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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

EFFECTS OF ACTH AND GONADOTROPINS ON URINARY 17-KETOSTEROID PRODUCTION IN THE MALE PSEUDOHERMAPHRODITE RAT

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1971

EFFECTS OF ACTH AND GONADOTROPINS ON URINARY 17-KETOSTEROID PRODUCTION IN THE MALE PSEUDOHERMAPHRODITE RAT

APPROVED BY

DISSERTATION COMMITTEE

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TO MY WIFE

Young Hee

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EFFECTS OF ACTH AND GONADOTROPINS ON URINARY 17-KETOSTEROID PRODUCTION IN THE MALE PSEUDOHERMAPHRODITE RAT

CHAPTER I

INTRODUCTION

Testicular Feminization in Man

There are many examples of heritable sexual dimorphism that have appeared in man (Melicow and Uson, 1964). One of these is a form of male pseudohermaphroditism called "testicular feminization" (Morris, 1953). Testicular feminization is an inherited form of male pseudohermaphroditism wherein the affected individual is genetically male, but exhibits a female phenotype. Inconclusive evidence indicates that it is sex-linked. At any rate, since the mutant gene is transmitted by the mother to one-half of her offspring and only the males receiving the gene are affected, it is safe to say that it is sex-limited (Grumbach and Barr, 1958). Adult males carrying the mutant gene lack Wolffian derivatives and a blind vagina is all that remains of the Müllerian structures that were present in embryos of males carrying the gene. The adults have very little axillary hair, they do have inguinal or labial testes, but no ovarian tissue.

These testes are immature or non-functioning and contain more than normal numbers of interstitial cells (Delageniere, 1899; Amann, 1906; Wagner, 1927; Krückmann, 1937; Novak, 1943; Hain and Schofield, 1947; Ward-McQuaid and Lennon, 1950; Wachstein and Scorza, 1951; Morris, 1953; Morris and Mahesh, 1963; Wilkins, 1965; Piver et al., 1966; Pjol-Amat et al., 1970; Weisberg et al., 1970). The seminiferous tubules are lined with Sertoli cells and spermatogonia. Characteristically, no spermatogenic elements beyond the primary spermatocyte are seen (Morris and Mahesh, 1963; David et al., 1965; Kase and Morris, 1965; Wilkins, 1965; Weisberg et al., 1970).

Although many patients may, among other things, exhibit some of the characteristics just described (Wachstein and Scorza, 1951; Morris, 1953; Jones and Scott, 1958; Overzier, 1963; Jones and Zourlas, 1965; Zourlas and Jones, 1965), complete testicular feminization must include all of the characteristics described in the previous paragraph (Morris and Mahesh, 1963).

Delageniere (1899), Chevassu (1906), Carmichael and Oldfield (1934), Novak (1943), Bleyer (1948), Wachstein and Scorza (1951), Scully (1953), Teter (1963), Pujol-Amat et al. (1970), Salle and Hedinger (1970) and others observed that the testes in adults with testicular feminization often become tumorous and even metastatic. Morris and Mahesh (1963), in their review, indicated that the testes become tumorous in about 22 percent of the adults with this syndrome.

The ontogenetic stage at which the mutant gene responsible for testicular feminization exerts its influence in man is not known. The cytological

picture seen in testes from one patient caused Goldberg and Maxwell (1948) to conjecture that testicular development had been arrested during the eighth week of gestation. Chambers (1879-80) was probably the first to describe a case of complete or true testicular feminization in man.

Administration of adrenocorticotropic hormone (ACTH) to these individuals causes an increase in the level of plasma 17-hydroxycorticosteroids and all urinary steroids except dehydroepiandrosterone (Salassa et al., 1961). Salassa and coworkers also noted that administration of chorionic gonadotropin results in elevated output of urinary 17-ketosteroids (17-Ks). After orchiectomy, their estrogen, as well as urinary 17-Ks titer, decreases to levels which are physiologically ineffective. This seems to indicate that gonads of such patients are the source of appreciable quantities of estrogens and urinary 17-Ks (Salassa et al., 1961).

French et al. (1965) also observed that the testes in individuals with testicular feminization produce substantial amounts of estrogen as well as testosterone. The effectiveness of the small amounts of estrogen produced by the testes in these patients is indicated by the normal development of the breast and, in some cases, relatively severe vasomotor menopausal symptoms (Evans and Riley, 1953). The urinary gonadotropins are generally elevated (Morris, 1953; Ikkos et al., 1959; Southren and Saito, 1961). The rate of urinary 17-Ks excretion is within or slightly above the normal male or female range (Evans and Riley, 1953; Salassa et al., 1961; Morris and Mahesh, 1963; Kase and Morris, 1965; David et al., 1965).

Patients with testicular feminization have higher than normal levels of dehydroepiandrosterone, androstenedione, testosterone, estradiol, and estrone in their gonadal venous blood than in their gonadal arterial or peripheral venous blood (Morris and Mahesh, 1963; Pion et al., 1965). Morris and Mahesh (1963) found that even though androgens are present in normal amounts throughout development and in the adult life of these individuals, for some reason these hormones cannot act on their target organs. These workers proposed that this tissue insensitivity to androgen and an existing inherent testicular Müllerian inhibiting factor are responsible for the origin of these abnormalities in fetal development. Likewise, androgen insensitivity and the action of testicular estrogens is thought to account for the kind of secondary sex characteristics seen in the adult. In summary, Morris and Mahesh suggested that the action of the mutant gene responsible for testicular feminization is manifested by an inability of the gonad to produce testosterone. This, in turn, may be caused by a deficiency or alteration of enzymes such as 3β -hydroxysteroid dehydrogenase or 17β -hydroxysteroid dehydrogenase. Ordinarily these enzymes are necessary for the conversion of dehydroepiandrosterone and androstenedione to testosterone. Because of these findings, it is particularly interesting that pateints with testicular feminization exhibit significantly increased urinary levels of dehydroepiandrosterone (Morris and Mahesh, 1963; Kase and Morris, 1965; David et al., 1965). In addition, these investigators suggested that certain target organs fail to respond to androgens (i.e. they show androgen insensitivity). They also proposed that in individuals so affected, there is possibly an accelerated metab-

olism of androgens to inactive metabolites and/or an ability to rapidly convert androgens to estrogens at peripheral sites such as the liver and kidney. However, French et al. (1965) found no detectable peripheral conversion of labeled testosterone to estrogen in patients with testicular feminization. It is believed that the entire sequence of events in testicular feminization from intrauterine development through adolescence may be fully explained by androgen target organ defects (French et al., 1965).

On the other hand, Northcutt et al. (1969) and Armstrong (1970) reported that in patients with testicular feminization, there exists an inherent abnormal defect in their cellular system which converts testosterone to dihydrotestosterone. This also may contribute to feminization in these individuals. Weisberg et al. (1970) reiterated the proposed existence in testicular feminization of a Müllerian inhibiting factor produced by the testis. They also suggested that the testicular Wolffian duct stimulator may be totally absent.

Even though most of the target organ complex of individuals with testicular feminization is unresponsive to testosterone, a specific biological effect of androgens has been demonstrated in these individuals. In this respect, Rivarola et al. (1967) found that their testosterone interstitial cell stimulating hormone homeostatic mechanism is normal.

