Albumin
4) Watterman paraffin; melting point 52°C
5) Bouin fixative: 1 gm of Picric acid crystals were dissolved in 75 ml of warm distilled water. The volume was brought to 105 ml by the addition of 25 ml of formalin and 5 ml of acetic acid.
6) Neutral buffered formalin; 4 gm of monosodium phosphate and 6.5 gm of disodium phosphate were dissolved in 900 ml of distilled water. The solution was brought to a volume of one liter by adding 100 ml of formalin.

7) Delafield's hematoxylin; 1 gm of hematoxylin dissolved in 10 ml of absolute ethanol was added to a saturated aqueous solution of ammonia alum. This mixture was exposed to air and light for 6 to 8 weeks, filtered and 25 ml of methanol and glycerin added.

8) Eosin counter stain; 0.5 gm of eosin crystals were dissolved in 100 ml of 95% ethanol.

9) Mounting medium: 50 gm of acacia were dissolved in 100 ml of distilled water at 55°C and to this was added 100 gm of thymol as a preservative.

Method of Embedding and Staining

Following fixation with Bouin's fixative or buffered formalin, the tissues were rinsed in tap water for 5 minutes and subjected to a dehydration process, using graded concentrations of ethanol. The dehydration process consisted of one rinse in 35%, 50% and 70% ethanol followed by 8 hours in 80% and 95% ethanol respectively. The final dehydration step involved several changes of absolute ethanol over a period of one hour. Embedding was accomplished by clearing the tissues in xylol for 2 hours followed by infiltration in 1/2 xylol - 1/2 paraffin for 4 hours. The tissues were transferred into melted paraffin and embedded in paper boxes.

Tissue blocks were mounted on a microtome and sectioned at 5μ. The sectioned ribbons were affixed to a glass slide smeared with albumin and water, and heated slightly to insure the removal of any wrinkles in the ribbon.

After rehydration the slides were placed in hematoxylin for two minutes and then rinsed in tap water for 15 minutes. The sections were counter-stained with eosin for 30 seconds and again dehydrated by a graded series of ethanol solutions. After clearing in xylol for 15 minutes the sections were mounted with Permount and cover slips affixed to the slides.

CHAPTER III

EXPERIMENTAL RESULTS

For convenience of interpretation, data appearing in this section are presented both in tabular and graphic form. Individual values plotted as a mean in any graph can be found by referring to the corresponding table. Attention is directed to comparative values, since interpretation of experimental results is dependent on these comparisons.

Assay of the Luteinizing Hormone

The first condition of the working hypothesis necessitated quantitation of the gonadotropin titer in the pseudohermaphrodite rat. With this in view, twelve separate assays were carried out on pituitary preparations and plasma from normal male, female and male pseudohermaphrodite rats. A total of 50 test rats were employed in each assay (10 animals per dose level), using two dose levels of pituitary extract or plasma and two dose levels of an NIH-LH reference standard, plus a saline control group. Data were recorded as micrograms of ascorbic acid from the ovary of each assay rat and converted to micrograms per 100 milligrams ovarian weight. Since a regression analysis was employed to compute the relative potency of each preparation, micrograms per 100 milligrams ovarian weight were converted to per cent depletion of ascorbic acid. This gave regression lines with a positive slope, since the most potent preparation gave a smaller ovarian ascorbic acid content, but resulted in the larger per cent depletion.

The relative potency of each test preparation was determined by computing the slopes of the dose-response curves for test and standard (NIH-LH) preparations and then expressing the slopes as a ratio. Regression lines were constructed between points obtained from the average per cent depletion of ovarian ascorbic acid at two dose levels. The lines crossed the ordinate at a fixed point computed from the regression equation. Validity tests were determined from the analysis of variance for the assays by dividing the error mean square into deviation from regression mean square. The calculated F value so obtained was then compared with the tabulated F value for the appropriate degrees of freedom. Assays showing significant deviation from regression were declared statistically invalid. Significant deviation from regression generally resulted from an underestimate of the unknown preparation at the low dose level. Since the relative potency computed from the slopes of the dose-response curves in non-linear assays either underestimated or overestimated the true relative potency, these cannot be taken as absolute values. However, the relative potencies in those assays showing non-linearity do indicate differences between experimental and control groups, since all data were collected and treated in the same manner.

Values of the per cent depletion of ovarian ascorbic acid from two separate LH assays of pseudohermaphrodite rat pituitary are listed in Tables 1 and 2. The average values with standard errors (Rα) produced by 25 μg and 100 μg dose levels of pituitary extract are listed at the bottom of columns 1 and 2, while those resulting from 0.4 μg and 1.6 μg

TABLE 1

ASSAY OF LH FROM PITUITARY GLANDS OF MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups			
	Pseudo Pituitary 25 μg	Pseudo Pituitary 100 μg	NIH-LH 0.4 μg	NIH-LH 1.6 μg
	Per cent Depletion of Ovarian Ascorbic Acid			
	30.1	50.1	24.9	39.0
	34.6	46.9	31.8	39.4
	16.9	38.5	31.8	26.0
	39.6	43.6	31.9	39.9
	19.2	41.4	26.6	37.4
	9.3	41.2	26.9	41.3
	9.8	41.0	24.9	25.0
	9.8	31.6	28.1	40.0
	9.1	42.0	23.3	38.7
	11.7	38.8	24.0	38.3
$\frac{\Sigma x_i}{n}$	19.0 ± 3.7	41.5 ± 1.7	27.4 ± 1.9	36.5 ± 1.1
Σx_i^2	4,823.9	17,453.2	13,445.0	7,619.8
$\frac{(\Sigma x_i)^2}{n}$	3,613.8	17,230.8	13,104.4	7,518.6
	$b_s = 12.3$	$b_t = 14.0$	R = 113.8%	Rα = 17.2%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

TABLE 2

ASSAY OF LH FROM PITUITARY GLANDS OF MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups			
	Pseudo Pituitary 25 μg	Pseudo Pituitary 100 μg	NIH-LH 0.4 μg	NIH-LH 1.6 μg
	Per cent Depletion of Ovarian Ascorbic Acid			
	29.1	35.9	12.4	24.2
	20.6	43.1	10.1	35.7
	20.7	43.7	12.3	36.7
	6.3	35.9	13.5	40.4
	17.5	31.9	20.0	35.7
	27.4	35.9	11.4	29.7
	7.0	42.9	10.4	39.1
	18.1	42.1	8.9	33.1
	22.5	44.1	8.4	30.0
	20.5	43.0	16.3	29.0
$\frac{\Sigma x_i}{n}$	19.0 ± 2.4	39.9 ± 1.4	12.4 ± 1.1	33.4 ± 1.6
Σx_i^2	4,093.6	16,057.9	1,642.9	11,361.2
$\frac{(\Sigma x_i)^2}{n}$	3,594.8	15,880.2	1,530.2	11,128.9
	b_s = 15.1	b_t = 19.2	R = 131.8%	Rα = 11.5%

Values representing per cent depletion have been calculated from Saline control treatment groups.

b_s - Slope of standard preparation

b_t - Slope of test preparation

TABLE 3

ASSAY OF LH FROM PITUITARY GLANDS OF MALE RATS

	Treatment Groups			
	Male Pituitary 50 μg	Male Pituitary 200 μg	NIH-LH 0.4 μg	NIH-LH 1.6 μg
	Per cent Depletion of Ovarian Ascorbic Acid			
	24.2	42.5	13.2	35.8
	26.0	41.4	19.8	37.2
	15.1	36.0	7.9	41.6
	21.4	41.2	15.9	33.4
	19.1	44.3	21.5	38.7
	18.1	43.4	13.6	39.2
	20.2	36.4	17.4	30.0
	22.3	33.7	7.0	42.7
	15.1	45.2	22.5	38.8
	26.7	30.4	20.6	40.5
$\frac{\Sigma x_i}{n}$	20.8 ± 1.3	39.4 ± 1.6	15.9 ± 1.7	37.8 ± 1.2
Σx_i^2	4,486.3	15,770.3	2,811.1	14,414.9
$\frac{(\Sigma x_i)^2}{n}$	4,334.7	15,547.3	2,540.8	14,280.8
	b_s = 16.0	b_t =17.7	R 110.6%	R_α = 8.3%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

TABLE 4

ASSAY OF LH FROM PITUITARY GLANDS
OF MALE RATS

	Treatment Groups			
	Male Pituitary 50 µg	Male Pituitary 200 µg	NIH-LH 0.4 µg	NIH-LH 1.6 µg
	Per cent Depletion of Ovarian Ascorbic Acid			
	29.3	44.9	26.0	42.6
	21.8	43.4	18.6	42.9
	18.8	39.6	19.8	40.5
	17.8	39.5	20.4	34.6
	9.9	39.6	26.4	37.8
	14.8	41 8	15.2	44.5
	18.2	35.6	21.2	41.7
	18.4	43.3	25.1	44.4
	15.6	44.1	19.0	44.6
	18.9	36.3	28.0	35.4
$\frac{\Sigma x_i}{n}$	18.4 $\pm$ 2.6	40.4 $\pm$ 1.1	22.0 $\pm$ 1.3	40.9 $\pm$ 1.2
Σx_i^2	3,951.4	16,447.3	4,982.6	16,854.2
$\frac{(\Sigma x_i)^2}{n}$	3,367.2	16,329.7	4,826.8	16,728.1
	b_s = 17.5	b_t = 16.6	R = 94.9%	R_α = 7.9%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

Regression Lines For The Assay of The Luteinizing Hormone From Pituitary Glands of Male Rats

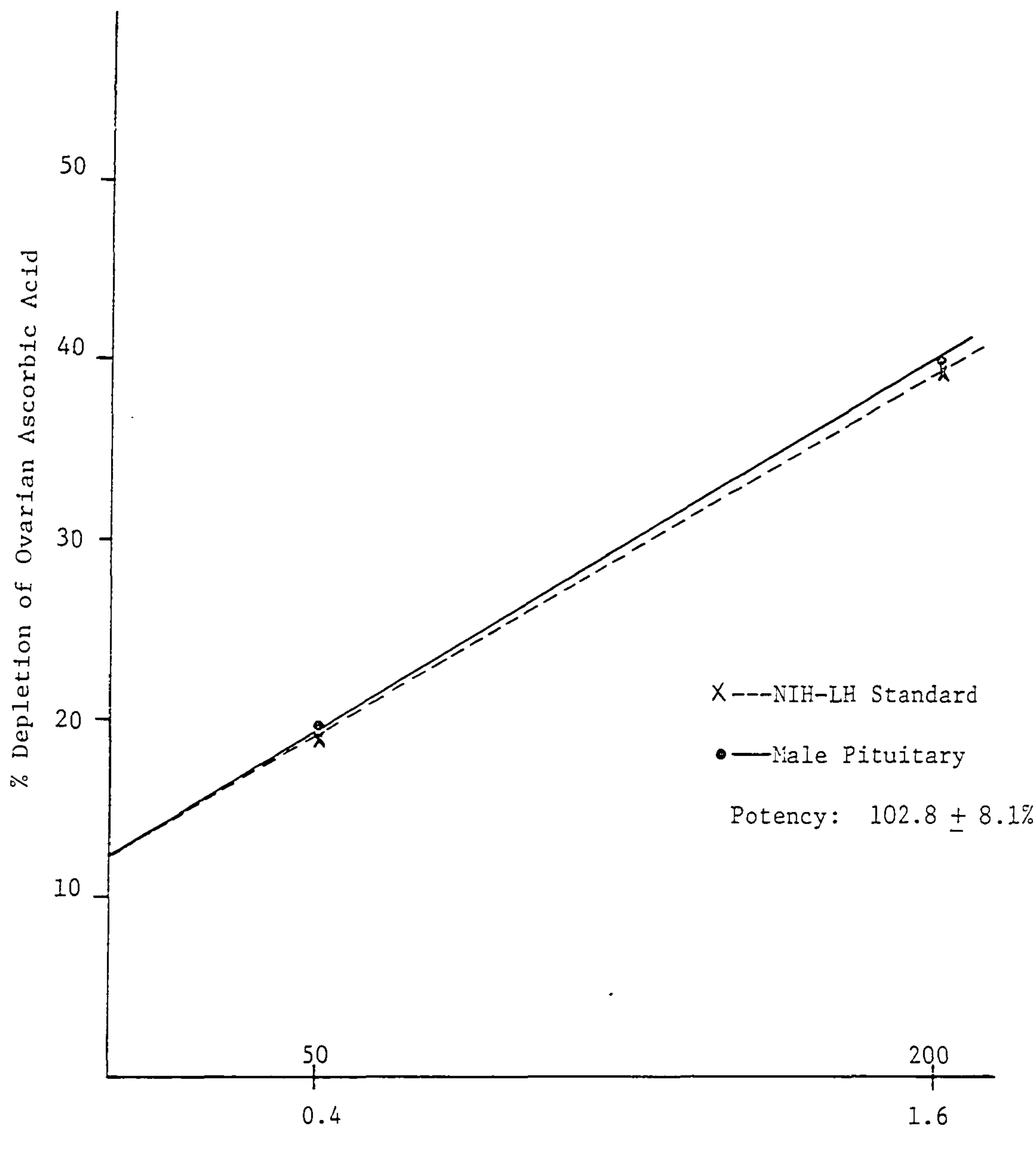

Figure 2. The pituitary preparation (50 μg and 200 μg) was compared to an NIH-LH reference standard (0.4 μg and 1.6 μg) by determining the slopes of the dose-response curve and expressing the potency as a ratio of the determined slopes.

TABLE 5

ASSAY OF LH FROM PITUITARY GLANDS
OF FEMALE RATS

	Treatment Groups			
	Female Pituitary 50 μg	Female Pituitary 200 μg	NIH-LH 0.4 μg	NIH-LH 1.6 μg
	Per cent Depletion of Ovarian Ascorbic Acid			
	11.9	33.7	24.1	35.1
	18.3	24.1	17.5	28.6
	10.9	43.6	27.6	40.2
	3.5	34.5	21.2	31.0
	2.2	30.7	32.7	40.1
	5.4	27.4	38.6	35.8
	12.5	34.4	16.8	42.8
	4.6	31.6	23.0	39.6
	6.9	32.9	21.1	41.6
	15.4	34.0	23.0	41.3
$\frac{\Sigma x_i}{n}$	9.2 ± 1.7	32.7 ± 1.6	24.6 ± 2.1	37.6 ± 1.5
Σx_i^2	1,103.7	10,921.3	6,442.9	4,352.9
$\frac{(\Sigma x_i)^2}{n}$	839.1	10,684.4	6,031.9	14,145.1
	b_s = 17.7	b_t = 12.6	R = 71.2%	R_α = 7.7%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

TABLE 6

ASSAY OF LH FROM PITUITARY GLANDS
OF FEMALE RATS

	Treatment Groups			
	Female Pituitary 50 μg	Female Pituitary 200 μg	NIH-LH 0.4 μg	NIH-LH 1.6 μg
	Per cent Depletion of Ovarian Ascorbic Acid			
	10.6	20.5	19.7	40.7
	14.6	26.1	11.8	30.2
	8.9	33.1	9.0	35.8
	6.2	27.5	16.3	23.7
	3.3	30.6	11.0	36.8
	6.6	23.8	3.6	38.8
	8.0	36.4	13.3	34.5
	2.1	32.3	9.1	39.5
	12.7	33.9	18.2	33.4
	0.9	31.5	13.1	36.8
$\frac{\Sigma x_i}{n}$	7.4 ± 1.4	29.6 ± 1.6	12.5 ± 1.5	35.0 ± 1.6
Σx_i^2	728.1	8,965.8	1,770.5	12,491.8
$\frac{(\Sigma x_i)^2}{n}$	546.1	8,743.9	1,565.0	12,264.0
	b_s = 20.5	b_t = 16.7	R = 81.5%	Rα = 6.0%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

Regression Lines For The Assay Of The Luteinizing Hormone From Pituitary Glands of Female Rats

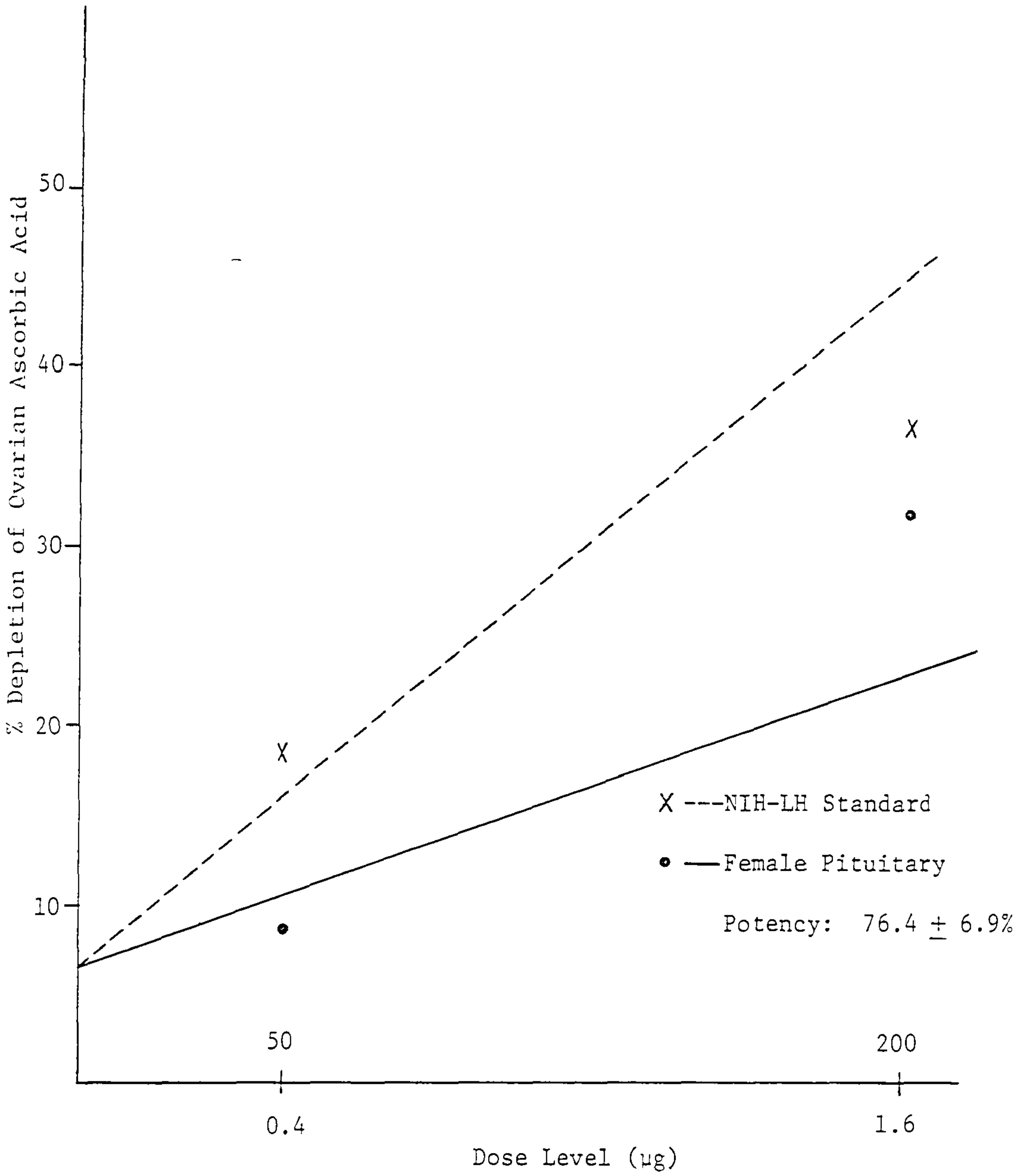

Figure 3. The pituitary preparation (50 μg and 200 μg) was compared to an NIH-LH reference standard (0.4 μg and 1.6 μg) by determining the slopes of the dose-response curve and expressing the potency as a ratio of the determined slopes.