Male Pseudohermaphroditism in Rats

Spontaneous intersexuality in the rat was described by D'Amour and Funk in 1941. These rats appeared to be females characterized by having a

blind vagina which opened at the age of 3 months, a pair of small cryptorchid testes lacking a tubular structure connecting them with the exterior, and interstitial tissue condensed into dense masses forming a collar around each tubule. The original parents of these sterile animals were lost. No attempt was made to mate the female siblings of the male mutants. This defect in rats, first described by D'Amour and Funk, appears to have been a form of male pseudohermaphroditism. It appeared in the Stanley-Gumbreck Colony with a single litter of 3rd generation King-Holtzman hybrids at the University of Oklahoma Medical Center in 1959. This male pseudohermaphroditism is believed to be a sex-linked character which is transmitted by the mother to one half of her male offspring, and the gene producing the defect is thought to be the X chromosome (Stanley and Gumbreck, 1964). With methods used to date, the karyotype and sex chromatin pattern of male pseudohermaphrodite rats appear to be characteristic of the normal male and the nuclei of hepatic cells are chromatin negative (Allison et al., 1965). Externally, such male pseudohermaphrodites resemble females, have mammary glands with well-developed nipple lines, and many have a very short blind vagina which varies from a dimple to about 2 mm in depth. Internally, they lack Wolffian and Müllerian derivatives. The reproductive tract is present on the 17th day of gestation but disappears by the 19th day (Allison, 1966). Affected individuals have a pair of testes situated in the inguinal region. The seminiferous tubules in these are surrounded by an abundance of interstitial cells (Stanley and Gumbreck, 1964; Easley, 1968; Goldman, 1970; Vanha-Perttula et al., 1970). The cell types in the seminiferous tubules consist of spermatogonia, Sertoli cells,

and large primary spermatocytes. Secondary spermatocytes, spermatids and spermatozoa are never observed (Easley, 1968; Chan, 1970; Vanha-Perttula et al., 1970; Sherins et al., 1971). The pituitary and adrenal glands of these animals are larger than those of the normal male rats (Stanley and Gumbreck, 1964; Easley et al., 1968), but their kidneys are smaller. The tissues of these rats are almost totally insenstive to androgen (Stanley et al., 1966; Stanley et al., 1969; Chan et al., 1969; Bardin et al., 1969; Bardin et al., 1970; Chan, 1970; Allison, 1970; Vanha-Perttula et al., 1970; Sherins et al., 1971; Chan and Allison, 1971). Deoxycorticosteroid levels are within normal range, indicating that ACTH production is normal. Higher than normal levels of luteinizing hormone (LH) are produced, but follicle stimulating hormone (FSH) concentrations are normal (Easley, 1968; Bardin et al., 1970). These animals produce less than normal amounts of testosterone. This is thought to be due to decreased 17β -hydroxysteroid dehydrogenase activity (Bardin et al., 1969; Schneider and Bardin, 1970; Goldman, 1970). These animals also produced significantly greater than normal amounts of urinary 17-Ks. The most abundant of these is dehydroepiandrosterone (Stanley et al., 1967; Easley et al., 1968; Easley, 1968). It is believed that male pseudohermaphrodites fail to masculinize in utero because of insufficient fetal testosteronogenesis, supposedly due to a genetic defect of 17β -hydroxysteroid dehydrogenase (Goldman, 1970).

From the preceding, one can see the amazing paralellism between testicular feminization in man and male pseudohermaphroditism in the rat. Since man cannot be placed in a cage, injected, bred, and laparotomized at will,

detailed knowledge obtained by these means, using the rat as a tool, will help us to better understand the etiology of this syndrome in man. Also indicated in the preceding paragraph were the observations that urinary 17-Ks in testicular feminization in man and male pseudohermaphroditism in rats are elevated, yet testosterone production from the testes of both averages lower than normal. These two facts later will be involved in setting forth the purpose of this paper.

17-Ketosteroids, Adrenal Cortex and Testes

Butenandt (1931) described the isolation of a crystaline steroid from male urine which in 1934, he identified as androsterone. This was the first of a class of substances to be isolated that are now known as 17-Ks. Since then, a number of structurally related compounds have been isolated and methods have been developed for their quantitative determination. Zimmermann (1935) described a colorimetric method for this based on the reaction of the group - CH_2CO- with m-dinitrobenzene in alkaline solution. This results in a purple color, and has been used most widely for the quantitative determination of 17-Ks. Many modifications of the Zimmermann reaction have been proposed by a number of investigators (Wu and Chou, 1937; Holtorff and Koch, 1940; Mason and Engstrom, 1950; Drekter et al., 1952; Munson and Kenny, 1954; Zimmermann, 1955; James and De Jong, 1961; Beale et al., 1962; Corker et al., 1962; Epstein, 1962). A comparison of the amounts of each urinary 17-Ks present in a patient's urine is often an aid in clinical diagnosis and interpretation of abnormal changes in testicular and adrenal cortical function (Mason and Engstrom, 1950).

A number of investigators have reported the mean value of the urinary

17-Ks of normal rats (Koch, 1937; Kowalewski and Bastenie, 1950; Kowalewski et al., 1951; Danford and Danford, 1951; del Greco et al., 1952; Langecker, 1952; Kullander, 1960; Tăceva and Pospíšil, 1967; Easley, 1968). These substances also have been measured in both sexes of castrated, adrenalectomized, hypophysectomized-adrenalectomized-castrated rats (Langecker, 1952).

Zizine (1951), reporting on the rat, Glenn and Heftmann (1951), on the dog, and Browne et al. (1943), on man, claim that administration of ACTH has no effect on the 17-Ks titer. On the other hand, Kowalewski et al. (1951) studying the rat and Thorn et al. (1947), Forsham et al. (1948), Thorn and Forsham (1948), Dingemanse and Huis in't Veld (1950), Howard et al. (1950), Dobriner et al. (1951), Landau et al. (1951), Marti (1951), Sprague et al. (1951), Gordon et al. (1954), Salassa et al. (1961), Hulka and Solomon (1966) and Norman et al. (1968), working with man, suggested that ACTH will cause an increase in 17-Ks. In man, LH or human chorionic gonadotropin (HCG) will stimulate increased urinary 17-Ks output from the adrenal and testis (Albright et al., 1942; Reifenstein et al., 1945; Howard et al., 1950; Reifenstein, 1950; Kyle and O'Donovan, 1950; Gómez Maestro, 1951; Plate, 1952; Moracci, 1953; Borell, 1954; Cerasuolo, 1954; Decio, 1955; Salassa et al., 1961; Pauerstein and Solomon, 1966), whereas stimulation of the adrenal cortex in the rat requires the combined action of ACTH and LH (Diczfalusy et al., 1950). Similarly, it is thought that in the male pseudohermaphrodite rat enlargement of the adrenal gland and increased adrenocortical

function is due to stimulation of the adrenal cortex by a normally occurring elevated titer of pituitary gonadotropins, primarily LH (Easley, 1968). In this case, the tropic influence of LH on the adrenal appears to be dependent on ACTH for maintenance of the adrenal function. FSH has no such effect (David et al., 1965).

Hypophysectomy leads to a rapid atrophy of the cortical cells of the adrenal gland in the rat and in other mammals (Smith, 1930; Crooke and Gilmour, 1938; Sarason, 1943; Mason et al., 1948; Diczfalusy et al., 1950; Wexler, 1963). Administration of ACTH stimulates the cells of the adrenal cortex to greater functional activity and causes hypertrophy of this organ in the hypophysectomized rat (Davidson, 1937; Moon, 1937; Astwood and Tyslowitz, 1942; Sayers et al., 1943; Li et al., 1943; Simpson et al., 1943; Ingle et al., 1944; Marx et al., 1943; Plzak, 1960; Wexler, 1963; Mikołajczky and Pawlikowski, 1965; Chung and Allison, 1971). In the mouse, the adrenal cortex possesses a well-developed juxtamedullary X-zone which shows variations with age and sex. This zone is believed to be the layer which produces androgens (Howard-Miller, 1927; Waring, 1935; Grollman, 1936). In mice, hypophysectomy causes the X-zone to atrophy and the cells thereof to exhibit a shrunken cytoplasm and irregular nuclei (Jones, 1948). Administration of ACTH to these animals does not cause repair of this X-zone, but does stimulate growth of the rest of the cortex. However, administration of LH to these animals will restore the X-zone to a point where it resembles that of a normal animal. On the other hand, it has no effect on the rest of the cortex. This appears to indicate that in

the mouse, LH is necessary for the maintenance of the X-zone or androgen producing zone (Jones, 1948).

Interstitial cells of Leydig produce androgens and gonadal failure following pituitary dysfunction is accompanied by altered activity of such cells, as well as of the adrenal and other endocrine glands (Turner, 1966). Such a defect is also reflected in abnormal excretion of urinary 17-Ks. Gonadectomy results in an increase in the gonadotropic activity of the pituitary gland (Smith et al., 1933; Hellbaum and Greep, 1943). Since gonadectomy at least temporarily removes some of the circulating androgens and thus decreases the feedback of androgens on the hypothalamus, it secondarily increases the gonadotropic titer in the blood (Everett, 1969). Castration also causes hypertrophy of the adrenal cortex (Anderson and Kennedy, 1933; Winter and Emery, 1936; Hashimoto, 1940; Selye, 1940; Wooley et al., 1941; Gardner, 1941).

From the preceding, it is seen that in normal animals, LH probably can directly stimulate adrenal hypertrophy and at the same time cause the adrenals to produce 17-Ks. If, in turn, LH will do this in hypophysectomized and castrated male pseudohermaphrodites and if more adrenal hypertrophy is caused and an even greater amount of 17-Ks are produced when ACTH and LH are given together, this would support the idea that adrenal hypertrophy and high production of adrenal androgen exhibited in the male pseudohermaphrodite rat are the result of tropic stimulation on the adrenal cortex by the high titer of pituitary gonadotropin normally present in these animals.

Thus, the present study was designed to investigate whether elevated

LH values in male pseudohermaphrodite rats are involved in producing the abnormally high levels of 17-Ks in their urine.

CHAPTER II

MATERIALS AND METHODS

Eighty-four 28 day-old King-Holtzman rats from the Stanley-Gumbreck Colony at the University of Oklahoma Medical Center were hypophysectomized and castrated. Prior to being operated, 42 of these were normal males (NM) and 42 were male pseudohermaphrodites (Ps). In addition, 6 NM and 6 Ps rats, age 28 days, were left unoperated and functioned as a control group. The operated animals were divided into 7 groups. Each group contained 6 operated normal males (ONM) and 6 operated male pseudohermaphrodites (O Ps). Seven days postoperatively, each operated animal in one group received 2 USP units of ACTH, in another 50 μg of LH, in a third 500 μg of LH, in a fourth 50 μg of FSH, in a fifth 2 USP units of ACTH and 50 μg of LH. A sixth group was administered 2 USP units of ACTH, 50 μg of FSH and 50 μg of LH at the same time. Subsequently, these amounts were administered daily to all members of each group for 7 days. Members of the seventh group received no hormones.

As these animals and the 12 unoperated controls reached 35 days of age they were placed in metabolism cages where they remained during the subsequent 7 days of the experiment. Twenty-four hour urine specimens were

collected from each of these animals during these 7 days in a 150 ml Erlenmeyer flask. Food was given to each animal only while the analyses for urinary 17-Ks were being conducted. After each daily procedure of analysis, all remnants of food were taken from the rats, the cages were cleaned, and urine collection flasks replaced. Thus, no urine was collected while there was a chance of food falling into the collection flasks.

Another group of 12 animals (6 NM and 6 Ps) were established. When they were 28 days old their urinary 17-Ks output was measured. This was repeated on each subsequent day for a total of 35 days. On the fourth day after this regimen was begun, all were hypophysectomized and castrated. Eleven days later they were administered 50 μg FSH. The 50 μg LH was given for 4 days, after which they received 500 μg of the same hormone for 4 more days. After the LH series, they received 2 USP units of ACTH for 4 days, followed by 4 days during which they were given all three hormones simultaneously for another 4 days. The LH in this last step was given at a dose-level of 50 μg per day and the FSH and ACTH in amounts as described above.

Thus, each animal in this series was carried through all the procedures used on the separate groups, except that LH and ACTH together were not given.

Initially, in order to determine any changes in adrenal gland weight of treated and untreated animals during the course of the experiment, these glands were removed from 6 NM and 6 Ps rats, age 28 days. They were weighed and their value recorded. At the end of each subsequent phase of the experiment, the adrenals of all other animals used were weighed. These weights were

compared with those from the 28 day-old animals.

Method of Hypophysectomy and Castration

Hypophysectomy

Hypophysectomies were performed by the parapharyngeal approach under ether anesthesia. The procedure applied was a modified method of Ingle and Griffith (1942), originally described by Smith (1930). Using a binocular dissecting microscope, a small parasagittal incision was made on the ventral surface of the neck. The subcutaneous tissues and salivary glands were retracted by means of a small hemostat. The inferior surface of the sphenoid bone was then reached by separating the sternohyoid and omohyoid muscles at the level of the thyroid gland. The trachea and esophagus also had to be carefully pushed to one side. Bony ridges, representing the ventral surface of the basisphenoid, mark the position of the underlying pituitary gland. These were exposed by clearing away the overlying muscle and then wiping the area dry with cotton pellets. A hole was drilled in the midline on the basisphenoid with a trephine which has a diameter of about 2 mm. The trephine was not allowed to rupture the dura mater which lies between the pituitary gland and the sphenoid bone. After the disk of bone was removed the dura was carefully lifted off with the sharp point of a dissecting needle that had been bent into a tiny hook. The tip of a Spemann pipet was inserted into the hole and the gland was removed by suction. After removal of the pituitary gland, the tissues and organs which had been pushed aside were placed back in their normal position and the skin incision was closed with skin-clips.

At autopsy, a dissecting microscope was used to examine the sella turcica for pituitary remnants. Animals were considered completely hypophysectomized only if they lost weight, stopped growing, their adrenal cortex atrophied and no remnants of glandular tissue remained in the sella turcica.

Castration

Each animal was castrated immediately following hypophysectomy. This was accomplished by making a ventral, midline incision through the skin of the abdominal wall about 1 cm rostral to the penis. This incision was large enough so that gentle forward pressure on the scrotum would permit the testis to emerge through the incision onto the surface of the body. As each testis was extruded, the spermatic cords were ligated and the organ was removed by cutting the spermatic cord between the ligature and testis. The abdominal muscles were then sutured and the skin incision closed with wound clips.

Administration of ACTH and Gonadotropins

Adrenocorticotropic Hormone

Five ml multiple dose vials of H. P. Acthar gel adrenocorticotropins containing 80 USP units per ml were obtained from the Armour Pharmaceutical Company, Chicago. The USP assay is essentially that devised by Sayers et al. (1948). It is based upon adrenal ascorbic acid depletion in the hypophysectomized rat (Parlow, 1961). The international unit of ACTH activity of this preparation has the biological activity of 1 mg of the international standard. One USP unit equals 1 international unit of activity. As indicated previously, the

different groups of rats were injected intramuscularly with daily doses of 2 USP units dissolved in 0.5 ml of 0.9% sodium chloride.