Relative Potency of The Luteinizing Hormone From Pituitary Glands
Of Male Pseudohermaphrodite, Male and Female Rats

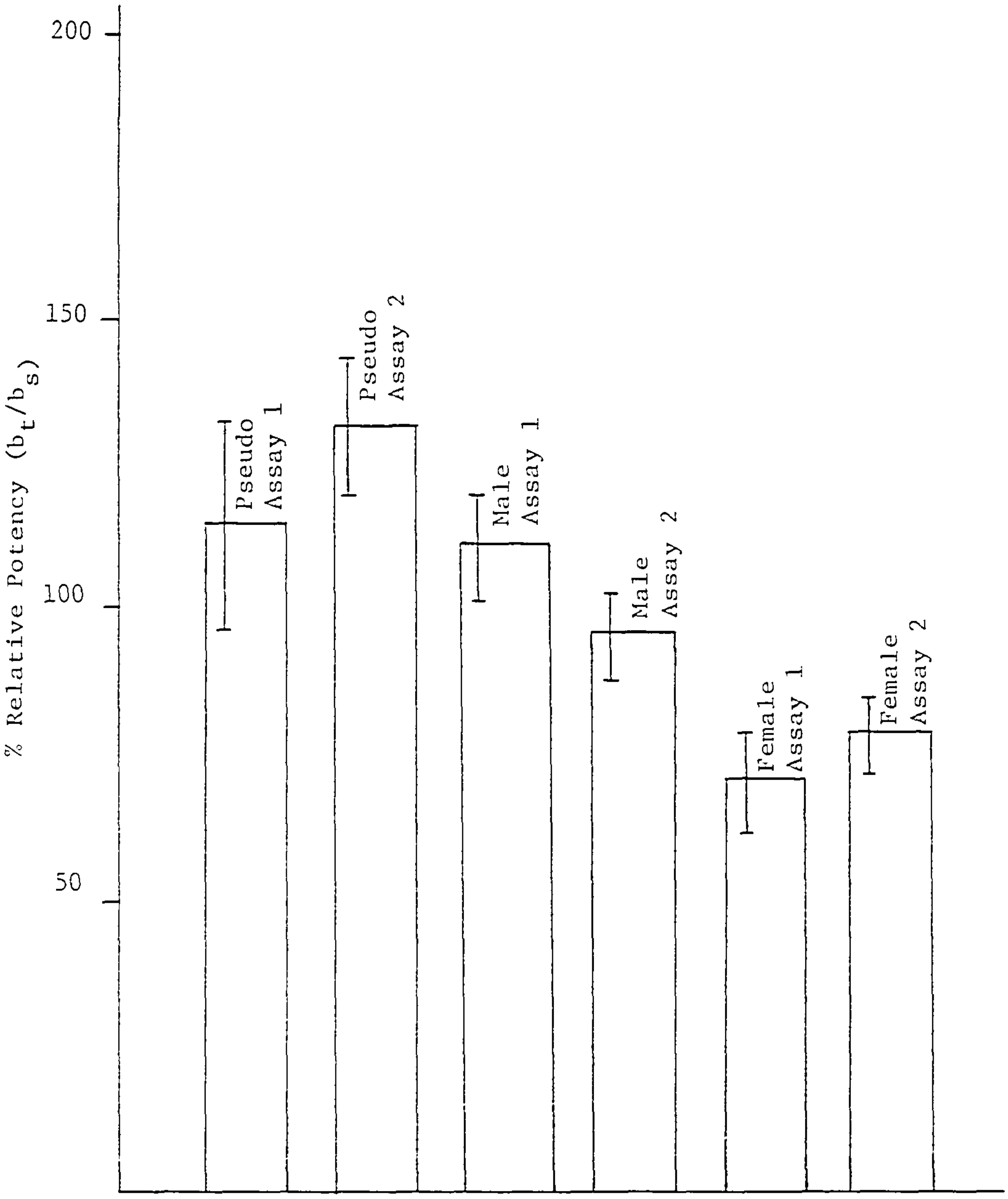

Figure 4. The relative potencies were determined by computing the slopes of the regression lines for test and standard preparations and then expressing the slopes as a ratio. The dose levels of pituitary preparations of pseudohermaphrodites must be taken into consideration when comparisons are made with control groups, since the dose levels were only 1/2 of that employed for controls.

pituitary.

Plasma samples from normal male, female and male pseudohermaphrodite rats employed in the LH assays caused a significant ovarian ascorbic acid depletion, especially at the high dose. However, the plasma assays all showed significant deviation from regression and therefore are considered statistically invalid. Values are shown in Tables 7 and 8 for the assay of pseudohermaphrodite rat plasma. Dose levels of 0.5 ml and 2.0 ml of plasma, and 0.4 μg and 1.6 μg of the reference standard resulted in relative potencies of 14.5 ± 3.5% and 13.9 ± 4.2%. In addition, the regression lines illustrated in Figure 5 gave an average relative potency of 14.2 ± 3.9%. Values for two assays of male plasma listed in Tables 9 and 10 and Figure 6 are only slightly below those produced by pseudohermaphrodite plasma, but the dose levels employed for the male plasma assays were one-third larger. Dose levels of 0.75 ml and 3.0 ml of male plasma, and 0.4 μg and 1.6 μg of the reference standard produced relative potencies of 12.8 ± 4.1% and 14.0 ± 4.6%. The average relative potency for male rat plasma shown in Figure 6 was 13.4 ± 4.4%. The relative potencies of female rat plasma shown in Tables 11 and 12, and Figure 7 were below those of the pseudohermaphrodites as well as those of normal males. Figure 7 shows an average relative potency of 9.7 ± 4.0% for female rat plasma. Again, the relative potencies are only estimates of the actual values due to curvilinear regression lines. However, Figure 8 shows that the pseudohermaphrodite plasma has a relative potency at least as large as that of males or females at only 2/3 the dose level. These comparisons indicate that the relative potency of female rat plasma $<$ male plasma $<$ pseudohermaphrodite plasma.

TABLE 7

ASSAY OF LH FROM PLASMA OF MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups			
	Pseudo Plasma 0.5 ml	Pseudo Plasma 2.0 ml	NIH-LH 0.4 μg	NIH-LH 1.6 μg
	Per cent Depletion of Ovarian Ascorbic Acid			
	4.4	18.4	18.6	37.0
	8.8	19.3	24.5	39.8
	5.6	16.9	21.9	44.2
	6.4	13.4	19.8	42.1
	5.3	15.2	16.7	38.9
	8.6	9.1	22.9	40.5
	6.9	14.7	21.6	41.6
	10.3	20.9	18.4	35.6
	4.9	17.3	20.8	39.9
	9.0	13.7	17.5	33.7
$\frac{\Sigma x_i}{n}$	7.0 ± 0.64	15.9 ± 1.8	20.3 ± 0.79	39.3 ± 1.0
Σx_i^2	529.8	2,629.9	4,165.2	15,558.2
$\frac{(\Sigma x_i)^2}{n}$	492.8	2,524.9	4,108.7	15,468.5
	b_s = 19.3	b_t = 2.8	R = 14.5%	Rα = 3.5%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

TABLE 8

ASSAY OF LH FROM PLASMA OF MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups			
	Pseudo Plasma 0.5 ml	Pseudo Plasma 2.0 ml	NIH-LH 0.4 μg	NIH-LH 1.6 μg
	Per cent Depletion of Ovarian Ascorbic Acid			
	6.0	17.1	22.9	41.1
	6.5	14.5	26.2	40.4
	10.5	8.1	22.0	45.9
	6.9	12.3	30.2	41.6
	9.6	16.9	31.0	40.2
	5.6	15.5	23.7	45.0
	4.0	19.3	21.2	44.8
	5.2	16.0	32.4	40.6
	8.4	15.7	22.0	42.2
	8.6	14.0	26.9	40.1
$\frac{\Sigma x_i}{n}$	7.1 ± 0.66	14.9 ± 0.97	25.9 ± 1.3	42.2 ± 0.71
Σx_i^2	547.2	2,316.4	6,836.4	17,844.0
$\frac{(\Sigma x_i)^2}{n}$	508.4	2,232.0	6,682.2	17,799.9
	b_s = 19.5	b_t = 2.7	R = 13.9%	Rα = 4.2%

Values representing per cent depletion have been calculated from Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

Regression Lines For The Assay Of The Luteinizing Hormone From Plasma Of Male Pseudohermaphrodite Rats

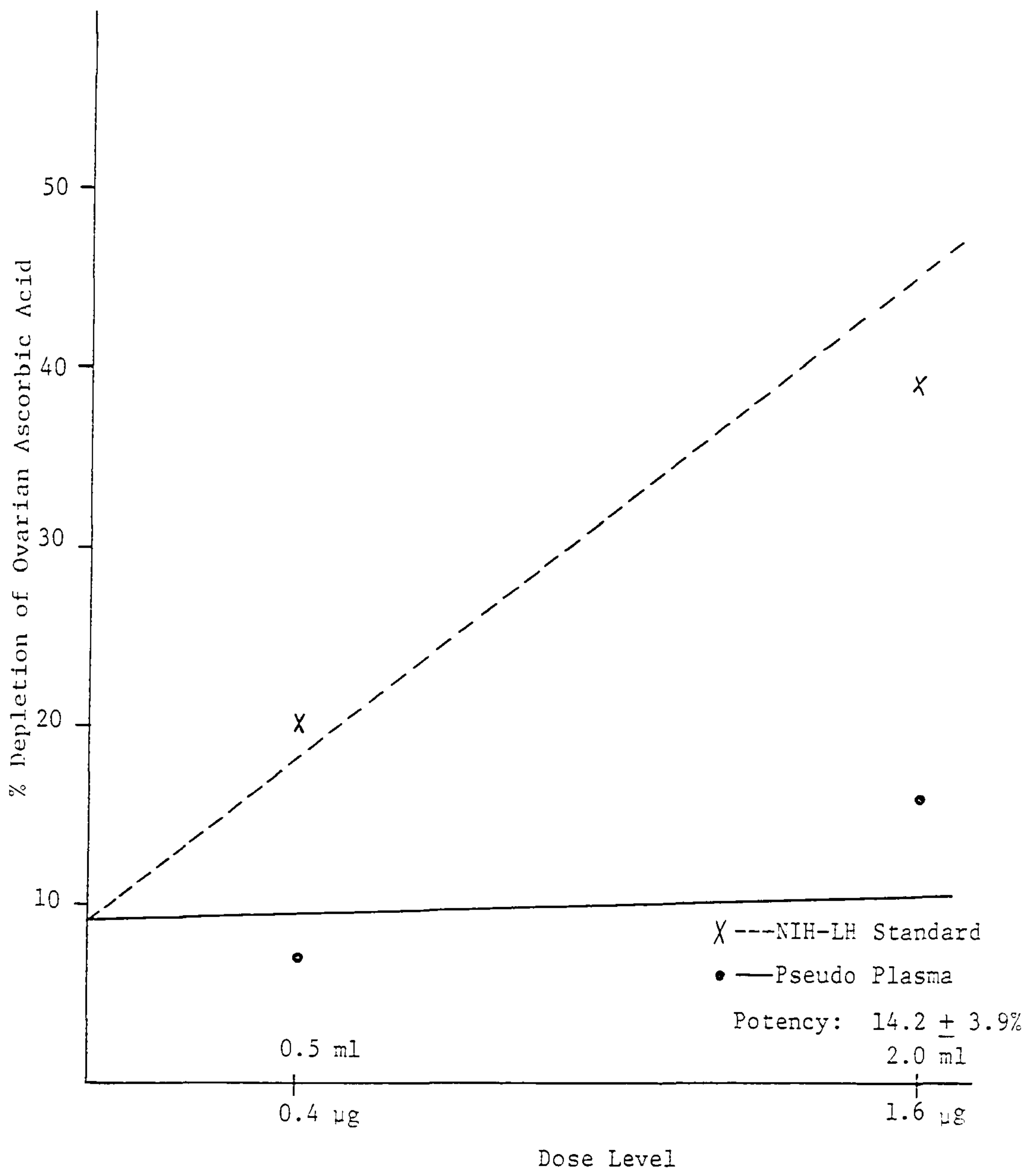

Figure 5. The plasma preparation (0.5 ml and 2.0 ml) was compared to an NIH-LH reference standard (0.4 µg and 1.6 µg) by determining the slopes of the dose-response curve and expressing the potency as a ratio of the determined slopes.

TABLE 10

ASSAY OF LH FROM PLASMA OF MALE RATS

	Treatment Groups			
	Male Plasma 0.75 ml	Male Plasma 3.0 ml	NIH-LH 0.4 µg	NIH-LH 1.6 µg
	Per cent Depletion of Ovarian Ascorbic Acid			
	11.2	20.5	17.1	33.6
	10.0	16.1	23.9	40.3
	6.1	18.1	19.8	46.4
	7.6	9.8	22.1	45.7
	9.7	13.0	25.5	39.5
	10.5	17.3	20.7	40.6
	3.1	14.7	20.0	35.1
	10.0	13.4	22.9	37.9
	5.1	15.6	22.1	43.8
	7.9	14.3	21.1	42.0
$\frac{\Sigma x_i}{n}$	8.1 ± 0.55	15.3 ± 0.95	21.5 ± 0.79	40.5 ± 1.33
Σx_i^2	722.8	2,414.9	4,686.8	16,553.9
$\frac{(\Sigma x_i)^2}{n}$	695.3	2,334.8	4,631.1	16,394.4
	b_s = 17.8	b_t = 2.5	R = 14.0%	Rα = 4.6%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

Regression Lines For The Assay Of The Luteinizing Hormone From Plasma Of Male Rats

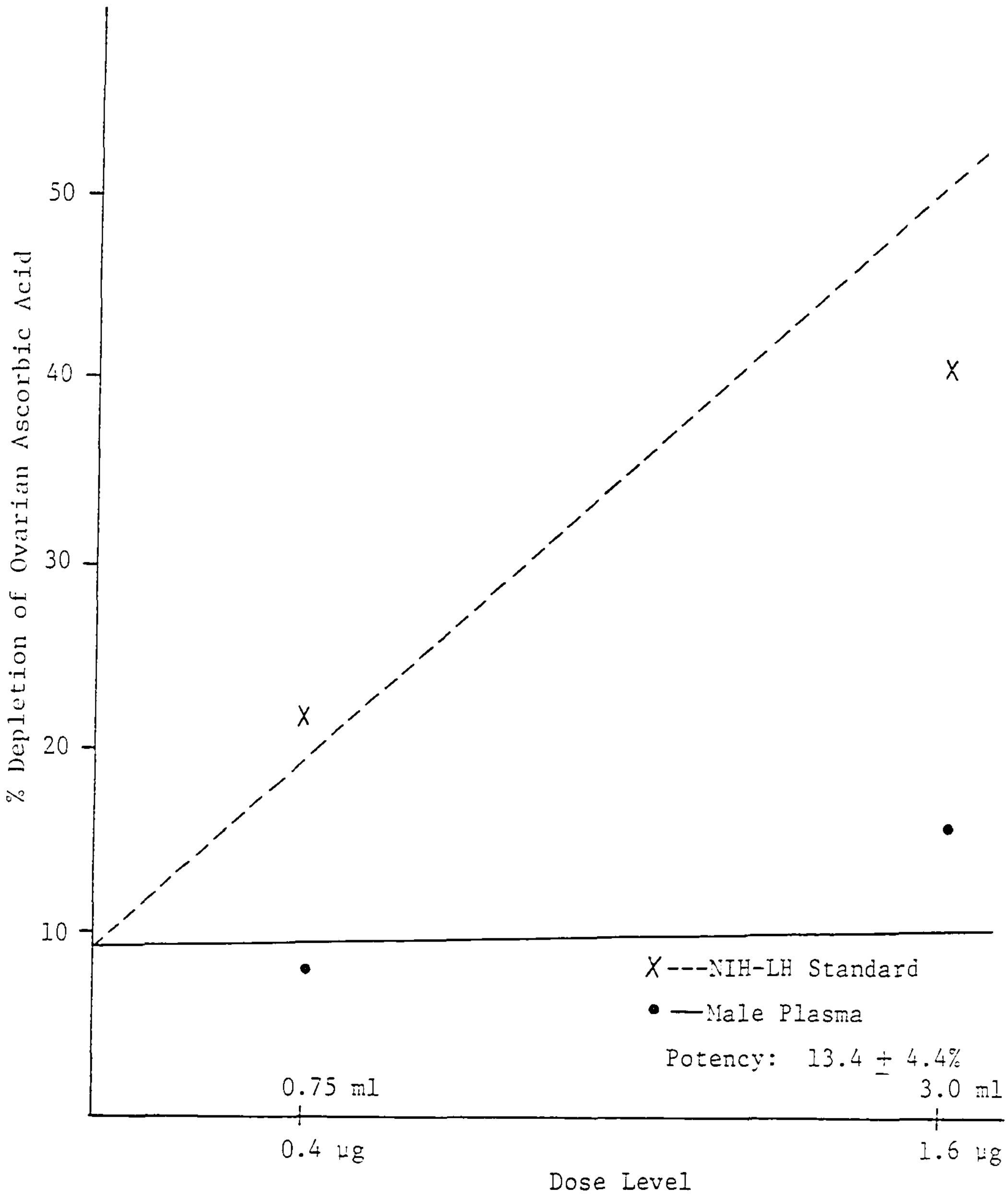

Figure 6. The plasma preparation (0.75 ml and 3.0 ml) was compared to an NIH-LH reference standard (0.4 µg and 1.6 µg) by determining the slopes of the dose-response curve and expressing the potency as a ratio of the determined slopes.