Gonadotropins

Lutenizing hormone and FSH were purchased from the Mann Research Laboratories, Division of Becton Dickinson and Company, New York. Injections were given intramuscularly in daily doses of 50 μg.

When these hormones were injected singly, each was dissolved in 0.5 ml of 0.9% sodium chloride. When 2 or 3 hormones were injected together, each was dissolved in 0.15 ml of saline. Thus, animals given 2 hormones received 0.3 ml of solution, where as those receiving 3, were injected with 0.45 ml of solution.

Determination of Urinary 17-Ketosteroids

This topic has been reviewed carefully by Dorfman (1962) and Loraine and Bell (1966). Most colorimetric methods for the determination of the total neutral 17-Ks are performed according to the process described by Zimmermann (1935). In this assay, m-dinitrobenzene is used. This yields a purple color with 17-Ks in an alkaline medium.

In the application of the Zimmermann method for the assay of 17-Ks, various modifications of the general procedure have come into use. These modifications primarily involve variations in the concentrations of solvents for potassium hydroxyide, concentrations of m-dinitrobenzene, and the duration and temperature of heating for the development of the color.

The method used in the present work for the determination of urinary 17-Ks was that of Drekter et al. (1952) as modified by Klindskoj et al. (1953).

Urine collected over each 24-hour period from all individuals in each group, was pooled by filtering it through a Whatman No. 42 filter paper into a single flask. Ten ml of urine (in duplicate) taken from a 24-hour specimen was pipetted into a 50 ml round bottom centrifuge tube with a Pyrex flathead stopper. In each case, a tube containing 10 ml of distilled water was used as a blank. Hydrolysis of both the test solution and the blank was carried out by adding 3 ml of concentrated hydrochloric acid to the tubes, then placing them in a boiling water bath for 10 minutes. The 17-Ks exist in urine as conjugates with glucuronidates or sulfates. Hydrolysis was carried out because acid hydrolysis of 17-keto-steroid conjugates breaks them down, thereby liberating the free steroids for extraction. After cooling the tubes, 10 ml of ethylene dichloride was added. In this way, intimate contact was obtained between solvent and solute. This is necessary in order to obtain complete removal of 17-Ks. Subsequently, the stoppers were removed to release pressure. The tubes were then restoppered and shaken on an automatic shaker for 15 minutes. They were then centrifuged for 10 minutes at 2000 rpm and the top aqueous layer was carefully aspirated with suction. The organic extract was filtered into 50 ml glass stoppered bottles through Whatman No. 1 filter paper. In order to remove any estrogens and other interfering substances, 20 sodium hydroxide pellets were added to each bottle. The preparations were then again placed in the automatic shaker for 15 more minutes, then the solvent was filtered into a dry test tube through Whatman No. 1

filter paper. A 2 ml aliquot from each tube was then pipetted into dry tubes and evaporated to dryness at 50^{0}C for about 48 hours.

The next several steps were carried out in a darkened room. Four-tenths ml of m-dinitrobenzene was added to each of the dry tubes prepared as described in the preceding paragraph. Three-tenths ml of 4 N-potassium chloride in methanol was then added. The contents of each tube were mixed well in order to dissolve the dried material and allowed to stand for sixty minutes at room temperature. Finally, 5 ml of ethylene glycol monomethyl ether was added to each tube, which was then gently shaken. Spectrophotometric analysis of these solutions was carried out on a Coleman Junior Spectrophotometer at 510 millimicrons and 100 percent transmission. A cuvette containing ethylene glycol monomethyl ether was placed in the spectrophotometer which was then set at zero. The cuvette was replaced with a color blank and the transmittance value recorded. Again the spectrophotometer was set at zero. The standard was placed in the cuvette well, and its transmittance value recorded. The standard was removed and replaced by the water blank. This was read and the value recorded. The spectrophotometer again was set at zero and the unknown was read. This procedure was repeated for each unknown. The concentration of urinary 17-Ks for 24 hours in micrograms is then obtained according to the following formula (Ingel, 1954):

$$\mu g\ 17\text{-Ks}/24\ hr = \frac{\text{Optical density of unknown tube}}{\text{Optical density of standard tube}} \times \mu g\ DHA\ in\ \text{standard tube} \times \frac{\text{total vol. extract}}{\text{vol. extract analyzed}} \times \frac{\text{total vol. urine}}{\text{vol. urine hydrolyzed}}$$

Chemicals and Equipment Used

Chemicals. The following chemicals were used in this study: (1) concentrated hydrochloric acid, A.C.S.; (2) ethylene dichloride, purified; (3) sodium hydroxide, A.C.S.; (4) m-dinitrobenzene - 1 percent solution of m-dinitrobenzene was made by placing 1 gm of the reagent in a 100 ml volumetric flask and filling to the mark with ethylene glycol monomethyl ether; (5) potassium hydroxide, U.S.P.; (6) ethylene glycol monomethyl ether, purified; (7) dehydroepiandrosterone (DHA) - a standard solution of 10 gamma per ml prepared in methanol. Stock standard solution was made by placing 10 mg of DHA in 100 ml of methanol. The working standard solution was prepared by adding 10 ml of stock solution to 90 ml of methanol; and (8) methanol, purified.

Equipment. The following types of equipment were used in this study: (1) Pyrex round bottom centrifuge tubes of 50 ml capacity, with ground-glass stoppers; (2) mechanical shaker; and (3) a Coleman Junior Spectrophotometer, Coleman Instruments, Inc., Maywood, Illinois.

Histological and Histochemical Preparation and Photography

Histological Preparation

Adrenal glands were removed from representative NM and Ps rats when some of each were 28 and 42 days old. When the urinary 17-Ks analyses were completed, adrenals were removed from all animals involved. All of these

glands were weighed and then fixed in Bouin's fluid or in 10 percent buffered formalin for at least 24 hours. The tissues were washed in tap water, dehydrated in ethanol, cleared in xylene, and embedded in paraffin for sectioning. The paraffin blocks then were serially sectioned at 7 μ and serially mounted on slides with albumin fixative. Subsequently, every other slide was deparaffinized and cleared in xylene, hydrated, stained with Delafield's hematoxylin and eosin, dehydrated in ethanol, cleared in xylene, and mounted with permount. Adjacent slides were stained with Mallory's trichrome method.

Histochemical Preparation

Preparations designed to demonstrate loci of general lipid were stained with the Sudan Black B technique recommended by Lison (1953). Five μ sections were made on a Cryostat maintained between -15° and -20°C. A short ribbon of these sections was mounted on the slides and allowed to dry at room temperature. These slides then were rinsed in 70 percent ethanol and stained in Sudan Black B for 3 minutes. The staining solution is 70 percent ethanol saturated with Sudan Black B. It is filtered while at 4°C and kept at this temperature during the staining procedure. Stained sections were rinsed in 70 percent ethanol, washed in tap water, and mounted in glycerin jelly. All tissue preparations were examined under a light microscope.

Photography

Photomicrographs were taken with a Wild MKa4 camera on a Wild M20 Research Microscope. Kodak Pantomic-X 135 mm film was exposed

using maximum light through a blue filter at 1/60th second. The film was developed in Kodak D-76 for 6 1/2 minutes at 70^{0}F, then placed in a 2 percent acetic acid stop bath for 30 seconds. It was then immersed in Kodak rapid fix for 5 minutes, followed by 30 minutes in a water bath. Prints are on single weight F-4 Kodabromide paper. (See Plates in Appendix).

CHAPTER III

RESULTS

In the following pages, all differences described as significant are based on the probability that they could have happened accidentally only less than once in one thousand instances ($P < .001$).

Urinary 17-Ketosteroid Production

Effect of Hypophysectomy and Castration

With the methods used, NM rats were shown to excrete an average of 28.6 and Ps animals 85.3 µg urinary 17-Ks in 24 hours. This difference is significant. Their operated, untreated counterparts exhibit an average daily output of 3.3 and 4.3 µg, respectively. Statistically, there is no difference between these lower values. Thus, even though the Ps normally produces considerably more urinary 17-Ks than the NM, removal of their pituitary gland and testes lowers the amount of this substance they produce to almost zero and its output in the two animal types becomes essentially the same. (See Tables 1, 2 and 3 and Fig. 1).

Effect of ACTH Alone

Of the 3 hormones administered separately, ACTH causes the most

TABLE 1

AVERAGE URINARY 17-KETOSTEROIDS PRODUCED BY
6 NORMAL MALE RATS (μg/24) HOURS

Age in Days	UO-UT	O-UT	O-FSH	O-LH(1)	O-LH(2)	O-ACTH	O-LH(1) +ACTH	O-3H
36	28.2	3.5	4.0	5.0	15.2	19.7	18.2	19.8
37	27.5	2.9	4.7	3.4	18.7	20.4	21.8	20.6
38	29.0	3.1	2.4	4.3	14.0	21.0	18.5	24.9
39	27.3	4.7	3.4	7.7	15.4	24.9	27.4	21.3
40	30.6	3.9	5.4	4.9	16.5	24.2	23.9	21.3
41	28.8	3.1	3.7	4.3	13.4	20.2	27.7	24.4
42	28.9	2.2	3.7	7.9	14.6	18.0	24.6	23.0
Ave.	28.6	3.3	3.9	5.4	15.4	21.2	23.2	22.2
S.D.	±2.7	±2.0	±4.6	±4.3	±6.1	±4.7	±9.8	±4.7

O = Operated
UO = Unoperated
UT = Untreated
FSH = 50 μg FSH
LH(1) = 50 μg LH
LH(2) = 500 μg LH
ACTH = 2 USP units ACTH
3 H = 3 Hormones

TABLE 2

AVERAGE URINARY 17-KETOSTEROIDS PRODUCED BY 6 MALE PSEUDOHERMAPHRODITE RATS (μg/24 HOURS)

Age in Days	UO-UT	O-UT	O-FSH	O-LH(1)	O-LH(2)	O-ACTH	O-LH(1) +ACTH	O-3H
36	81.5	3.7	3.0	12.9	15.7	25.2	31.8	29.5
37	85.9	2.6	6.3	17.5	18.8	29.1	37.8	36.0
38	87.2	6.3	5.3	13.2	21.9	31.1	34.1	36.2
39	87.3	4.2	6.0	22.4	19.0	27.1	35.8	38.7
40	79.3	6.7	3.1	16.6	16.8	27.3	32.1	40.4
41	93.9	3.5	5.3	14.8	18.2	30.9	35.7	37.6
42	82.2	3.1	4.6	15.7	18.1	27.1	35.7	38.3
Ave.	85.3	4.3	4.8	16.2	18.4	28.3	34.7	36.7
S.D.	±11.9	±3.9	±3.2	±7.9	±4.8	±5.4	±5.3	±8.6

O = Operated
UO = Unoperated
UT = Untreated
FSH = 50 μg FSH
LH(1) = 50 μg LH
LH(2) = 500 μg LH
ACTH = 2 USP units ACTH
3 H = 3 Hormones

TABLE 3

SUMMARY OF CHANGES IN URINARY 17-KETOSTEROIDS

Type of Operation	Treatment	No. of days treated	Average U 17-Ks in μg/day N	Ps
UO	None	None	28.6	85.3
HC	None	None	3.3	4.3
HC	50 μg FSH	7	3.9	4.8
HC	50 μg LH	7	5.4	16.2
HC	500 μg LH	7	15.4	18.4
HC	2 USP units ACTH	7	21.2	28.3
HC	2 USP units ACTH 50 μg LH	7	23.2	34.7
HC	2 USP units ACTH 50 μg LH 50 μg FSH	7	22.2	36.7

UO = Unoperated
HC = Hypophysectomized and Castrated

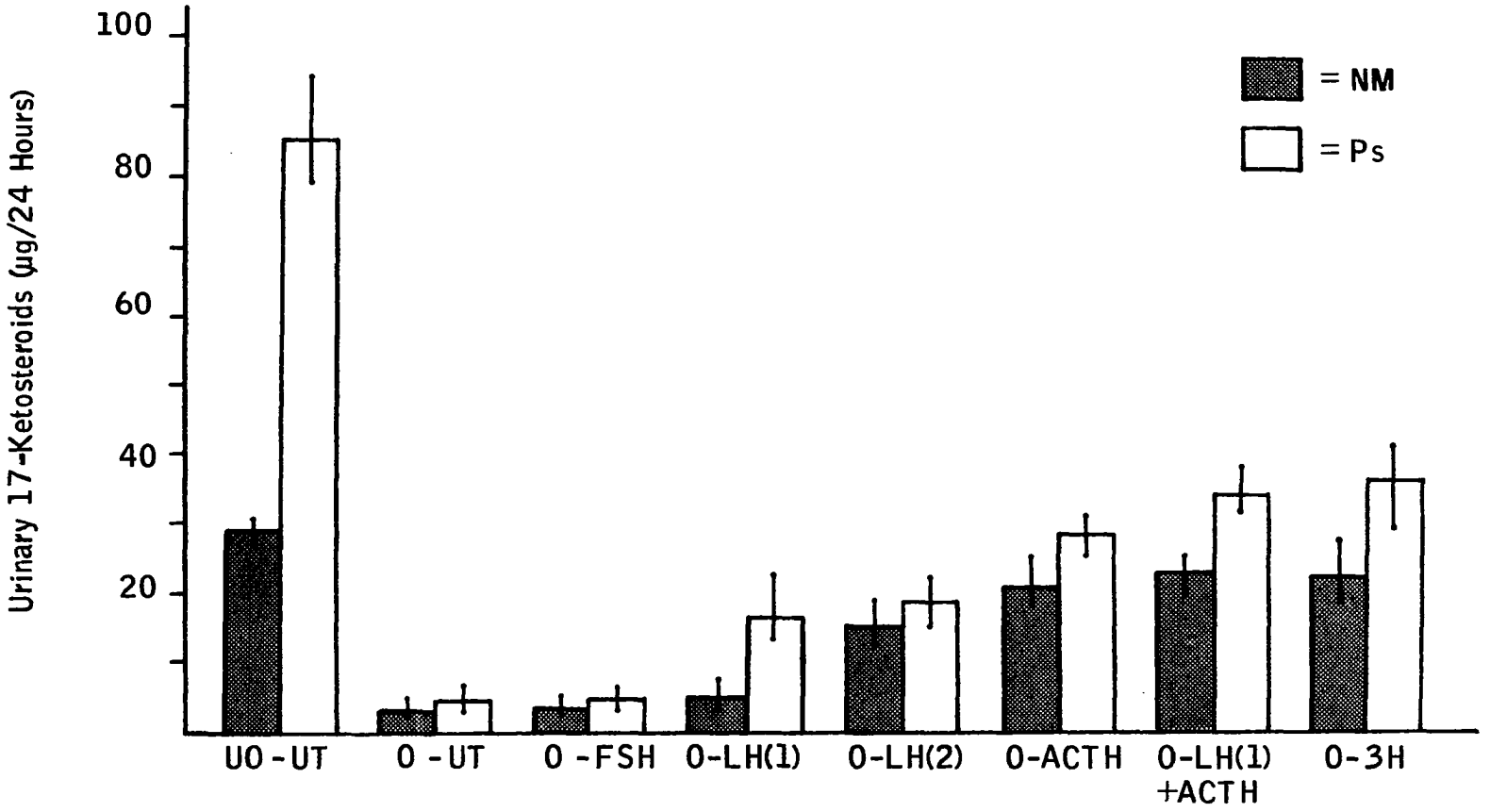

Figure 1. Average daily urinary 17-Ks produced by 6 normal and 6 pseudohermaphrodite rats. O=operated; UO=unoperated; UT=untreated; FSH=50 µg FSH; LH(1)=50 µg LH; LH(2)=500 µg LH; ACTH=2 USP units of ACTH; 3H=3 Hormones.