TABLE 11

ASSAY OF LH FROM PLASMA OF FEMALE RATS

	Treatment Groups			
	Female Plasma 0.75 ml	Female Plasma 3.0 ml	NIH-LH 0.4 μg	NIH-LH 1.6 μg
	Per cent Depletion of Ovarian Ascorbic Acid			
	4.4	10.6	20.4	42.4
	1.6	8.5	18.6	40.8
	0	17.3	24.0	45.5
	0.8	11.6	19.5	39.6
	1.1	15.8	21.6	41.1
	7.0	12.2	17.4	40.2
	0	14.4	25.6	38.6
	10.2	9.6	22.2	44.4
	4.7	10.1	16.6	40.5
	0	18.5	20.3	43.3
$\frac{\Sigma x_i}{n}$	3.0 ± 1.1	12.9 ± 1.1	20.6 ± 0.89	41.6 ± 0.70
Σx_i^2	198.9	1,760.7	4,323.5	17,382.5
$\frac{(\Sigma x_i)^2}{n}$	88.8	1,653.8	4,251.8	17,338.9
	b_s = 22.6	b_t = 2.1	R = 9.3%	Rα = 3.8%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

TABLE 12

ASSAY OF LH FROM PLASMA OF FEMALE RATS

	Treatment Groups			
	Female Plasma 0.75 ml	Female Plasma 3.0 ml	NIH-LH 0.4 µg	NIH-LH 1.6 µg
	Per cent Depletion of Ovarian Ascorbic Acid			
	0	12.8	25.1	38.1
	0	8.9	18.0	42.0
	1.6	11.1	22.9	43.1
	9.0	15.6	18.3	35.3
	3.7	14.2	15.8	44.9
	0	11.7	21.9	41.3
	1.9	14.9	21.2	37.9
	4.9	18.0	23.7	41.1
	2.1	17.8	17.2	42.5
	1.3	12.5	15.4	40.7
$\frac{\Sigma x_i}{n}$	2.5 ± 0.89	13.8 ± 0.93	20.0 ± 1.1	40.7 ± 0.90
Σx_i^2	131.0	1,967.3	4,086.7	16,629.4
$\frac{(\Sigma x_i)^2}{n}$	60.0	1,890.6	3,980.0	16,556.8
	$b_s = 19.9$	$b_t = 2.0$	R = 10.0%	Rα = 4.3%

Values representing per cent depletion have been calculated from a Saline control treatment group.

b_s - Slope of standard preparation

b_t - Slope of test preparation

Regression Lines For The Assay Of The Luteinizing Hormone From Plasma of Female Rats

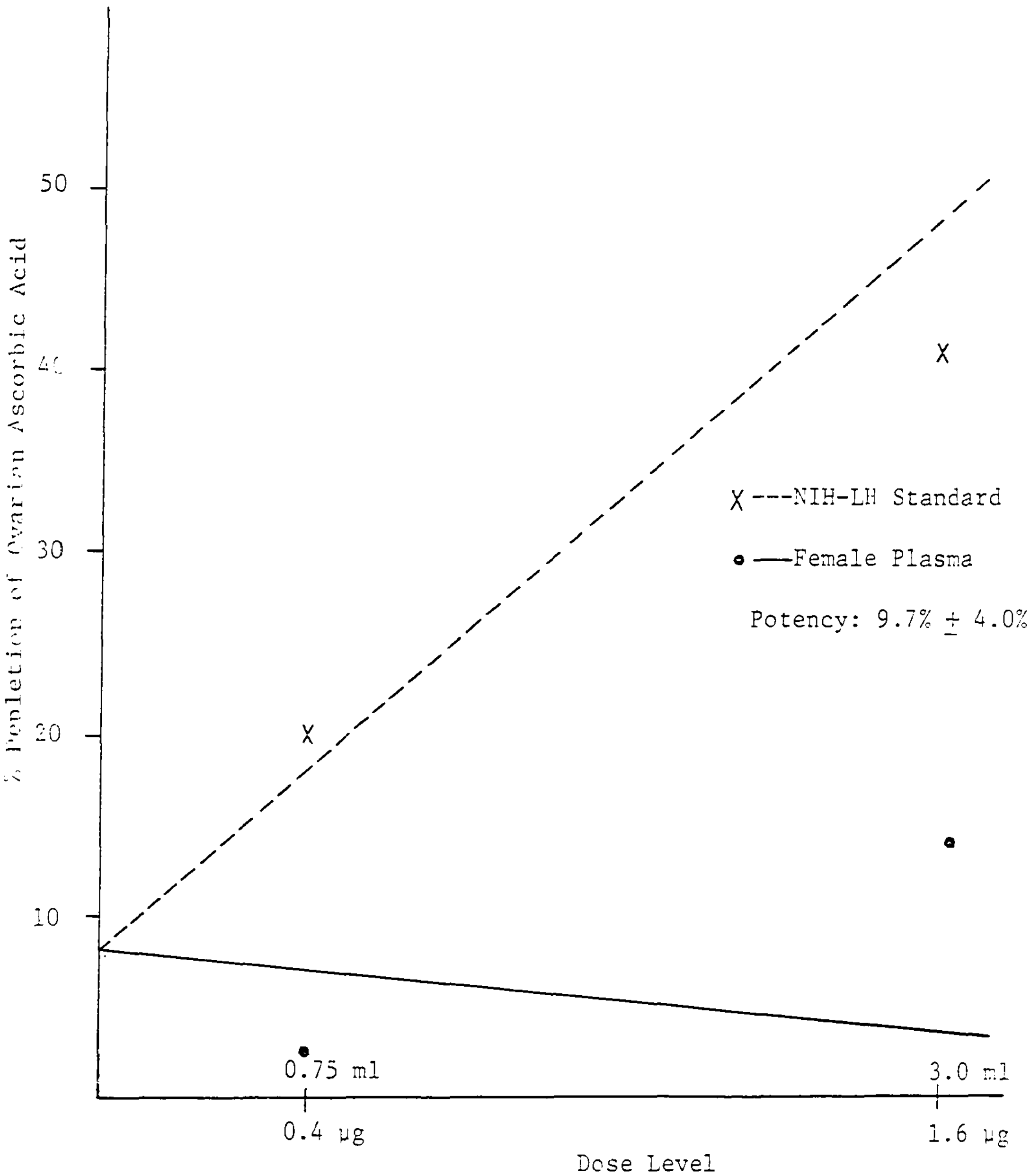

Figure 7. The plasma preparation (0.75 ml and 3.0 ml) was compared to an NIH-LH reference standard (0.4 μg and 1.6 μg) by determining the slopes of the dose-response curve and expressing the potency as a ratio of the determined slopes.

Relative Potency Of The Luteinizing Hormone From The Plasma Of Male Pseudohermaphrodite, Male and Female Rats

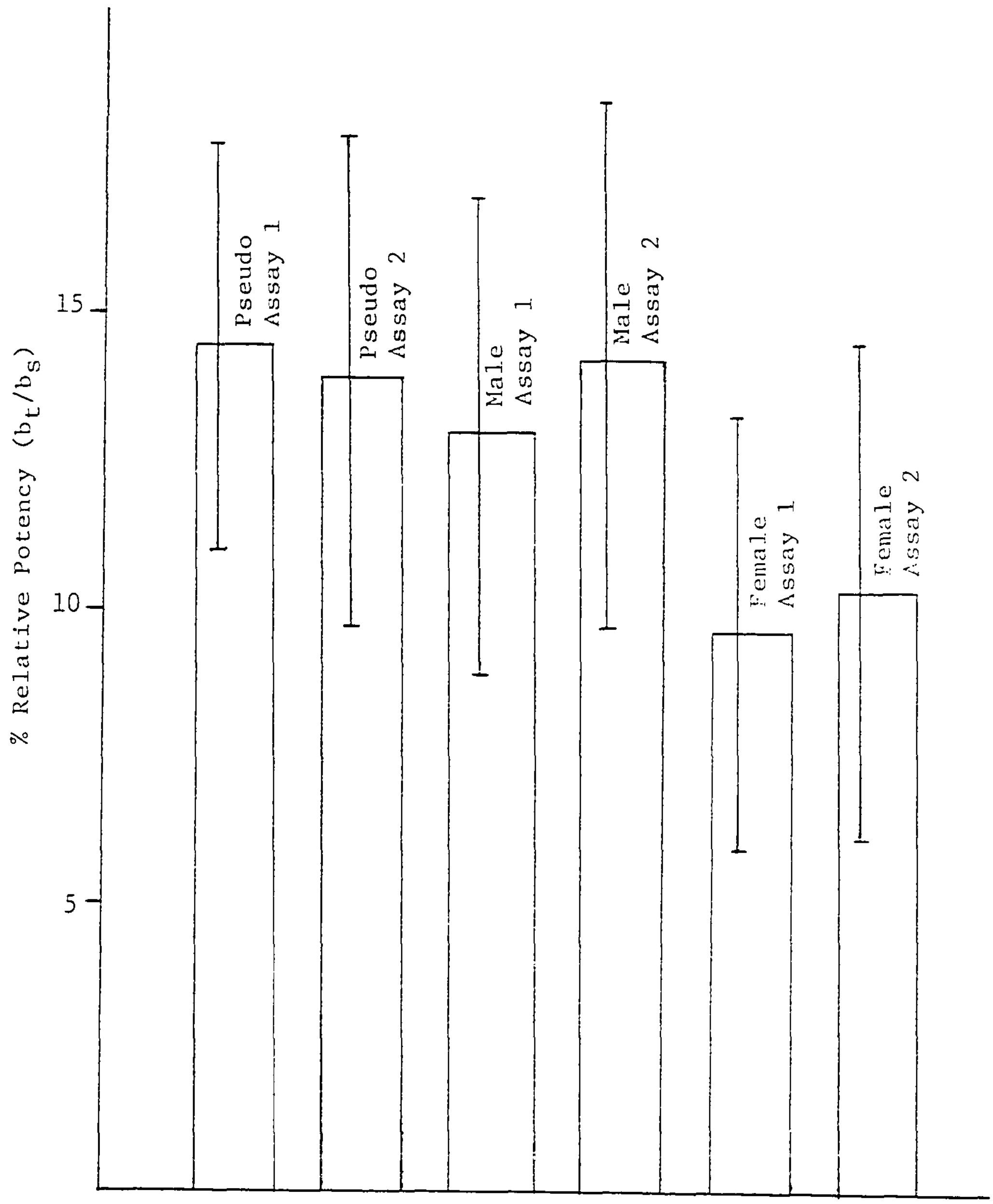

Figure 8. The relative potencies were determined by computing the slopes of the regression line for test and Standard Preparations and then expressing the slopes as a ratio. The dose levels of plasma preparations of pseudohermaphrodites must be taken into consideration when comparisons are made with control groups, since the dose levels were only 2/3 of that employed for controls.

Assay of the Follicle-Stimulating Hormone

To further quantitate the gonadotropin titer in male pseudohermaphrodite rats, forty pairs of rats were surgically united in parabiosis for the assay of plasma FSH. Of the 40 pairs originally united, 33 per cent developed parabiosis intoxication and were subsequently discarded. Female assay rats were surgically united at 30 to 33 days of age with male and pseudohermaphrodite siblings. The parabiotics were sacrificed 15 days following surgical union. Table 13 lists the ovarian weights of female assay rats 15 days after surgical union. The pseudohermaphrodite X female pairs listed in Column 2 resulted in an average ovarian weight of 66.6 $\pm$ 5.5 mg in contrast to 29.7 $\pm$ 1.9 mg for male X female unions listed in Column 4. The increase in ovarian weights of female parabiotics shown in Figure 9 indicates that a significant increase of plasma FSH exists in the pseudohermaphrodite rat.

In order to investigate the secretory output of FSH after castration, normal male and pseudohermaphrodite rats were castrated at the time of surgical union to female siblings. Columns 1 and 3 of Table 13 lists the ovarian weights of castrate pseudohermaphrodite X female and castrate male X female pairs. Average ovarian weights of 101.1 $\pm$ 14.4 mg and 101.3 $\pm$ 14.0 mg respectively, demonstrate that the secretory output of FSH increases following castration. Likewise, Figure 9 shows that the pituitary of the pseudohermaphrodite, after castration, further increases the secretory output of FSH to levels comparable to that of the castrate male.

Determination of Total Neutral Urinary 17-Ketosteroids

Determinations of urinary 17-ketosteroids were undertaken to

TABLE 13

OVARIAN RESPONSE OF FEMALE RATS IN PARABIOSIS
WITH MALE AND PSEUDOHERMAPHRODITE SIBLINGS

	Castrate Pseudo X Normal Female	Normal Pseudo X Normal Female	Castrate Male X Normal Female	Normal Male X Normal Female	Normal Female
	Ovarian Weight of Female Assay Rats				
	mg	mg	mg	mg	mg
	98.0	67.4	146.0	30.0	27.4
	42.0	64.6	57.0	29.5	26.0
	110.0	76.4	105.0	32.4	28.2
	94.0	82.0	85.4	20.0	28.6
	95.0	73.0	96.6	28.0	31.0
	176.0	37.0	66.0	32.0	26.4
	93.0	65.5	153.0	36.0	28.0
$\frac{\Sigma x_i}{n}$	101.1*	66.6*	103.3*	29.7	27.9
Σx_i^2	80,954.0	32,265.1	79,979.7	6,324.0	5,481.9
$\frac{(\Sigma x_i)^2}{n}$	71,609.1	31,009.0	71,811.6	6,174.6	5,465.6
$S\bar{x}$	14.4	5.5	14.0	1.9	0.62

Test and assay rats were surgically united in parabiosis at 30-33 days of age and sacrificed 15 days subsequent to surgical union.

*Significantly different at the .01 level from normal female control rats.