significant increase in output of urinary 17-Ks by ONM and OPS rats. The amounts produced by each under these circumstances is 21.2 and 28.3 μg, respectively. Since the difference between these two values is also significant, it appears that the Ps adrenal gland is more sensitive to ACTH than is that of the NM. (See Tables 1, 2 and 3 and Fig. 1).

Effect of LH Alone

The increase in urinary 17-Ks from ONM and OPs rats treated with 50 and 500 μg of LH follows a pattern similar to that seen in these animals after ACTH. The higher dose level produces a greater increase in urinary 17-Ks in both animal types. Again, this increase is significantly greater from the OPs than the ONM animals. Consequently, as with ACTH, the Ps adrenal gland appears to be more sensitive to LH than does that of the NM rat. (See Tables 1, 2 and 3 and Fig. 1).

Effect of FSH Alone

Follicle stimulating hormone, in the amounts given, has no apparent effect on urinary 17-Ks output in either animal type. (See Tables 1, 2 and 3 and Fig. 1).

Effect of Simultaneous Administration of LH and ACTH

Luteinizing hormone and ACTH given simultaneously raise the urinary 17-Ks output from both animal types to a level greater than when either was given singly. In ONM rats, this increase is significantly greater than that

produced by LH but not for that produced by ACTH. However, the increased output in OPs animals given both hormones together is significantly greater than for either given individually. Since in this case LH was given in the lesser amount (50 μg/day), it appears that in the OPs animals LH may be more effective when adrenal function is maintained by ACTH. (See Tables 1, 2 and 3 and Fig. 1).

Effect of Simultaneous Administration of LH, ACTH and FSH

Results of administering the three hormones at the same time are essentially the same as those produced by LH and ACTH together. In the light of this result and the previous observation that FSH given by itself has no effect on urinary 17-Ks output in any animal type studied, it is concluded that under the conditions of this experiment, FSH does not stimulate the adrenal gland to produce urinary 17-Ks. (See Tables 1, 2 and 3 and Fig. 1).

Hormones Administered in a Continuous Series

Animals administered all the procedures in one continuous series react much as do the individuals given each procedure separately (Fig. 2). As expected, hypophysectomy and castration causes a marked drop in the average daily urinary 17-Ks output from both NM and Ps animals. FSH is ineffective in raising these values in either animal type.

Fifty ug of LH causes a significantly greater increase in urinary 17-Ks production from Ps than from NM rats. On the other hand, 500 μg of LH raises the daily output in both types to almost the same level. However, in this case the average daily amount produced over the 4-day period by the Ps is greater than

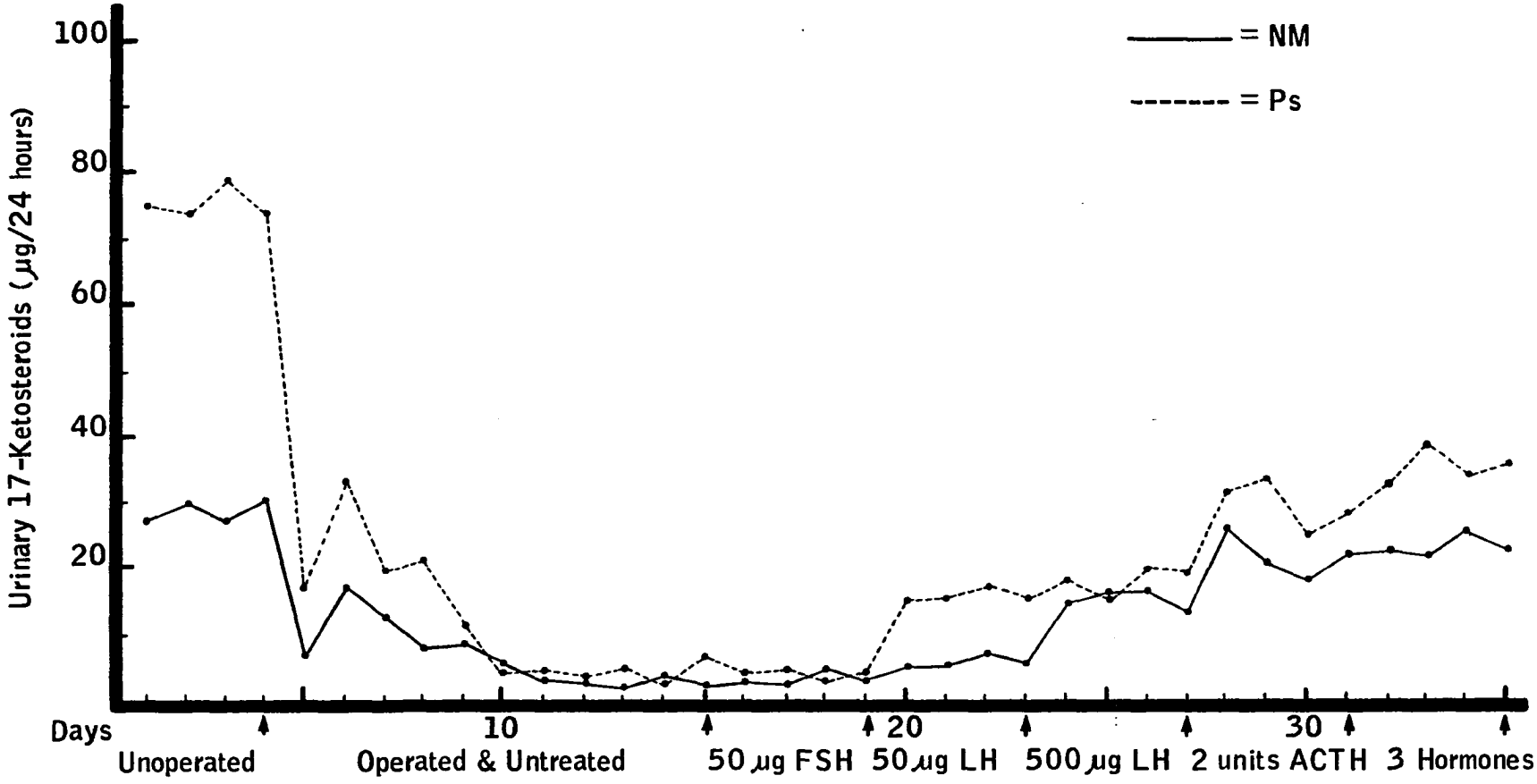

Figure 2. Average daily excretion of urinary 17-Ks produced by the same group of 6 animals at different ages and with different treatments.

that for the NM. These results again appear to indicate that the Ps adrenal is more sensitive to LH than is that of the NM.