Ovarian Weights of Female Rats In Parabiosis With Normal And Castrate Male and Pseudohermaphrodite Rats

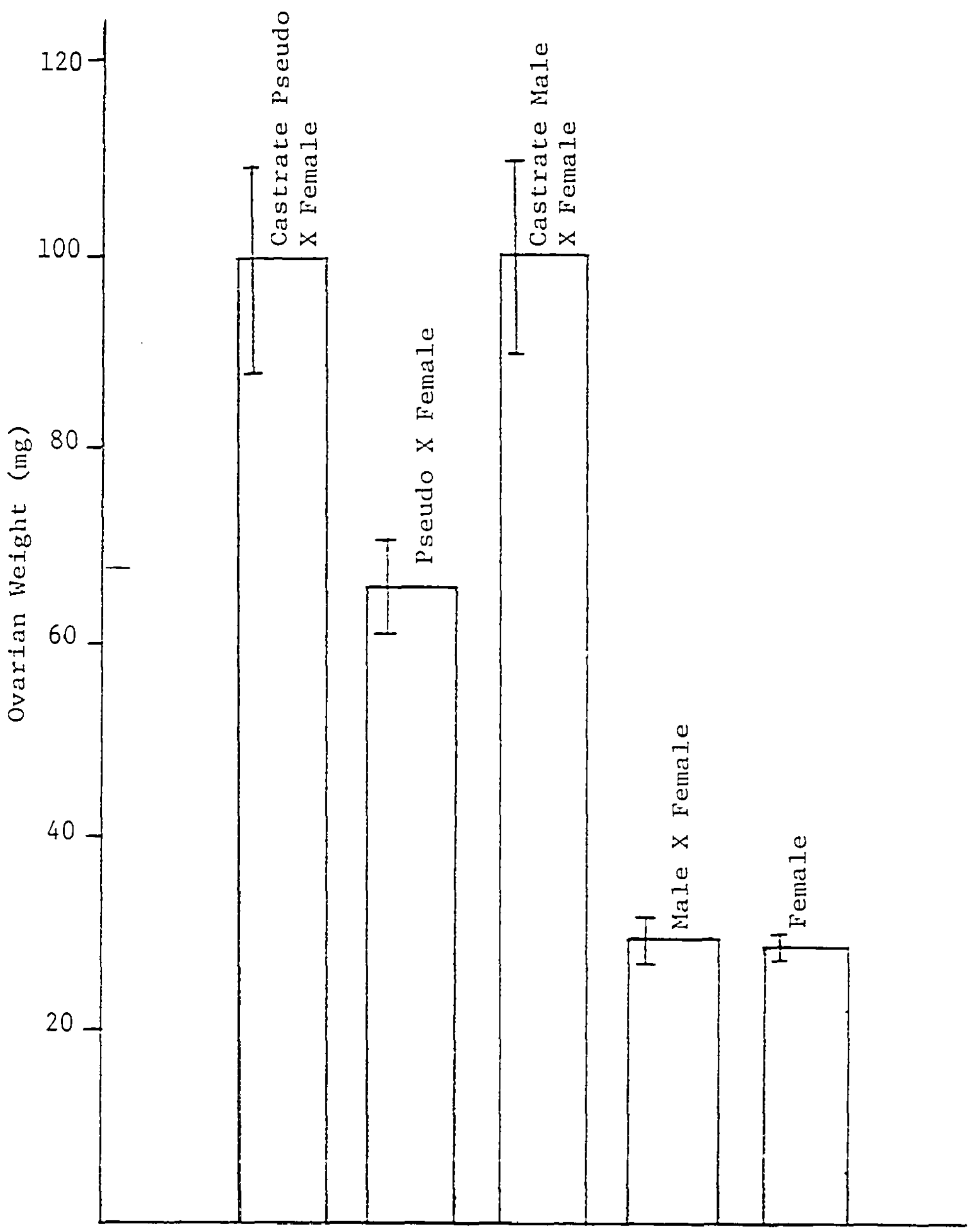

Figure 9. Female Rats were surgically united in parabiosis with male and pseudohermaphrodite rats at 30-33 days of age and sacrificed 15 days subsequent to surgical union.

quantitate the production of these steroids in the pseudohermaphrodite and control rats and also, to establish a baseline value from which the adrenal contribution could be assessed under various conditions. A total of 128 separate urinary 17-ketosteroid determinations were carried out on urine collections from male, female and male pseudohermaphrodite rats. Each determination was carried out on a pooled 250 ml aliquot of a 24-hour urine collection and individual rat values were calculated from the value of the total aliquot. Since each determination was carried out on a pooled urine collection, individual values actually represent an average. Table 14 lists the results of 30 analyses conducted on urine from normal male, female and pseudohermaphrodite rats with values reported in μg per 24 hours. The average of 10 determinations each for males, females and pseudohermaphrodites were 18.0 ± 0.71 μg, 33.6 ± 0.60 μg and 105.2 ± 1.3 μg respectively. The data summarized in Figure 10 demonstrate that the male pseudohermaphrodite rat has a markedly elevated output of urinary 17-ketosteroids in comparison with those of male and female controls. In addition, the female values are considerably above those of males.

In order to investigate the source of 17-ketosteroid production in pseudohermaphrodite and control rats, 30 separate determinations were carried out on urine from castrate males, females and pseudohermaphrodites. These findings are listed in Table 15. The 24-hour values dropped from 105.2 ± 1.3 μg for the normal pseudohermaphrodite to 44.7 ± 1.0 μg for the castrate pseudohermaphrodite and from 18.0 ± 0.71 μg to 13.1 ± 0.62 μg for the castrate male, while the female values remained essentially equivalent to the pre-castration values. Thus in the case of the pseudohermaphrodite, the testes accounted for more than one-half of the

TABLE 14

DETERMINATION OF TOTAL NEUTRAL URINARY 17-KETOSTEROIDS FROM THE URINE OF NORMAL MALE, FEMALE AND MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups		
	Normal Male	Normal Female	Normal Pseudo
	24 Hour Urinary 17-Ketosteroid Values		
	μg/24 hr	μg/24 hr	μg/24 hr
	16.0	33.5	114.0
	18.7	35.6	116.0
	13.9	30.6	92.4
	16.9	33.8	101.0
	19.8	33.0	86.0
	21.3	34.0	92.4
	16.9	30.0	112.0
	17.8	35.2	116.0
	20.7	34.8	110.0
	17.8	35.0	112.0
$\frac{\Sigma x_i}{n}$	18.0	33.6	105.2
Σx_i^2	3,278.0	11,288.5	111,768.5
$\frac{(\Sigma x_i)^2}{n}$	3,232.8	11,256.0	110,628.3
$S\bar{x}$	0.71	0.60	1.3

Steroid determinations were carried out on pooled 24 hours urine collections and individual rat values were calculated from the total aliquot.

TABLE 15

DETERMINATION OF TOTAL NEUTRAL URINARY 17-KETOSTEROIDS FROM THE URINE OF CASTRATE MALE, FEMALE AND MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups		
	Castrate Male	Castrate Female	Castrate Pseudo
	24 Hour Urinary 17-Ketosteroid Values		
	μg/24 hr	μg/24 hr	μg/24 hr
	12.6	33.6	43.3
	14.2	36.4	48.4
	10.4	32.5	43.0
	11.3	36.4	46.5
	12.3	33.4	40.0
	15.0	35.0	41.5
	13.0	34.3	42.5
	16.9	36.4	46.5
	11.3	33.6	45.0
	14.2	37.2	50.0
$\frac{\Sigma x_i}{n}$	13.1	34.9	44.7
Σx_i^2	1,756.5	12,189.9	20,044.5
$\frac{(\Sigma x_i)^2}{n}$	1,721.3	12,166.1	19,954.1
$S\bar{x}$	0.62	0.52	1.0

Steroid determinations were carried out on pooled 24 hour urine collections and individual rat values were calculated from the total aliquot.

Average Urinary 17-ketosteroid Values Of Normal And Castrate Male, Female and Male Pseudohermaphrodite Rats

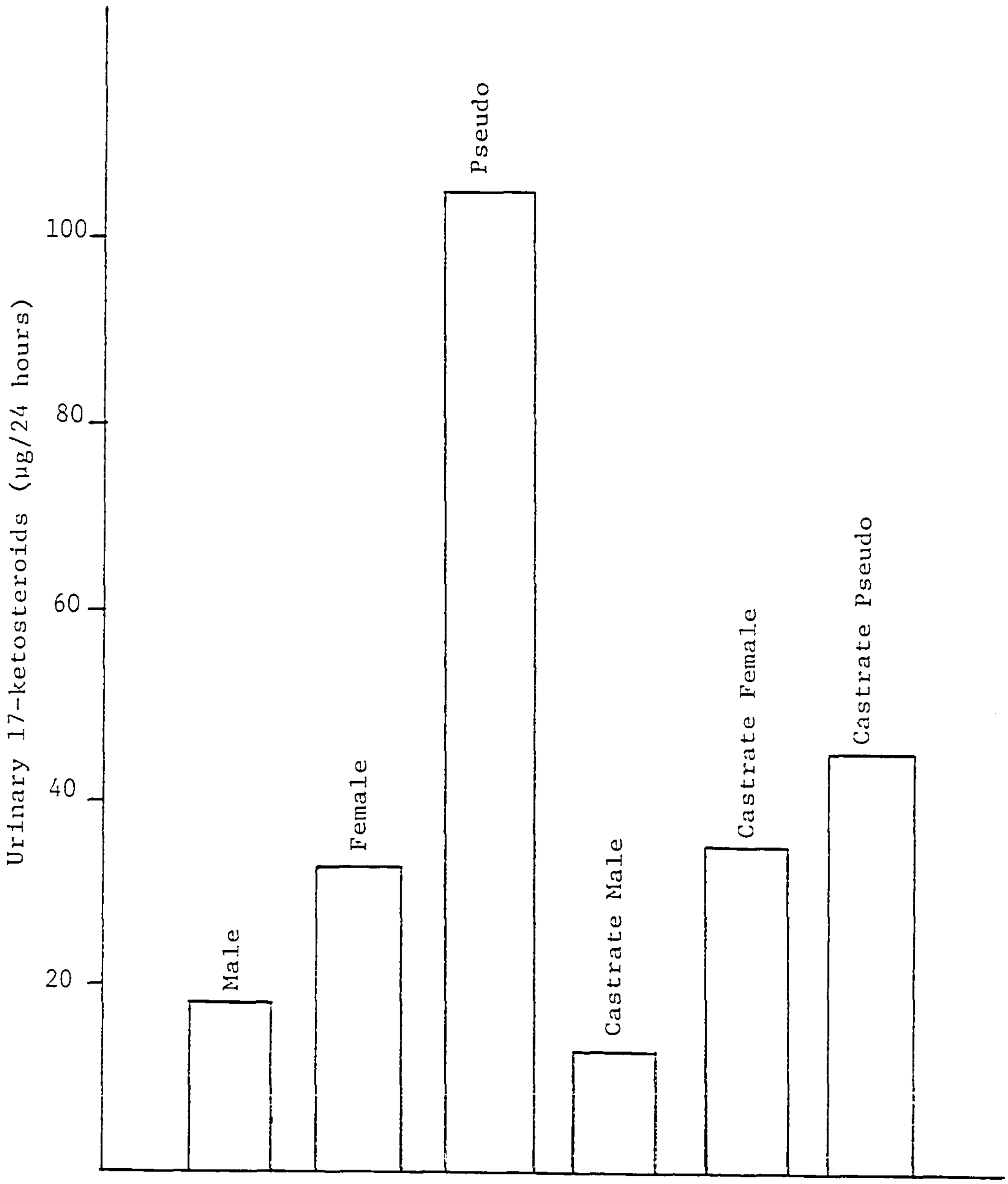

Figure 10. The above values represent an average of 10 separate determinations. Steroid determinations were carried out on pooled 24 hour urine collections and individual rat values were calculated from the value of the total aliquot.

17-ketosteroid values, while in the male and female, the adrenal contribution was preponderant. The data included in Figure 10 demonstrates that the adrenal output of 17-ketosteroids in the pseudohermaphrodite is considerably above that of the male or female controls.

With baseline values established and the sites of production identified and quantitated, the adrenal contribution to the urinary 17-ketosteroids was studied under various conditions. The principle point of the working hypothesis considered the gonadotropic hormones to be tropic to the adrenal cortex; accordingly, the adrenal contribution to the urinary 17-ketosteroids was studied under the condition of gonadotropin suppression in order to evaluate this postulate. The gonadotropic hormone secretion was suppressed by administering 50 μg of diethylstilbestrol per day to pseudohermaphrodite rats, and to male and female control rats. Table 16 lists 24-hour urinary 17-ketosteroid values from determinations carried out under the above conditions. In contrast to an average value of 44.7 $\pm$ 1.0 μg for castrates, the pseudohermaphrodite value fell additionally to a mean value of 32.1 $\pm$ 0.52 μg, while the male value fell to that of the castrate condition. The female output dropped slightly from an average value of 33.6 $\pm$ 0.60 μg to 30.0 $\pm$ 0.62 μg. The data included in Figure 11 indicate that suppression of gonadotropic hormone secretion decreased not only the testicular value in the pseudohermaphrodite but also the adrenal contribution.

In order to further test the effect of gonadotropin suppression, diethylstilbestrol was administered to castrate pseudohermaphrodite, and male and female control rats. The values listed in Table 17 and Figure 11 demonstrate that the adrenal contribution to the urinary 17-ketosteroids

TABLE 16

DETERMINATION OF TOTAL NEUTRAL URINARY 17-KETOSTEROIDS FROM THE URINE OF STILBESTROL AND DEXAMETHASONE TREATED NORMAL MALE, FEMALE AND MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups			
	Stilbestrol Treated Normal Male	Stilbestrol Treated Normal Female	Stilbestrol Treated Normal Pseudo	Dexamethasone Treated Normal Pseudo
	24 Hour Urinary 17-Ketosteroid Values			
	μg/24 hr	μg/24 hr	μg/24 hr	μg/24 hr
	10.8	28.6	30.0	16.6
	12.3	31.3	33.3	18.3
	11.8	27.0	30.8	17.5
	13.1	30.6	32.5	20.0
	10.3	29.2	33.3	15.8
	12.3	31.8	30.8	17.5
	10.5	30.0	33.3	16.6
	11.2	33.5	31.6	19.2
	11.2	27.6	34.9	
	13.6	30.6	30.3	
$\frac{\Sigma x_i}{n}$	11.7	30.0	32.1	17.7
Σx_i^2	1,382.3	9,047.3	10,314.9	2,516.8
$\frac{(\Sigma x_i)^2}{n}$	1,371.2	9,012.0	10,291.3	2,502.8
$S\bar{x}$	0.35	0.62	0.52	0.40

Steroid determinations were carried out on pooled 24 hour urine collections and individual rat values were calculated from the total aliquot.

TABLE 17

DETERMINATION OF TOTAL NEUTRAL URINARY 17-KETOSTEROIDS FROM THE URINE OF STILBESTROL TREATED CASTRATE MALE, FEMALE AND MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups		
	Stilbestrol Treated Castrate Male	Stilbestrol Treated Castrate Female	Stilbestrol Treated Castrate Pseudo
	24 Hour Urinary 17-Ketosteroid Values		
	μg/24 hr	μg/24 hr	μg/24 hr
	13.7	29.0	29.2
	16.0	32.7	30.3
	10.8	30.6	31.2
	13.3	33.4	31.7
	11.3	27.6	29.7
	14.0	32.0	30.3
	12.2	31.3	30.8
	13.7	32.3	32.9
	11.3	31.3	28.2
	13.7	33.4	30.0
$\frac{\Sigma x_i}{n}$	13.0	31.4	30.4
Σx_i^2	1,712.8	9,866.2	9,275.5
$\frac{(\Sigma x_i)^2}{n}$	1,690.0	9,834.5	9,259.8
$S\ \bar{x}$	0.51	0.60	0.42

Steroid determinations were carried out on pooled 24 hour urine collections and individual rat values were calculated from the total aliquot.

Average Urinary 17-ketosteroid Values Of Stilbestrol And Dexamethasone Treated Male, Female And Male Pseudohermaphrodite Rats

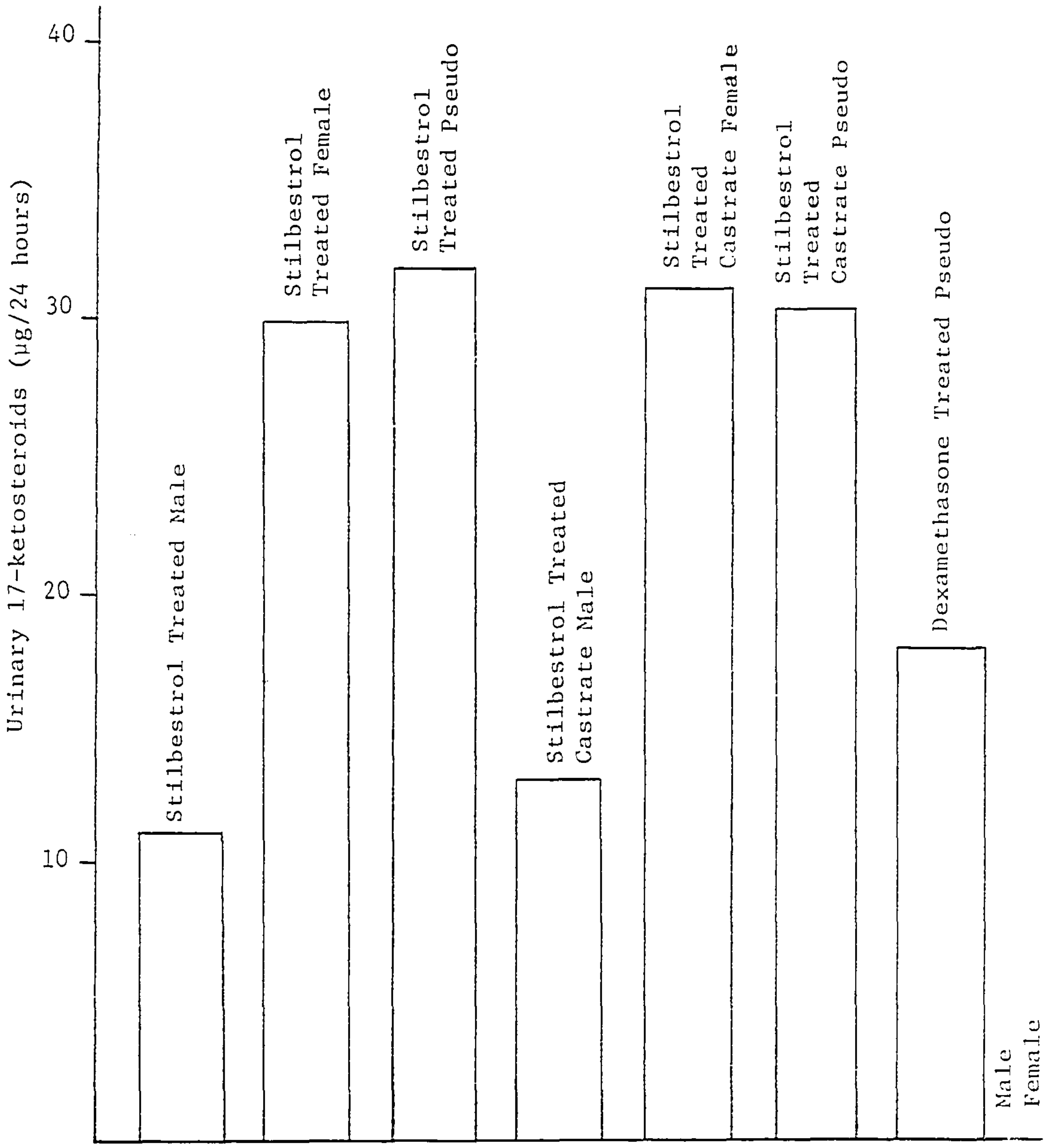

Figure 11. The above values represent an average of 10 separate determinations. Steroid determinations were carried out on pooled 24 hour urine collections and individual rat values were calculated from the value of the total aliquot.

was depressed in the pseudohermaphrodite rat from an average castrate value of 44.7 $\pm$ 1.0 µg to a value of 30.4 $\pm$ 0.42 µg. The male and female control values were essentially the same as the previous castrate values. These results demonstrate that suppression of gonadotropic hormone secretion leads to a depression of the adrenal output of 17-ketosteroids in the male pseudohermaphrodite rat.