Adrenocorticotropin alone results in a greater increase in the daily average production of urinary 17-Ks from both animal types than does LH. This increase in the Ps is significantly greater than that in the NM. When all three hormones are given at once, this increase is maintained and the difference between the two slightly enhanced. This supports the previously stated idea that urinary 17-Ks production by the rat adrenal gland is aided by the tropic action of LH if such activity is at the same time maintained by ACTH. (See Tables 1, 2 and 3 and Figs. 1 and 2).

Morphology of Adrenal Cortex

Adrenal Cortex of Animals with Intact Pituitary and Testes

Under the light microscope, the histological structure of the adrenal cortex of NM and Ps rats appear identical except that the cortex is wider in the Ps. The greater width is particularly apparent in the fasciculata and reticular zones. The adrenal cortex from unoperated NM and Ps rats exhibits a well differentiated zona glomerulosa, zona fasciculata, and zona reticularis (see Plate I, Figs. 4 and 5 in Appendix). The zona glomerulosa is composed of cuboidal cells arranged in cords. The cells of the wide fascicular zone are polyhedral in shape and arranged in long parallel columns. In the narrow reticular zone the cells stain more intensely with hematoxylin and eosin. The cells therein are arranged in anastomosing cords.

In Sudan Black B preparations, cells of the zona glomerulosa contain lipid droplets that are small and uniform in size. A layer between the glomerulosa and fasciculata contains little or no lipid and is called the sudanophobe or lipid-free zone. Cells in the outer portion of the zona fasciculata are occupied by relatively large lipid droplets but, both the cells and their contained droplets become reduced in size toward the inner margin of this zone. The lipid droplets in the reticular zone are so small that they are difficult to see under high power (see Plate II, Figs. 8 and 9 in Appendix).

Adrenal Cortex of Castrated and Hypophysectomized Animals

Fourteen days after the 28-day-old animals (NM and Ps) were castrated and hypophysectomized, the width of the cortex is greatly reduced (see Plate I, Figs. 6 and 7 in Appendix). The capsule is thicker, but the zona fasciculata and zona reticularis are much narrower than they are in intact animals. The zona glomerulosa seems to undergo the least alteration in width. In operated animals it is as wide as, and in some cases wider than, it is in normal animals (see Plate I, Figs. 6 and 7 in Appendix). The zona reticularis in these animals appears to consist of a few layers of squamous-like cells enmeshed in a network of connective tissue fibers (see Plate I, Figs. 6 and 7 in Appendix). These cells contain pycnotic nuclei and a highly vacuolated cytoplasm. Cells in the inner portion of the zona fasciculata and outer portion of the zona reticularis also exhibit vacuoles. These vacuoles probably represent loci of a lipid droplet, since they stain heavily with Sudan Black B (see Plate II, Figs. 10 and 11 in

Appendix). Thus, it appears that in both animal types castration and hypophysectomy is followed by atrophy of cells in the zona reticularis and zona fasciculata and coalescence of the lipid inclusions normally present. In adrenals of operated animals of both types, the sudanophobic zone at the outer margin of the zona fasculata is more prominent (see Plate II, Figs. 10 and 11 in Appendix).

Adrenal Cortex of Hypophysectomized and Castrated Animals Following Administration of ACTH and Gonadotropins

Of the 3 hormones administered, FSH has no significant restorative effect (see Plate III, Fig. 12; Plate IV, Fig. 16; Plate V, Fig. 20; and Plate VI, Fig. 24 in Appendix). Likewise, 50 μg of LH does very little to rebuild the normal structure of the cortex (see Plate III, Fig. 13; Plate IV, Fig. 17; Plate V, Fig. 21; and Plate VI, Fig. 25 in Appendix). Five-hundred μg of LH cause a significant widening of the zona fasciculata and zona reticularis (see Plate III, Fig. 14; Plate IV, Fig. 18; Plate V, Fig. 22; and Plate VI, Fig. 26 in Appendix). However, when LH and ACTH are administered together or the 3 hormones are given at the same time the cortex is restored to a degree equivalent to normal (see Plate III, Fig. 15; Plate IV, Fig. 19; Plate V, Fig. 23; Plate VI, Fig. 27; Plate VII, Figs. 28-31; and Plate VIII, Figs. 32-35 in Appendix). With the latter two, the lipid droplets are redistributed uniformly throughout the cortex and the normal cellular structure of all three layers reappears. The Ps adrenal is more sensitive to the combined action of LH and ACTH than is that of NM animals (see Plate VII, Figs. 28 and 30; and Plate VIII, Figs. 32

and 34 in Appendix). These hormones also reduce the abnormally wide chromophobic zone in the operated animals to the condition seen in normal animals. Finally hemorrhagic areas are seen in the cortex following ACTH. Most of these are in the zona fasciculata, although some are seen in the other two zones.

Adrenal Weight

The average weight of adrenal glands from NM rat, age 28-days, is 18.6 mg and that of a Ps the same age is 23.8 mg. Fourteen days later, the average weight of adrenal glands from similar animals is 26.6 and 34.9 mgs, respectively. Fourteen days after the NM and the Ps rats are hypophysectomized and castrated, the average adrenal weights of each is 9.8 mg and 11.5 mg, respectively.

These changes correspond to the destructive alterations seen in the histologic sections. Likewise there is a direct correlation between the repair seen in the sections following hormone treatment and weight changes of the gland. (See Tables 4, 5 and 6 and Fig. 3).

TABLE 4

AVERAGE ADRENAL WEIGHTS OF 6 NORMAL MALE RATS (mg)

	UO(1)-UT	UO(2)-UT	O-UT	O-FSH	O-LH(1)	O-LH(2)	O-ACTH	O-LH(1)+ACTH	O-3H
	16.3	26.2	8.4	9.3	13.4	16.8	22.5	22.6	25.7
	21.4	29.2	10.2	10.5	14.5	17.3	24.2	23.8	22.4
	20.5	22.4	8.2	8.7	12.8	15.8	21.2	27.5	32.6
	19.6	29.7	10.9	7.8	10.3	19.5	28.3	28.6	23.3
	16.5	27.5	9.5	8.4	9.6	16.2	23.6	25.3	24.3
	17.3	24.5	11.6	12.6	14.5	13.0	25.3	19.2	25.9
Ave.	18.6	26.6	9.8	9.6	12.5	16.4	24.2	24.5	25.7
S.D.	±4.9	±6.3	±3.0	±3.9	±4.7	±4.7	±5.5	±7.7	±8.1

Unless indicated, all aminals were 42 days old

O = Operated
UO(1) = Unoperated 28 days old rat
UO(2) = Unoperated
UT = Untreated
FSH = 50 μg FSH
LH(1) = 50 μg LH
LH(2) = 500 μg LH
ACTH = 2 USP units ACTH
3 H = 3 Hormones

TABLE 5

AVERAGE ADRENAL WEIGHTS OF 6 MALE PSEUDOHERMAPHRODITE RATS (mg)