In another series of experiments, ACTH titers were suppressed in pseudohermaphrodite and control rats by administering dexamethasone in a dose of 50 µg per day. This treatment completely abolished urinary 17-ketosteroid output in male and female control rats, and depressed values in the pseudohermaphrodite far below the previous testicular or adrenal values. These results are listed in the last column of Table 16 and in Figure 11. Thus, dexamethasone not only abolished the adrenal output, but also markedly depressed the testicular contribution.

Determination of Urinary 17-Deoxycorticosteroids

Determinations of urinary 17-deoxycorticosteroids were undertaken to assess the adrenal function of male pseudohermaphrodite and control rats with respect to corticosteroid production. Each determination was carried out on a pooled 120 ml aliquot of a 24-hour urine collection and individual rat values were calculated from the value of the total aliquot. Table 18 lists values in µg per 24 hours of urinary 17-deoxycorticosteroids. The average of 9 determinations each for males, females and pseudohermaphrodites were 9.2 $\pm$ 12 µg, 16.0 $\pm$ 0.5 µg and 16.7 $\pm$ 0.31 µg respectively. These figures demonstrated that the urinary corticosteroid output of the pseudohermaphrodite was in the normal female range, while that of the male was considerably below the female range of values.

TABLE 18

DETERMINATION OF URINARY 17-DEOXYCORTICOSTEROIDS FROM THE URINE OF NORMAL MALE, FEMALE AND MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups		
	Normal Male	Normal Female	Normal Pseudo
	24 Hour Urinary 17-Deoxycorticosteroid Values		
	µg/24 hr	µg/24 hr	µg/24 hr
	9.6	17.7	17.8
	9.8	15.5	17.2
	9.6	19.4	16.7
	9.0	15.5	15.5
	9.2	17.0	18.0
	9.4	14.6	15.8
	8.8	17.3	15.7
	9.0	18.1	16.5
	8.8	16.7	17.3
$\frac{\Sigma x_i}{n}$	9.2	16.9	16.7
Σx_i^2	770.2	2,578.1	2,530.5
$\frac{(\Sigma x_i)^2}{n}$	769.1	2,560.4	2,523.4
$S\bar{x}$	0.12	0.50	0.31

Steroid determinations were carried out on pooled 24 hour urine collections and individual rat values were calculated from the total aliquot.

Likewise, Table 19 lists urinary 17-deoxycorticosteroid values of 27 analyses of the urine of castrate pseudohermaphrodite and control rats. Figure 12 shows that these values do not differ significantly from those of normal rats.

Although administration of diethylstilbestrol depressed the urinary 17-ketosteroid output of adrenal origin in the pseudohermaphrodite rat, this did not establish whether the effect was due to gonadotropin suppression or simply to an adverse effect of diethylstilbestrol on the adrenal. In order to clarify this point, determinations of 17-deoxycorticosteroids were carried out on the urine of pseudohermaphrodites, and males and females during the course of diethylstilbestrol treatment. The values listed in Table 20 for castrate pseudohermaphrodite and castrate control rats demonstrated that adrenal function was not affected adversely by diethylstilbestrol. In fact, comparison of Figures 12 and 13 shows that these values were slightly elevated during the course of stilbestrol treatment.

Histological Study

The adrenal glands of pseudohermaphrodite rats were studied histologically to investigate the possibility of a correlation between adrenal enlargement and zonation and an elevated adrenal production of 17-ketosteroids. Figure 14 illustrates a low power view of the pseudohermaphrodite rat adrenal in comparison with those of male and female controls. The most obvious feature of the pseudohermaphrodite adrenal is a marked enlargement of an area in juxtaposition to the medulla. This area corresponds to the zona reticularis of the male and female adrenals. Figures 15 through 23 illustrate the same adrenal glands at high

TABLE 19

DETERMINATION OF URINARY 17-DEOXYCORTICOSTEROIDS FROM THE URINE OF CASTRATE MALE, FEMALE AND MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups		
	Castrate Male	Castrate Female	Castrate Pseudo
	24 Hour Urinary 17-Deoxycorticosteroids		
	μg/24 hr	μg/24 hr	μg/24 hr
	11.3	14.0	20.3
	11.5	15.5	16.3
	9.8	15.4	15.8
	10.2	17.7	16.5
	10.9	16.8	18.3
	11.3	18.4	17.0
	10.0	16.5	16.7
	10.9	15.4	18.8
	11.8	17.5	17 3
$\frac{\Sigma x_i}{n}$	10.8	16.4	17.5
Σx_i^2	1,062.3	2,424.0	2,760.3
$\frac{(\Sigma x_i)^2}{n}$	1,058.2	2,408.2	2,743 7
$S\bar{x}$	0.24	0.47	0.49

Steroid determinations were carried out on pooled 24 hour urine collections and individual rat values were calculated from the total aliquot.

Average Urinary 17-Deoxycorticosteroid Values Of Male, Female And Male Pseudohermaphrodite Rats

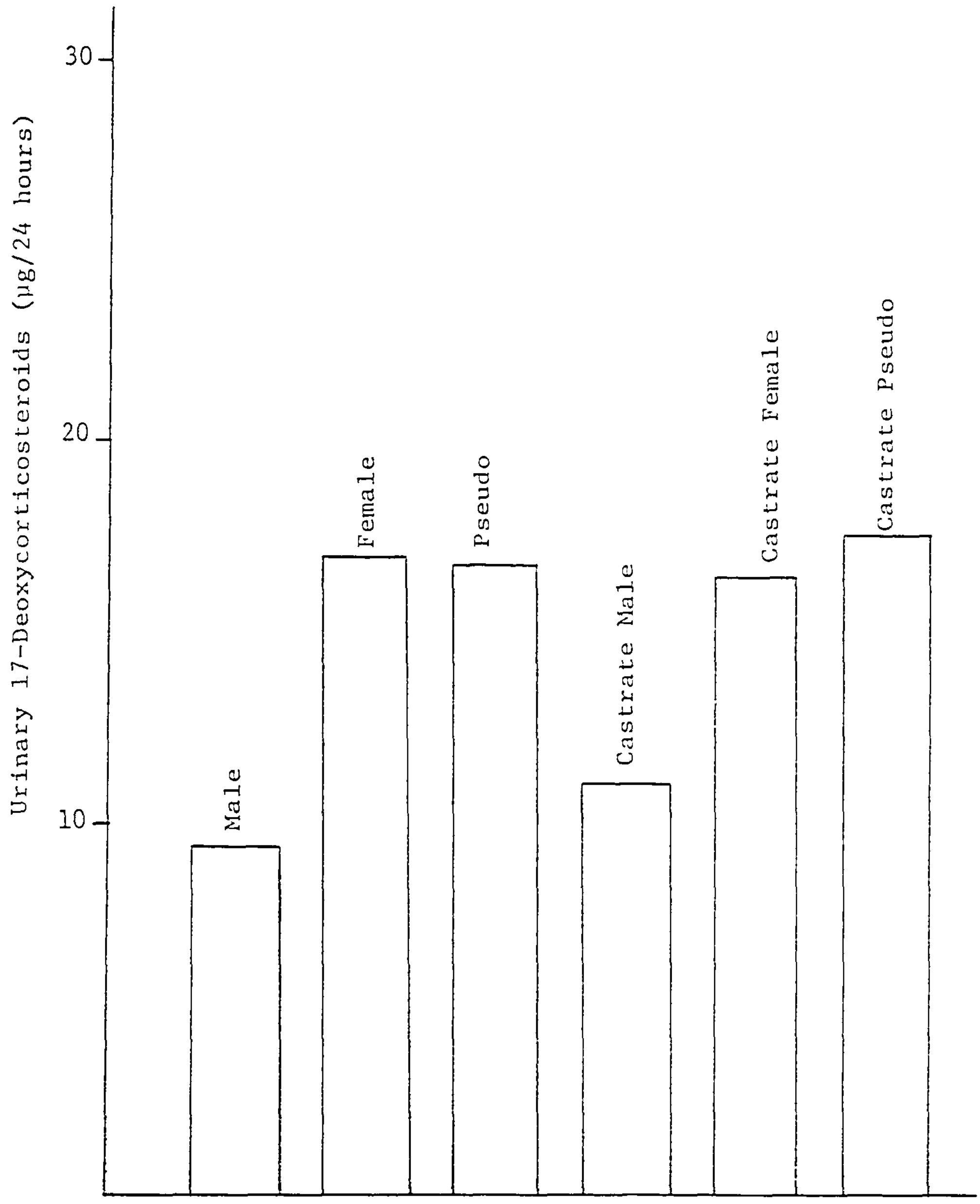

Figure 12. The above values represent an average of 9 separate determinations. Each determination was carried out on a pooled 24 hour urine collection and individual rat values were calculated from the value of the total aliquot.

TABLE 20

DETERMINATION OF URINARY 17-DEOXYCORTICOSTEROIDS FROM THE URINE OF STILBESTROL TREATED CASTRATE MALE, FEMALE AND MALE PSEUDOHERMAPHRODITE RATS

	Treatment Groups		
	Stilbestrol Treated Castrate Male	Stilbestrol Treated Castrate Female	Stilbestrol Treated Castrate Pseudo
	24 Hour Urinary 17-Deoxycorticosteroid Values		
	μg/24 hr	μg/24 hr	μg/24 hr
	11.3	19.3	20.8
	12.0	18.7	20.2
	12.8	18.8	19.7
	11.8	18.50	19.8
	11.1	18.7	19.0
	11.5	19.7	19.3
	9.6	20.2	19.5
	12.4	18.3	20.5
	11.8	17.8	17.8
$\frac{\Sigma x_i}{n}$	11.6	18.9	19.6
Σx_i^2	1,216.2	32.1	3,473.9
$\frac{(\Sigma x_i)^2}{n}$	1,209.4	3,207.7	3,467.6
$S\bar{x}$	0.31	0.24	0.29

Steroid determinations were carried out on pooled 24 hour urine collections and individual rat values were calculated from the total aliquot.

Average Urinary 17-Deoxycorticosteroid Values of Male, Female And Male Pseudohermaphrodite Rats

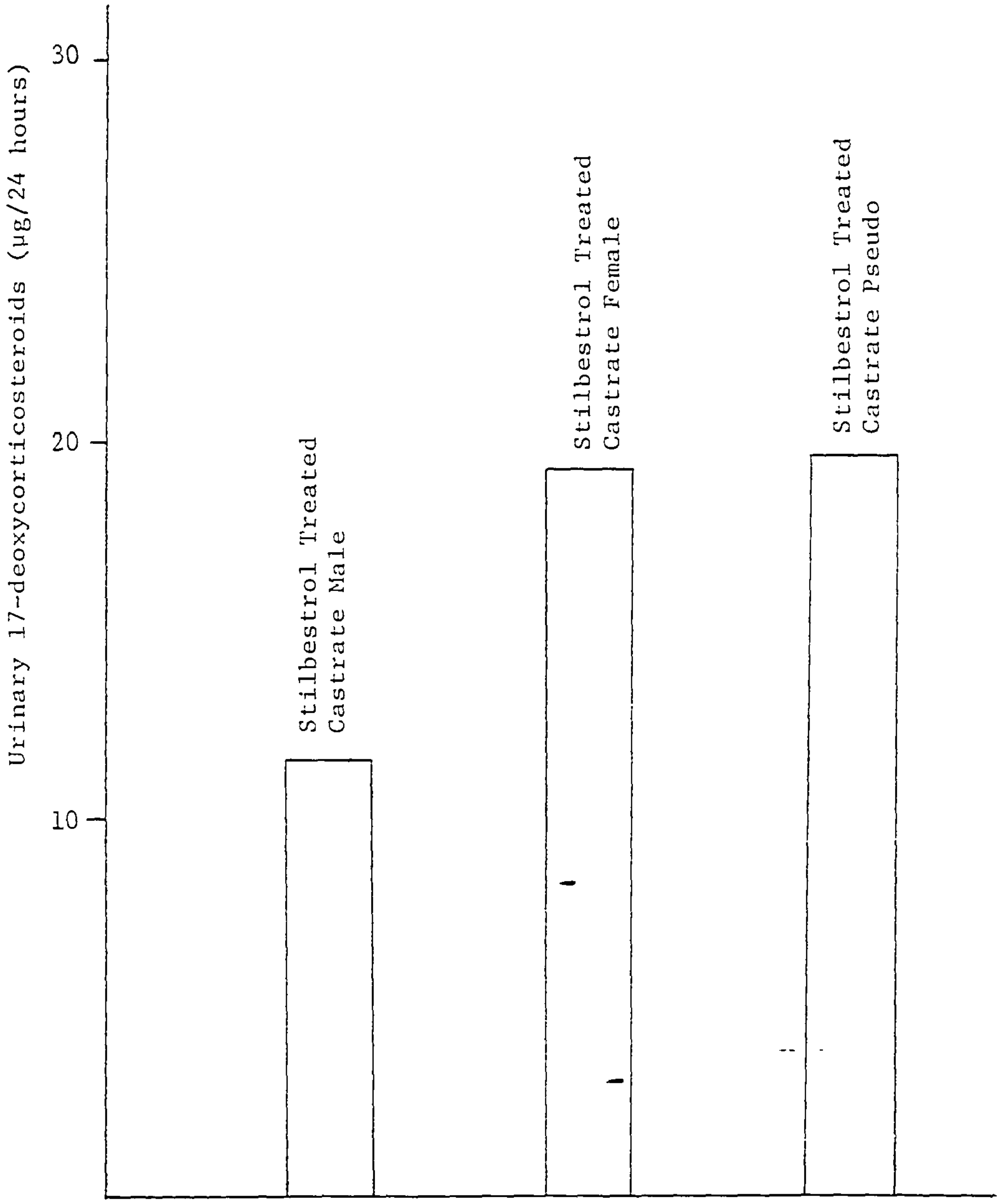

Figure 13. The above values represent an average of 9 separate determinations. Each determination was carried out on a pooled 24 hour urine collection and individual rat values were calculated from the value of the total aliquot.

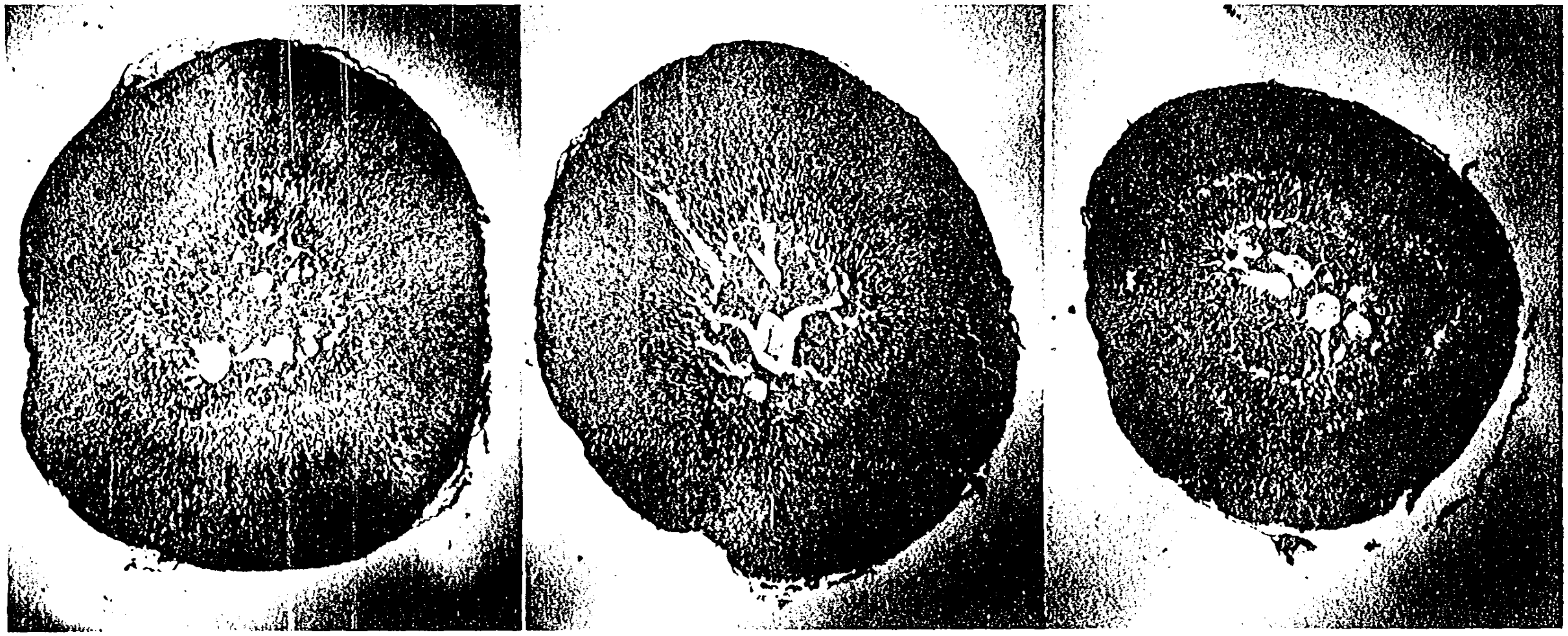

Figure 14. Cross-section of adrenal glands from male pseudohermaphrodite (left), female (middle) and male (right) rats. Note the marked enlargement of an area adjacent to the medulla in the adrenal of the pseudohermaphrodite. This area appears broken up by sinusoids and corresponds to the zona reticularis of the female and male adrenals. Delafield's hematoxylin. X 40.

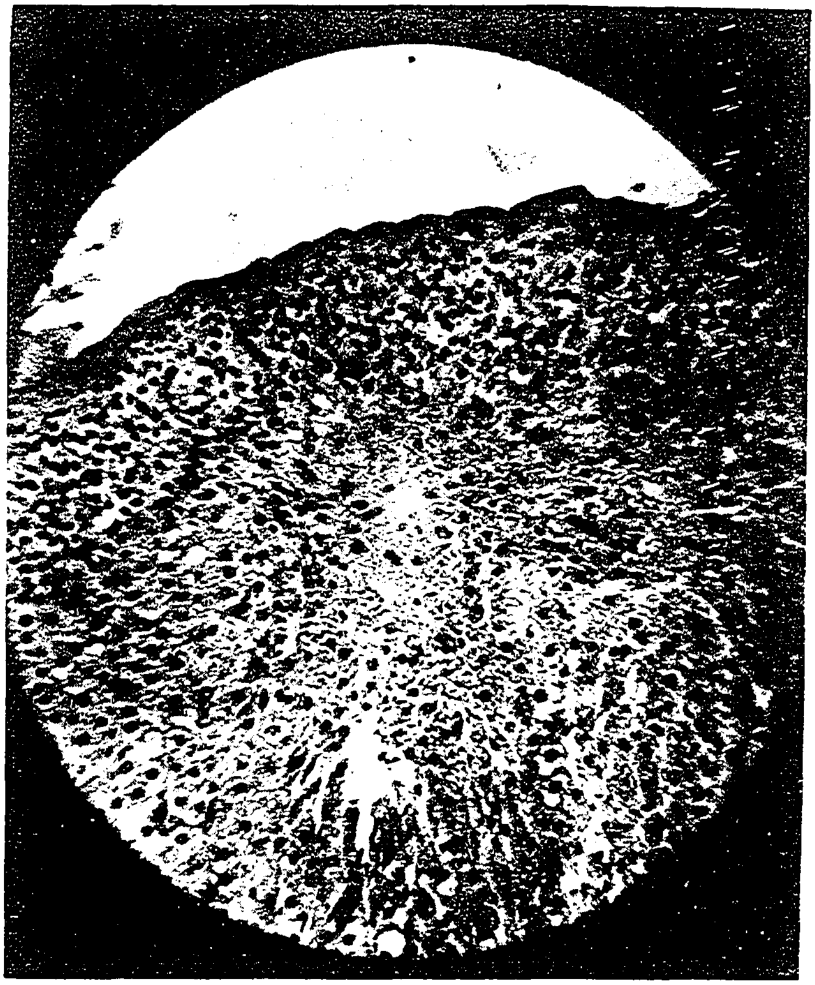

Figure 15. Cross-section of an adrenal gland from a male pseudohermaphrodite rat showing both glomerular and fascicular zones. The zona fasciculata does not retain the radial symmetry found in the female and male adrenals. Delafield's hematoxylin. X 400.

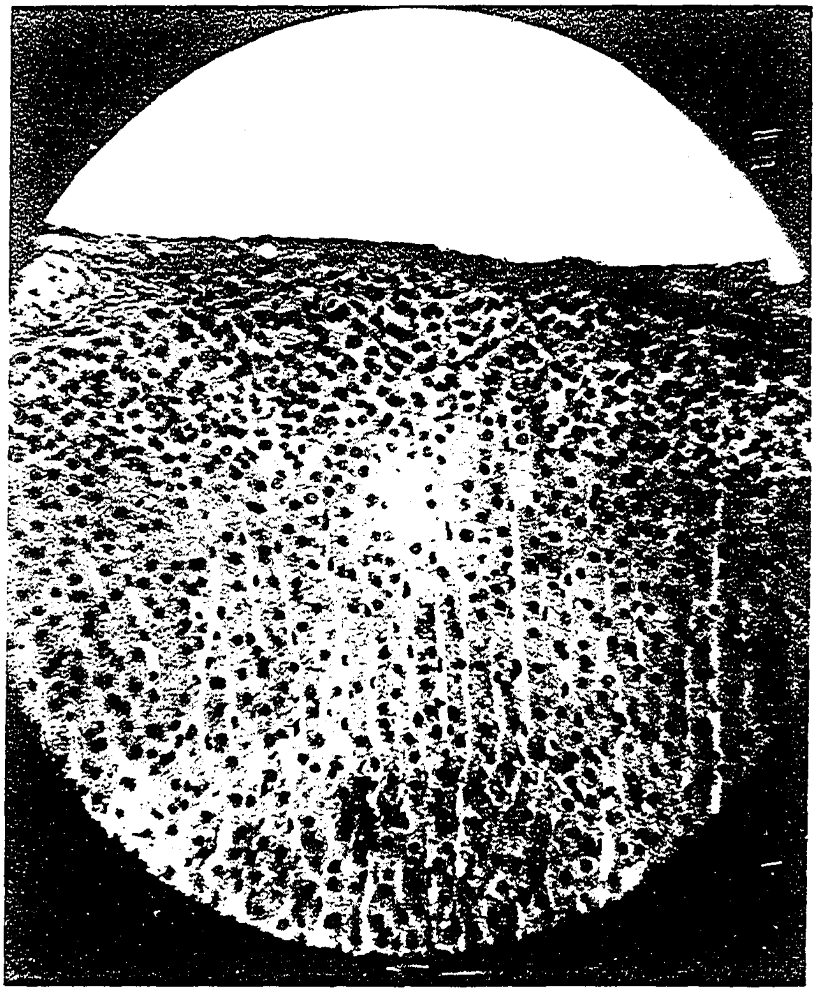

Figure 17. Cross-section of an adrenal gland from a male rat showing both glomerular and fascicular zones. Note the radial symmetry of the zona fasciculata. Delafield's hematoxylin. X 400.

Figure 18. Cross-section of an adrenal gland from a male pseudohermaphrodite rat illustrating the reticulo-fascicular junction. The cellular cords appear to be disrupted by numerous sinusoids, an appearance not found in the adrenals of males or females. Delafield's hematoxylin. X 400.

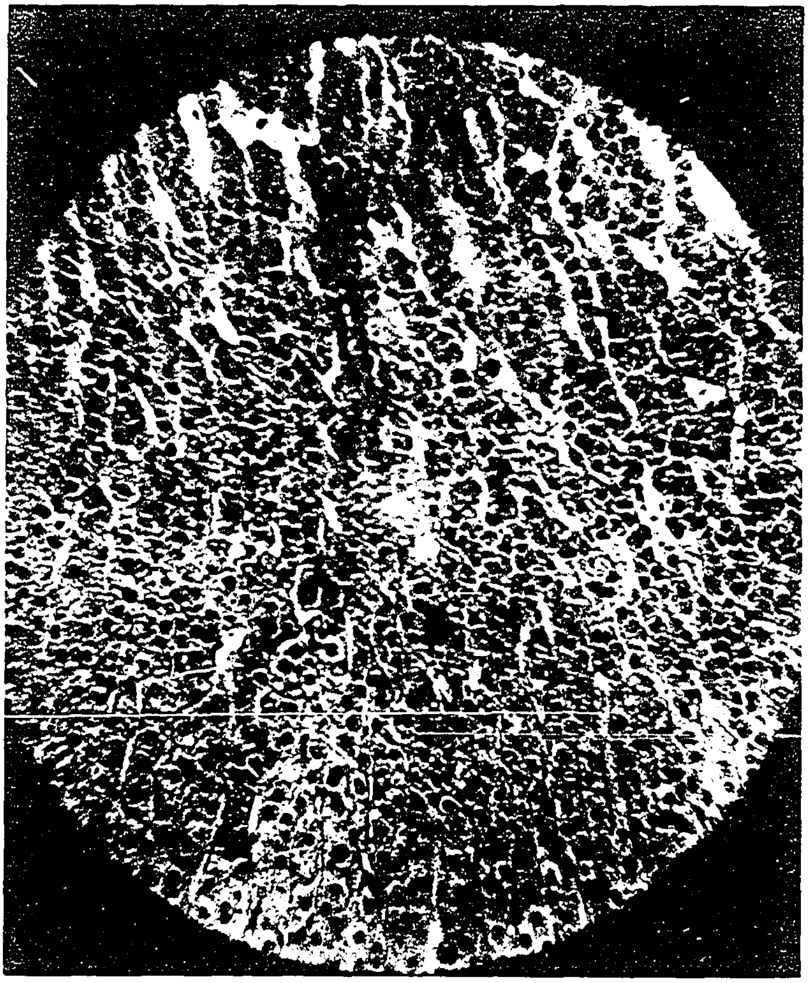

Figure 19. Cross-section of an adrenal gland from a female rat showing the reticulo-fascicular junction. Note the symmetry of the cellular cords in the zona fasciculata. Delafield's hematoxylin. X 400.

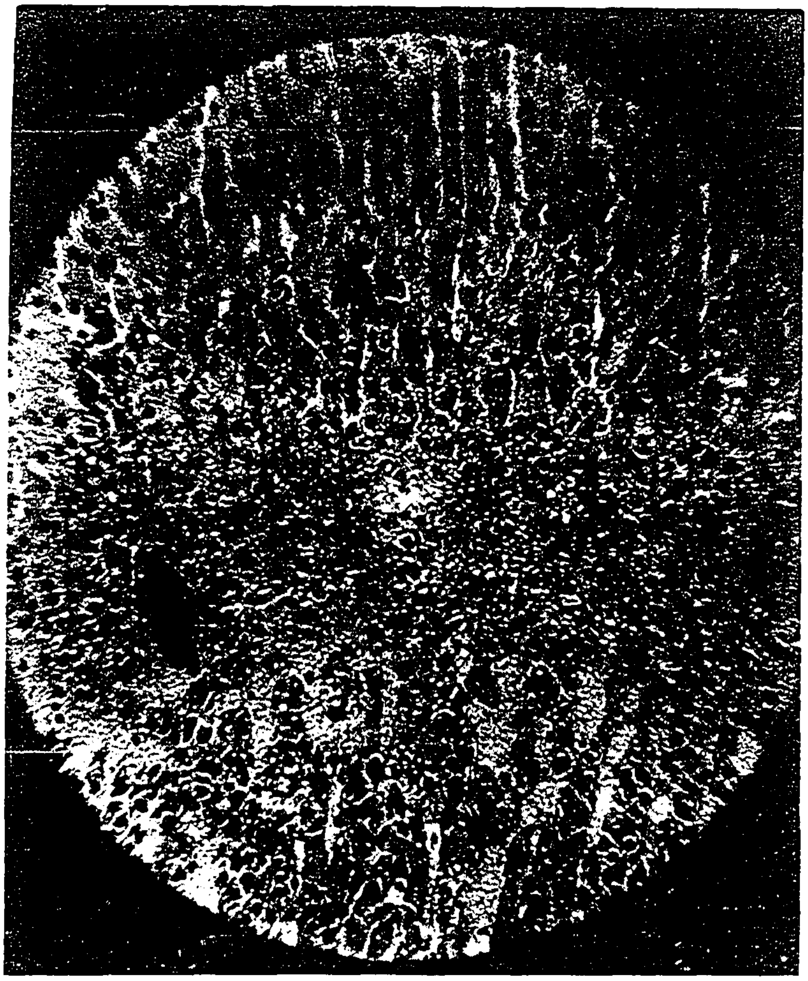

Figure 20. Cross-section of an adrenal gland from a male rat showing the reticulo-fascicular junction. Note the symmetry of the cellular cords in the zona fasciculata. Delafield's hematoxylin. X 400.

Figure 21. Cross-section of an adrenal gland from a male pseudohermaphrodite rat. The section illustrates the rich sinusoidal vascularity of the zona reticularis. Delafield's hematoxylin. X 1000.

Figure 22. Cross-section of an adrenal gland from a female rat showing the zona reticularis and parts of the medulla (upper right). Delafield's hematoxylin. X 1000.

Figure 23. Cross-section of an adrenal gland from a male rat showing the zona reticularis (lower) and parts of the medulla (upper). Delafield's hematoxylin. X 1000.

magnification. The outer zone or zona glomerulosa (Figures 15, 16, 17) is distinct with deeply staining nuclei and light basophilic cytoplasm in all three adrenals. However, the middle or fascicular zone differs to some extent in the pseudohermaphrodite adrenal. In contrast to palisades of radial cellular cords found in the male and female adrenals (Figures 16 and 17), some areas of the fascicular zone in the pseudohermaphrodite adrenal are less symmetrical (Figure 15). Sinusoids appear to cut across cellular cords in these areas rather than coursing radially, while in other areas the radial symmetry is retained.

The most conspicuous changes are found in the zona reticularis of the pseudohermaphrodite adrenal. This area is characterized by enlargement in thickness and increase in sinusoidal vascularity (Figures 18 and 21). In addition, cellular cords are broken up by anastomosing sinusoids. Cells in this region retain a spongy appearance due to lipid extraction during fixation, an appearance normally restricted to the zona fasciculata. In short, these changes are indicative of increased metabolic activity. Corresponding areas in the male and female adrenals retain a radial symmetry, cell nuclei are compact and the cytoplasm stains acidophilic (Figures 19, 20, 22 and 23). The above findings support the view that increased metabolic activity of the adrenal cortex in the pseudohermaphrodite rat involves mainly the zona reticularis and furthermore, supports the view of a trophic stimulation by LH.

Summary of Experimental Findings

The experimental findings demonstrated by the present study are as follows:

1) The gonadotropin content of the pituitary and plasma of male

pseudohermaphrodite rats was shown to be greater than that of male or female control rats.

2) Urinary 17-ketosteroid values in normal and castrate pseudohermaphrodite rats were greater than corresponding values in male and female control rats. The testicular contribution accounted for more than one-half of the 17-ketosteroid value in the pseudohermaphrodite, and in addition, the adrenal contribution was found to be greater than that of male and female control rats.

3) Suppression of gonadotropic hormone secretion in the castrate pseudohermaphrodite rat resulted in a marked decrease in urinary 17-ketosteroids without affecting the 17-deoxycorticosteroid values.

4) Urinary 17-deoxycorticosteroid values in pseudohermaphrodite rats were found to be in the normal female range.

5) Comparative histological studies of adrenal glands from pseudohermaphrodite and control rats demonstrated enlargement of the zona reticularis and increased sinusoidal vascularity in the adrenal of the pseudohermaphrodite.

CHAPTER IV

DISCUSSION

The techniques employed will be appraised before presenting an interpretation of the results.

Appraisal of Methods

The Ovarian Ascorbic Acid Method for the Bioassay of LH

In the original description of this method, Parlow (1958; 1961) showed that anterior pituitary hormones other than luteinizing hormone (LH) were not active in the ovarian ascorbic acid depletion (OAAD) assay except in doses large enough to indicate contamination with LH. Even when follicle-stimulating hormone (FSH), luteotropic hormone (LTH) or somatotropic hormone (STH) were added to LH, the response was not altered, thus indicating that the method was reliable in the presence of a mixture of hormones. These results were confirmed by McCann and Taleisnik (1960). These investigators found that vasopressin will cause ovarian ascorbic acid depletion, but the quantities required were extremely large. Moreover, endogenous vasopressin release (evoked by hemorrhage in rats) did not result in depletion of ovarian ascorbic acid. Guiliani _et al._ (1961) reported that a variety of drugs and stress procedures were without effect in this test. Schmidt-Elmendorff and Loraine (1962) appraised the assay and concluded that it had the advantages of sensitivity and specificity, but a large proportion of the assays were invalid due to a lack of parallelism. The problem of non-linearity was also encountered in the present investigation which employed per cent depletion of ovarian ascorbic acid and the slope-ratio form of regression analysis. A variety of standard LH test materials were used by Persky and Norton (1964) to compare the stability of the dose-response relationship of the OAAD assay and the hypophysectomized rat prostate method. Both assays yielded stable slopes with all standards, but the OAAD method was recommended because of its greater sensitivity.

Significant diurnal variation in ovarian ascorbic acid content of rats prepared for bioassay has been demonstrated by Stevens et al. (1964). Likewise Lawton and Schwartz (1965) demonstrated a 24-hour periodicity in ovarian ascorbic acid to standard doses of LH. Both groups recommended that OAAD assays begin at a standard time each day, preferably in the morning when ascorbic acid levels are high, in order to minimize this effect. Thus, despite some limitations, the OAAD assay is generally accepted as a convenient and sensitive method for the assay of LH potency in pituitary extracts. The OAAD method for estimating plasma LH activity employed in the present study either underestimated or overestimated the actual potency because of curvilinear regression lines. Statistical validity was determined by testing for deviation from regression. Those assays which showed significant deviation from regression were considered statistically invalid. Consequently, only inferences involving differences between experimental groups can be made, since the relative potencies are estimated from non-linear regression lines. Figures 4 and 8 show a greater pituitary and plasma LH potency for pseudohermaphrodites when the dose levels employes are considered.

The Method of Parabiosis for the Bioassay of FSH

Investigators have used many methods to quantitate follicle-stimulating hormone (FSH) from extracts of the anterior pituitary. Evans et al. (1939) used histological criteria based on the production of non-atretic follicles with small antra in the ovaries of hypophysectomized 21-day old female rats. In addition, increase in uterine weight was employed by the above investigators as an endpoint for the estimation of FSH activity. Fevold, Lee, Hisaw and Cohn (1940) demonstrated the effectiveness of an increase in ovarian weight in the immature 21-day old female rat as an assay procedure for FSH. Greep, Van Dyke and Chow (1940) utilized the increase in testicular weight, in the absence of prostate and seminal vesicle hypertrophy. Steelman and Pohley (1953) employed the increase in ovarian weight when the ovary was synergized with human chorionic gonadotropin, while Payne, Runser and Hagans (1959) recommended the increase in ovarian weight following augmentation with stilbestrol for estimation of FSH potency. The last two methods are the most sensitive, but are effective only for pituitary extracts and not for plasma.

Radio-immunological assays have been shown recently to be sensitive to plasma levels of gonadotropins (Faimen and Ryan, 1967). These methods depend on a reaction between hormone labeled with I-131 (labeled antigen) and an antibody specific to the hormone. This reaction results in the formation of a labeled antigen-antibody complex. If unlabeled hormone is added it competes for the fixed amount of antibody and forms an unlabeled antigen-antibody complex, thereby reducing the amount of antibody which can participate in the formation of the labeled complex.

Thus the ratio of I-131 hormone bound to antibody to the free I-131 hormone falls progressively in proportion to the increase in concentration of the unlabeled hormone. The major criticism of the radio-immunological assay deals with the fact that other anterior pituitary hormones cross-react with the antibodies for the gonadotropins.

The use of parabiosis in the present study has the disadvantage of demonstrating only elevated titers of FSH. In addition, the results are not quantitative in nature. However, the assay did demonstrate an elevated FSH titer in the pseudohermaphrodite rat as evidenced by an increase in ovarian weight of female parabionts. Normal plasma titers of FSH were readily metabolized by control animals and therefore significant amounts of FSH did not enter the circulation of the test animals. In the case of the pseudohermaphrodite, FSH titers were so high that significant quantities did enter the circulation of the test parabiont and stimulated ovarian enlargement.

Determination of Total Neutral Urinary 17-Ketosteroids

All 17-ketosteroids have in common an oxygen atom attached at C_{17} and all are derivatives of two parent hydrocarbons, 5α-androstane and 5β-androstane, which differ only in the spatial configuration of the hydrogen atom at C_5. Epiandrosterone and dehydroepiandrosterone are the principal 17-ketosteroids with the 3β-hydroxy group. However, the major portion of the total urinary 17-ketosteroids show the 3α-hydroxy configuration (Loraine and Bell, 1966). From a quantitative standpoint the two most important compounds in this fraction are androsterone and etiocholanolone. The estrogenic steroid estrone is also a 17-ketosteroid

However, this compound, due to its phenolic A ring, is acidic in nature and is removed from urinary extracts when these are washed with a base.

As a result of reactions which occur during the process of hydrolysis and extraction, a number of chemical changes called artifacts are produced in the steroids of urine (Dorfman and Shipley, 1956). The production of these artifacts may distort the true values of the urinary steroids by causing an apparent increase or decrease in the concentration. Artifacts arise as a result of reactions of substitution, dehydration, rearrangement or epimerization occurring during the process of hydrolysis and extraction (Dorfman and Shipley, 1956). Under special circumstances, these artifacts can be extinguished by various correction formulae. In the present study, the best results were obtained by correcting the unknown with a urine blank and calculating the unknown value from a calibration curve. Per cent transmittance was employed in the construction of calibration curves rather than absorbance, because small changes in concentration at the upper end of the transmittance scale result in relatively large changes in transmittance, but only small changes in absorbance. This is due, in part, to the difficulty in reading absorbance from a logarithmic scale and also to the exponential increase in transmittance with arithmetically increasing concentrations. The transmittance scale is linear over the entire range, but per cent transmittance measurements cannot be used for direct calculation of unknown concentrations, since transmittance increases or decreases exponentially. However, by plotting the logarithm of transmittance against concentration, a linear relationship is obtained and a direct proportion can be used in the calculation of unknown values. Thus, the linearity of the calibration curve was improved at the lower ranges of concentration by using transmittance. Despite the obvious limitations of the method employed for the determination of urinary 17-ketosteroids, results were consistent. The method was able to demonstrate differences in males, females and pseudohermaphrodites and differences following castration, and stilbestrol and dexamethasone treatments. Finally, the specificity of the method and magnitude of the results obtained in the present work were corroborated by plasma determinations of steroids from pseudohermaphrodite rats reported by Stanley and co-workers (1967). These results are listed in APPENDIX II, page 136).

Determination of Urinary 17-Deoxycorticosteroids

The methods available for the group determination of 17-deoxycorticosteroids are limited in number and lacking in specificity. Attempts at the group determination of urinary 17-deoxycorticosteroids on formaldehydrogenic substances proved unsuccessful, when it was shown that cortisone and other 17:21 diol-20-ones significantly contributed to the determination (Tompsett, 1953). The method employed in the present work is considered specifically to determine 17-deoxycorticosteroids which on consecutive treatment with borohydride and with sodium bismuthate yield non-volatile aldehydes (Exley, et al., 1961). The excellent correlation found between the excretion of 17-deoxycorticosteroids by normal untreated rats and by those whose adrenal function was suppressed by treatment with dexamethasone indicates adrenal origin of the compounds determined by the present method.

One of the primary criticisms directed against the method of aldehyde conversion for the determination of urinary 17-deoxycorticosteroids was stated by Exley and co-workers (1961) in their original description of the procedure. These investigators were able to show that large and variable reagent blanks resulted from surplus benzenesulphohydroxamic acid and formed sulphinic acid. However, when the formed hydroxamic acid was extracted with ethyl acetate, the interfering substances remained in the aqueous phase. Exley et al.,(1961) demonstrated that the ferric hydroxamate complex could be concentrated by adding petroleum ether to the ethyl acetate extract. This procedure quantitatively transferred the complex from the organic to the aqueous phase.

Interpretation of Results

Gonadotropin Assays

Evidence of a relationship between gonadotropins, particularly LH, and the adrenal was assembled by Reifenstein et al. (1945), Albright (1947), and Jones (1948). With the lapse of more than 20 years, the evidence has not been sufficiently substantiated to warrant a general theory. In a few species of animals there are seasonal changes in the reticular zone of the adrenal with particular reference to the sexual cycle (Zalesky, 1934). These few reports constitute the basis for the previously held notion which considered the gonadotropins to be tropic to the zona reticularis of the adrenal gland. The above reports have been criticized so severely that the relationship between gonadotropins and adrenal cortex has gone essentially uninvestigated for more than 20 years.

The present study has reopened the question of the relationship of the gonadotropins to the adrenal cortex with new evidence accumulated from experiments conducted on male pseudohermaphrodite rats. Among the various points considered is the status of the gonadotropin titer in the male pseudohermaphrodite rat. These animals exhibit a chronic high titer of LH both in the pituitary gland and plasma (Figure 4 and 8, Pages 56,67). Indeed, this very finding makes the entire study unique because the adrenal cortex of these animals is under the continuous influence of a high gonadotropin titer. A similar condition may be mandatory for the demonstration of androgenic stimulation in the absence of increased corticosteroid production in normal animals. The pseudohermaphrodite rat has a pituitary LH potency considerably greater than that of normal male and female rats. A high pituitary content of LH is associated generally with an increased secretory output. This is reflected in the findings from normal male and female rats. The male rat has a larger pituitary LH content and also a higher plasma titer of LH than does the female. Although interpretation of plasma results was difficult in the present study, plasma LH assays did demonstrate an elevated LH titer in the pseudohermaphrodite rat in comparison to that of male and female controls.

The gonadotropin titer in the pseudohermaphrodite rat was characterized further with the finding of an increase in the FSH fraction (Figure 9, Page 70). This finding was reflected in an increased ovarian weight of female rats united in parabiosis with pseudohermaphrodites Although these results cannot be quantitated, they do demonstrate that either FSH is not metabolized by the pseudohermaphrodite rat, or that the FSH titers are markedly elevated due to an increased pituitary secretion. The finding of a low pituitary FSH content in the pseudohermaphrodite rat pituitary supports the latter view (Easley, 1966). The significance of an elevated plasma FSH in relation to adrenal function is not as readily explained as that of LH, but certainly synergism between FSH and LH is known to exist with respect to gonadal function. In summary, the findings discussed in this section substantiate the first condition of the working hypothesis; namely, gonadotropin titers are elevated in the male pseudohermaphrodite rat.

Urinary 17-Ketosteroids

Acute experiments employing bolus injections of gonadotropins, exemplified by the work of Gemzell, Diczfalusy and Tillinger (1958), and Apostalokis, Bettendorf and Voigt (1962), have failed to demonstrate significant increases in urinary 17-ketosteroids. These findings are not surprising when the half-life of gonadotropin preparations are taken into account. In all probability, a continuous chronic presence of gonadotropins is necessary before the adrenal cortex becomes responsive to tropic stimulation. Three methods could be employed to meet the above conditions: in vitro incubation of adrenal slices with gonadotropins, perfusion of the adrenal with gonadotropins or a long-acting gonadotropin preparation. The male pseudohermaphrodite rat is a good experimental model, since the continuous high gonadotropin output in these animals produces chronic stimulation of the adrenal cortex.

Baseline values for the 24-hour urinary 17-ketosteroid excretion, established by the present study, demonstrated a urinary output in the pseudohermaphrodite rat 3 to 5 times greater than that of male or female controls (Figure 10, page 74). The adrenal and testicular contributions to the 17-ketosteroids were assessed by determinations carried out on castrate animals. Values of urinary 17-ketosteroids from castrate animals were considered to be entirely of adrenal origin. Accordingly, the testes were found to contribute more than one-half of the normal value in the pseudohermaphrodite and, in addition, the adrenal contribution was shown to be considerably above that of male or female control rats. The marked contribution of the testes to the 17-ketosteroids in the pseudohermaphrodite was reflected in the hyperplasia of the Leydig cells of these gonads. This was interpreted to be the result of an increased quantity of LH acting on the interstitial cells. Likewise, an increased adrenal contribution has been attributed to the high circulating titer of LH.

Several lines of evidence suggest that the adrenal cortex of the pseudohermaphrodite is stimulated by LH. It will be recalled from a previous section (Page 10) that the adrenals of the pseudohermaphrodite are significantly larger than those of males and females of the same age (APPENDIX II, page 135). This suggests some kind of increased metabolic activity, but does not indicate the mediator. On the assumption that LH was the mediator of adrenal enlargement and increased testicular and adrenal androgen production, experiments were conducted whereby pituitary gonadotropin secretion was suppressed by treatment with diethylstilbestrol. Coincident with gonadotropin suppression, the 17-ketosteroids of the pseudohermaphrodite rat fell considerably below the castrate values (Figure 11, page 78). This finding indicated that both testicular and adrenal function were dependent on the previously existing levels of LH. Stilbestrol treatment of male and female rats resulted in a moderate depression of 17-ketosteroids in the male, while the female values were essentially unaffected.

The adrenal function was assessed independently of the gonads by treating castrate pseudohermaphrodites with diethylstilbestrol. Again, the 17-ketosteroids fell below the castrate values, indicating a previous stimulation by LH. Adrenal function in relation to 17-ketosteroid production was not totally dependent on a tropic stimulation by LH, because gonadotropin suppression decreased the values only to the normal female range. Thus the greater part of the adrenal contribution in the pseudohermaphrodite was probably mediated via ACTH, as in the case of the male and female rats. The relationship between ACTH and LH was further demonstrated by results from experiments in which adrenal function was suppressed with dexamethasone. Under dexamethasone treatment, the 17-ketosteroid output in the pseudohermaphrodite fell to one-fifth of the normal value, while 17-ketosteroids were undectable in both male and female controls. The same experiments conducted on castrate pseudohermaphrodites completely abolished 17-ketosteroid production. Thus, in addition to completely abolishing the adrenal contribution, the testicular output of 17-ketosteroids was depressed by dexamethasone. It is concluded from these results that LH is effective only when adrenal function is maintained by ACTH.

A final line of evidence which suggests that the pseudohermaphrodite adrenal is influenced by LH comes from the histology of the adrenal. The relationship between LH and the zona reticularis of the mouse and thirteen-lined ground squirrel has been previously discussed (Zalesky, 1934; Jones, 1948). In this connection, the adrenal of the pseudohermaphrodite rat shows hypertrophy of the zona reticularis (Figure 14, Page 86). Thus the physiological data are supported by anatomical findings.

17-Deoxycorticosteroids

The striking enlargement of the pseudohermaphrodite adrenal gland (30% larger than that of control rats) gave rise to numerous speculations regarding the cause of its enlargement. Evidence seemed insufficient to suggest a cause for enlargement consistent with the well-established concepts of adrenal function. The commonly accepted view concerning adrenal function holds that adrenocorticotropin (ACTH) is the sole pituitary factor which can mediate increased metabolic activity of the adrenal cortex. Increase in adrenal function generally arises as the result of one of the following conditions: autonomous hyperfunction such as is found in tumorous conditions of the adrenal, increased tropic stimulation by ACTH due to a pituitary neoplasm, or increased tropic stimulation by ACTH in association with insufficient negative feedback on the hypothalamo-hypophyseal axis. Any of these conditions would impair the health of an affected individual, a situation not seen in the pseudohermaphrodite rat.

The possibility of ACTH being the mediator of adrenal hyperfunction in relation to 17-ketosteroid production was obviated in the present study by the demonstration of 17-deoxycorticosteroid values in the pseudohermaphrodite rat within the normal female range (Figure 12, page 83). Both pseudohermaphrodite and female 24-hour urinary 17-deoxycorticosteroid values were considerably above those of normal males, a finding further reflected in the small adrenal weight per 100 grams body weight and low 17-ketosteroid output of the normal male rat. It is unlikely that ACTH could mediate an increase in androgen production without causing a concomitant increase in the corticosteroid production. Conversely, LH does stimulate 17-ketosteroid production, but not 17-deoxycorticosteroids and is therefore considered to be the tropic factor involved.

Previous mention has been made of the fall in 17-ketosteroid production of the castrate pseudohermaphrodite following gonadotropin suppression. However, these experiments failed to establish whether the effect was due to gonadotropin suppression or to an adverse effect of stilbestrol on the adrenal. The latter possibility was examined experimentally by analyses of 17-deoxycorticosteroids in the urine from animals during stilbestrol treatment. Results from these experiments indicated that the 17-deoxycorticosteroid production was slightly increased during stilbestrol treatment. If stilbestrol was acting adversely on the adrenal, the effect was not reflected in the corticosteroid values. It seems unlikely that stilbestrol could suppress the 17-ketosteroids without having a similar effect on the corticosteroid production. These results support the interpretation that a fall in adrenal 17-ketosteroids in the pseudohermaphrodite rat is due to a suppression of the tropic stimulation by LH.

Histological Findings

In 1938, Howard described a special area in the adrenal of the new-born rat characterized by a poorly demarcated zone of cells at the cortico-medullary boundary which disappeared in both sexes with increasing age. It was removed, not by degeneration, but by transition into the adult reticular zone. The period during which the rat juvenile cortex is prominent bears a close relationship to certain changes in male accessory sex organs. Castration of male rats more than 30 days of age was followed by regressive changes in the prostate and seminal vesicles. Castration during the first week of life, however, was not followed by these changes, but instead, the accessory organs continued to grow. Howard (1938) interpreted these findings to mean that the juvenile

3) Corticosteroid production in the pseudohermaphrodite is comparable to and in the range of that of female controls.

4) Suppression of gonadotropin secretion decreased adrenal 17-ketosteroid production in the pseudohermaphrodite without affecting the corticosteroid values.

These findings indicate that the cause of adrenal enlargement and hyperfunction of the adrenal androgen biosynthetic pathway in the male pseudohermaphrodite rat is due to a tropic stimulation of the adrenal cortex by an elevated titer of pituitary gonadotropins.

CHAPTER V

SUMMARY

The rationale for the approach of the present study was based on a working hypothesis which considered the gonadotropic hormones, primarily LH, to be tropic to the adrenal cortex of the male pseudohermaphrodite rat. This hypothesis was based on numerous preliminary findings, including adrenal enlargement, increased plasma 17-ketosteroids, indications of an elevated titer of gonadotropins (Leydig cell hyperplasia) and absence of Cushingoid pathology. Accordingly, four conditions were set forth to test the validity of the hypothesis based on the effects expected from a tropic stimulation of the adrenal by LH. The present study has fulfilled all requirements of the experimental design in the findings listed below:

1) Bioassay of the gonadotropin content (LH and FSH) of pituitaries and plasma of male pseudohermaphrodite rats has demonstrated greater relative potencies, when compared with NIH reference standards, than corresponding potencies of male or female rats.

2) Determinations of the 24-hour urinary 17-ketosteroid excretion in pseudohermaphrodites and control rats demonstrated a value in the pseudohermaphrodite 3 to 5 times greater than values for control rats. The testicular contribution accounted for more than one-half of the 17-ketosteroids in the pseudohermaphrodite, and in addition, the adrenal

contribution was found to be greater than that of male or female control rats.

3) Suppression of gonadotropic hormone secretion in the castrate pseudohermaphrodite rat resulted in a sharp decrease in urinary 17-ketosteroids without affecting the 17-deoxycorticosteroid values. This finding was interpreted to mean that the pre-treatment values of 17-ketosteroids were partially dependent on a tropic stimulation by LH. Furthermore, the relationship between ACTH and LH with respect to adrenal function was shown by the fact that 17-ketosteroid production was abolished when ACTH secretion was suppressed by dexamethasone treatment. It is concluded that the tropic influence of LH on the adrenal is dependent on ACTH for maintenance of adrenal function.

4) Determinations of urinary 17-deoxycorticosteroids in the pseudohermaphrodite rat produced values in the normal female range. This finding indicated that ACTH was not the mediator of increased adrenal 17-ketosteroid production, since corticosteroid values were not elevated.

In addition to the above experimental findings, comparative histological studies of the adrenal glands of pseudohermaphrodite and control rats revealed enlargement of the zona reticularis and increased sinusoidal vascularity in the adrenal of the pseudohermaphrodite. Similar histological findings have been observed in the mouse and thirteen-lined ground squirrel after administering gonadotropic preparations (Zalesky, 1941; Jones, 1948). Hence the histological make-up of the pseudohermaphrodite adrenal supports the physiological data and indicates that enlargement of the zona reticularis is due to a tropic stimulation by LH.

In view of the findings presented in this dissertation, it is concluded that adrenal enlargement and increased 17-ketosteroid production of the adrenal glands of the male pseudohermaphrodite rat is due to a tropic stimulation by an increased titer of gonadotropins.

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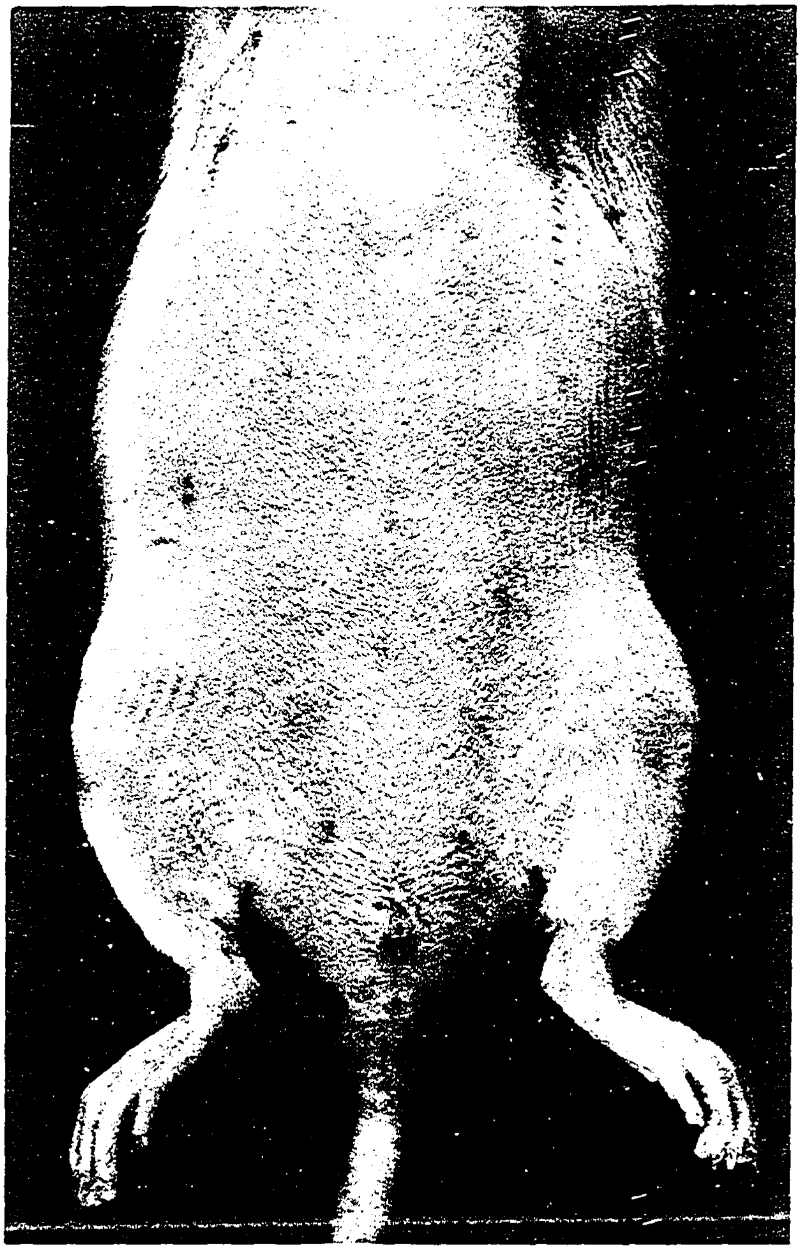
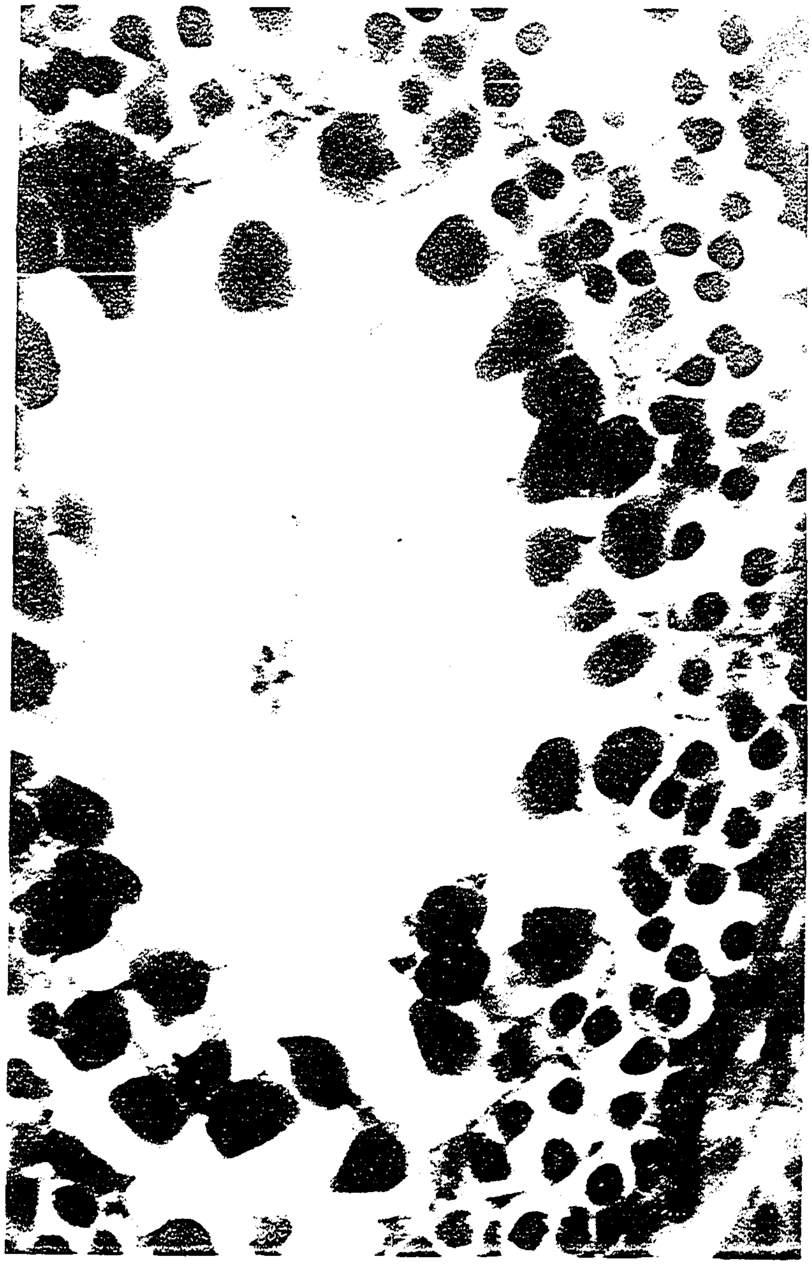
APPENDIXES

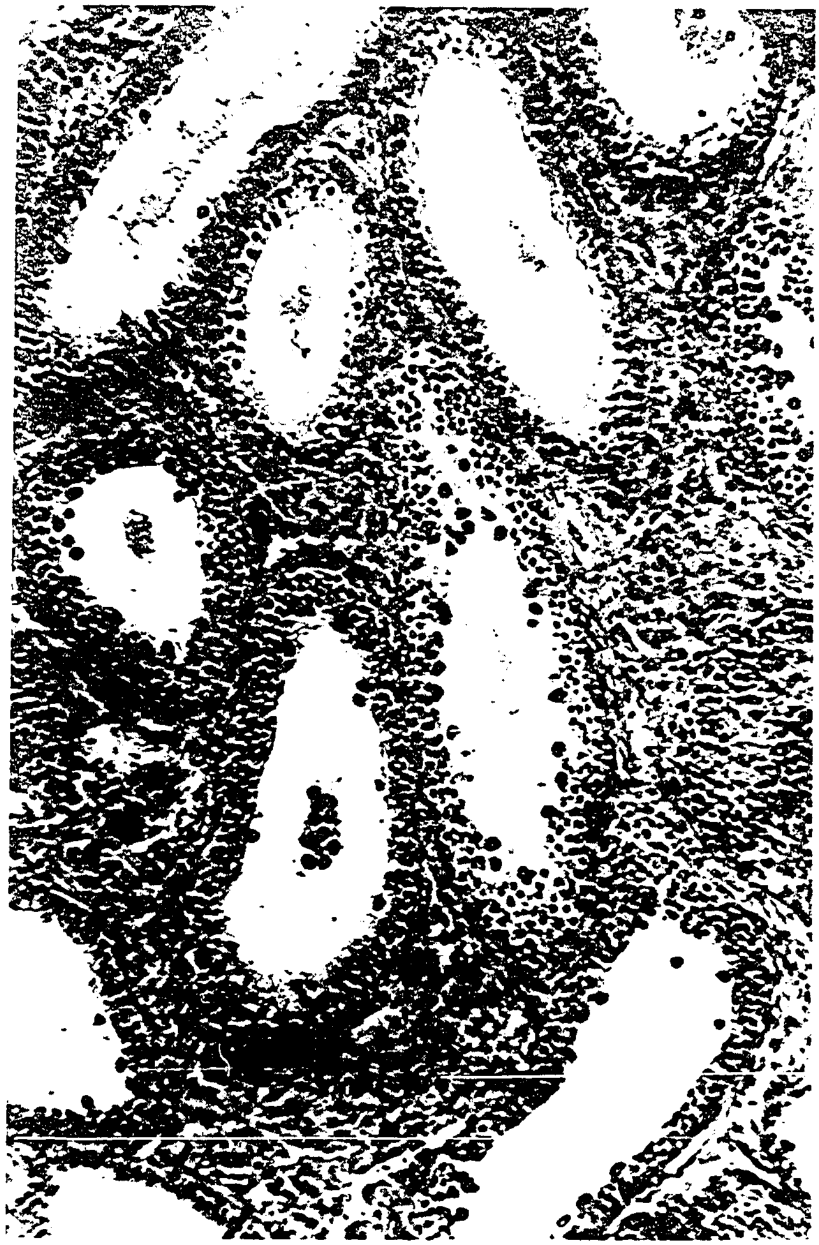
APPENDIX I



The above illustration shows the phenotypic make-up of a male pseudohermaphrodite rat. Note the mammary line and vaginal orifice. The bilateral protrusions on each side of the vagina are testes which have descended to the perineal region.

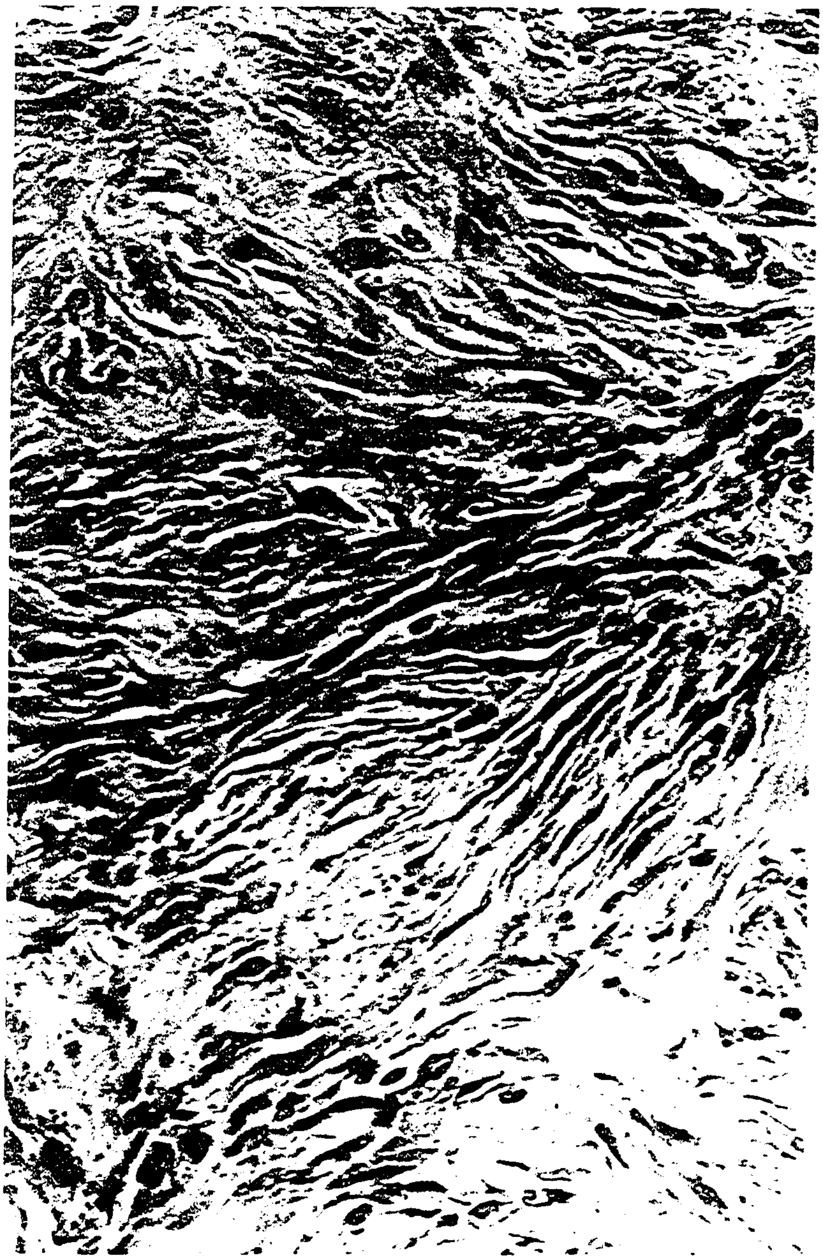


Cross-section of a testis from a male pseudohermaphrodite rat showing spermatogonia, large primary spermatocytes and Sertoli cells. Note the absence of secondary spermatocytes, spermatids and spermatozoa. Hematoxylin and eosin. X 1200.



Cross-section of a testis from a male pseudohermaphrodite rat showing hyperplasia of the interstitial cells. Hematoxylin and eosin. X 400.

Section through a Sertoli cell tumor from the testis of a male pseudohermaphrodite rat showing numerous Sertoli cells. Hematoxylin and eosin. X 1200.



Section through a Sertoli cell tumor from the testis of a male pseudohermaphrodite rat. This area bears no resemblence to the parent tissue. Hematoxylin and eosin. X 1000.

APPENDIX II

AVERAGE BODY AND ORGAN WEIGHTS AND AVERAGE PROPORTIONAL WEIGHTS OF ORGANS WITH CALCULATED STANDARD ERRORS OF THE MEANS OF FEMALE, MALE AND MALE PSEUDOHERMAPHRODITE RATS

No. Animals	Sex	Age Mos.	Body Wt. mg.	Adrenal Wt. mg.	Thymus Wt. mg.	Thyroid Wt. mg.	Pituitary Wt. mg.	Testis Wt. mg.	Kidney Wt. mg.
15	♀	3- 6	222 ± 4	45 ± 2	223 ± 18	14.5 ± .12	13.5 ± .13		1,597 ± 50
15	♀	6-12	239 ± 6	41 ± 2	85 ± 6	16.5 ± .18	14.3 ± . 2		1,742 ± 48
15	♂	3- 6	358 ± 16	44 ± 2	280 ± 23	18.3 ± .66	10.0 ± .37	3,105 ± 179	2,516 ±109
15	♂	6-12	414 ± 11	38 ± 2	123 ± 9	21.5 ± 1.0	11.1 ± .1	2,953 ± 77	2,787 ± 55
15	⚥	3- 6	292 ± 12	62 ± 2*	323 ± 35	16.3 ± .7	14.9 ± .7*	956 ± 45*	1,796 ± 64*
15	⚥	6-12	317 ± 12	57 ± 3*	126 ± 11	18.3 ± 1.4	17.2 ± .9*	679 ± 45*	1,936 ± 76*
AVERAGE PROPORTIONAL ORGAN WEIGHTS (mg/100 gm body weight)									
15	♀	3- 6	222 ± 4	21 ± .81	101 ± 9	6.5 ± .14	6.1 ± .17		720 ± 14
15	♀	6-12	239 ± 6	17 ± .46	36 ± 2	6.9 ± .21	6.0 ± .25		728 ± 8
15	♂	3- 6	358 ± 16	12 ± .43	81 ± 3	5.2 ± .20	2.8 ± .25	885 ± 11	704 ± 11
15	♂	6-12	414 ± 11	9 ± .34	30 ± 2	5.3 ± .23	2.71± .086	719 ± 19	679 ± 12
15	⚥	3- 6	292 ± 12	22 ± .70*	119 ± 13	5.6 ± .15	5.1 ± .14*	330 ± 13*	617 ± 11*
15	⚥	6-12	317 ± 12	18 ± .66*	40 ± 3	5.7 ± .37	5.4 ± .24*	218 ± 16*	610 ± 20*

*Significantly different, to the .01 level, from males of same age.

STEROID DETERMINATIONS OF PLASMA FROM NORMAL MALE AND MALE PSEUDOHERMAPHRODITE RATS

	Littermate Normal Male Control Rat Plasma				Pseudohermaphroditic Rat Plasma	
	Steroid added µg/100 ml	Steroid found after addition µg/100 ml	Steroid found (none added) µg/100 ml	Calculated Steroid content µg/100 ml	Steroid found µg/100 ml	Calculated Steroid content µg/100 ml
Estrone	10	11.5	6.0	11	9.9	18
Estriol	10	10.2	5.1	10	8.7	17
Estradiol-17β	10	11.1	5.6	11	11.8	24
Dehydroepi-Androsterone	10	8.2	2.0	3	11.1	18
Androsterone	10	34.6	28.2	44	26.2	41
Adrenosterone	10	9.6	4.2	8	13.7	25
Etiocholanolone and other 17-ks	10	7.8	2.9	6	5.9	12

Calculations of Steroid have been reported in the nearest whole number of µg; all calculations are based on percentage recovery of steroid added to control rat plasma.