	UO(1)-UT	UO(2)-UT	O-UT	O-FSH	O-LH(1)	O-LH(2)	O-ACTH	O-LH(1)+ACTH	O-3H
	21.7	28.6	11.8	12.9	17.8	15.8	24.2	27.7	25.6
	21.4	33.5	9.3	13.5	9.4	17.3	27.6	26.6	23.8
	28.6	29.6	8.3	12.2	11.5	19.8	22.3	25.4	29.3
	29.4	36.4	16.6	14.5	16.4	21.3	30.8	26.6	30.5
	17.5	42.8	10.6	9.3	13.6	18.0	22.6	23.3	26.8
	24.3	38.6	12.3	14.4	17.0	12.1	20.3	35.2	23.7
Ave.	23.8	34.9	11.5	12.8	14.3	17.4	24.6	27.5	26.6
S.D.	±10.2	±12.2	±6.5	±4.3	±7.5	±7.2	±8.7	±9.1	±6.3

Unless indicated, all animals were 42 days old

O = Operated
UO(1) = Unoperated 28 days old rat
UO(2) = Unoperated
UT = Untreated
FSH = 50 μg FSH
LH(1) = 50 μg LH
LH(2) = 500 μg LH
ACTH = 2 USP units ACTH
3 H = 3 Hormones

TABLE 6

SUMMARY OF ADRENAL WEIGHT CHANGES

Type of Operation	Treatment	No. of days treated	Average U 17-Ks in µg/day N	Ps
UO	None	None	18.6-26.6	23.8-34.9
HC	None	None	9.8	11.5
HC	50 µg FSH	7	9.6	12.8
HC	50 µg LH	7	12.5	14.3
HC	500 µg LH	7	16.4	17.4
HC	2 USP units ACTH	7	24.2	24.6
HC	2 USP units ACTH 50 µg LH	7	24.5	27.5
HC	2 USP units ACTH 50 µg LH 50 µg FSH	7	25.7	26.6

UO = Unoperated
HC = Hypophysectomized and Castrated

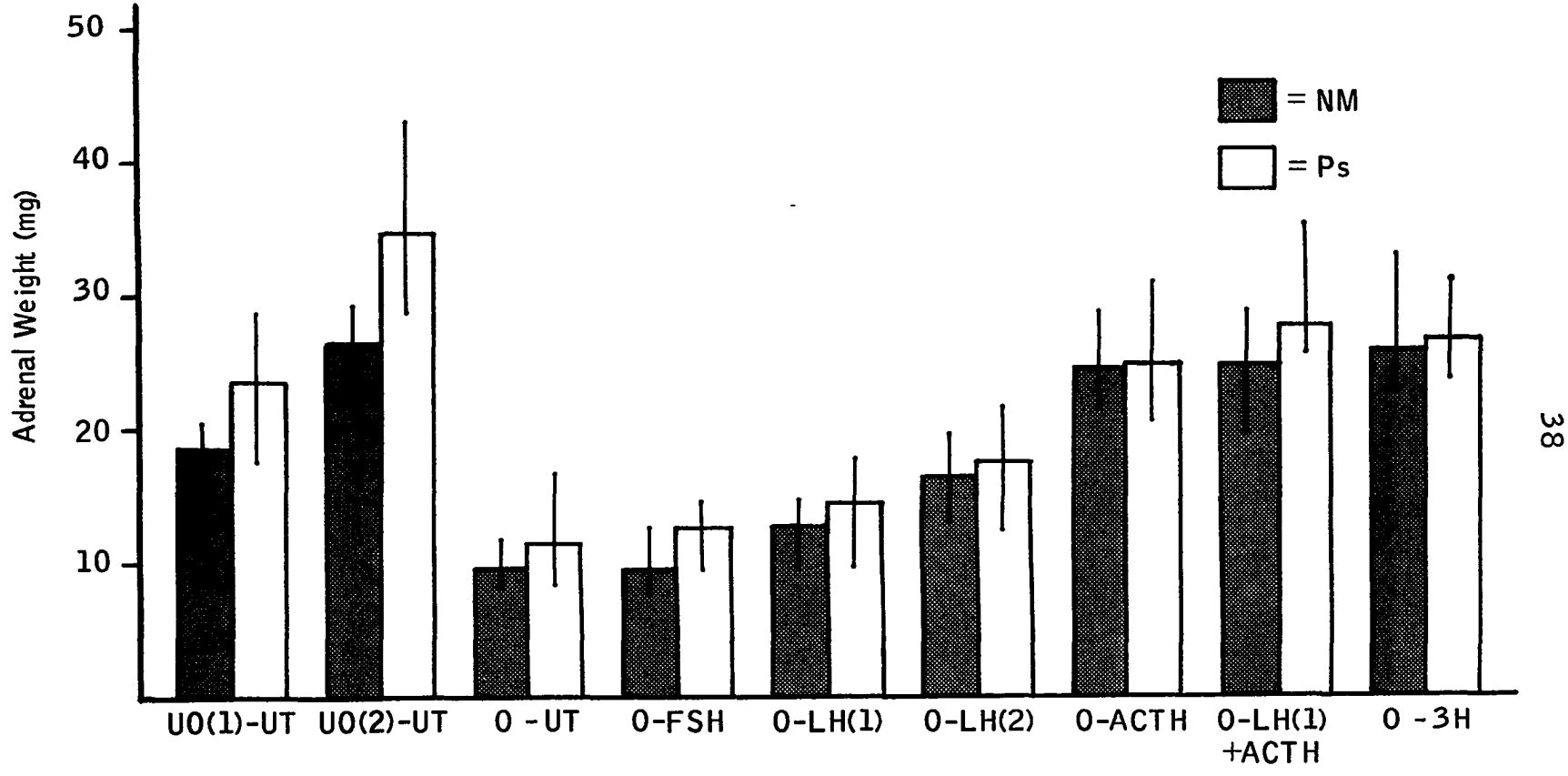

Figure 3. Average adrenal weights of 6 animals. Unless indicated, all animals were 42 days old rats; O=operated; UO(1)=unoperated 28 days old rat; UO(2)=unoperated 42 days old rats; UT=Untreated; FSH=50 μg FSH; LH(1)=50 μg LH; LH(2)=500 μg LH; ACTH=2 USP units of ACTH; 3H=3 Hormones.

CHAPTER IV

DISCUSSION

The amount of urinary 17-Ks produced by an individual under a given set of circumstances will reflect such parameters as the functional state of the gonads, adrenal cortex, and pituitary gland, as well as the presence or absence of other less apparent conditions related to hormone imbalance.

Adrenocorticotropic hormone stimulates the cells of the zona reticularis of the adrenal cortex to produce testosterone and 17-Ks (Mason, et al., 1948; Forsham et al., 1948; Bartter, 1948; McAlpine et al., 1948). Salassa et al., (1961) measured the urinary 17-Ks (etiocholanolone, adrosterone, 11-oxygenated 17-Ks, and dehydroepiandrosterone) produced by a patient with testicular feminization before and after administration of ACTH. Following ACTH, all of these compounds increased, except dehydroepiandrosterone which was unaffected. When ACTH was given to a patient with virilization associated with male pseudohermaphroditism, it caused an exaggered increase in his total urinary 17-Ks as well as in androsterone and etiocholanolone. After gonadectomy, this patient's total urinary 17-Ks production fell to normal levels and reacted normally to ACTH, but his androsterone titer still exhibited exaggerated increases when ACTH was

given (Green, 1969). In cultures of human fetal adrenocortical explants, Milner et al. (1969) and Milner and Villee (1970) found that ACTH induces the formation of microvilli, proliferation of the membranes of the smooth endoplasmic reticulum, changes in the mitochondria of adrenocortical cells, and an increase in sterioid biosynthetic activity.

The mechanism by which ACTH acts on the adrenal cortex is still not understood. According to Sawin (1969), it is possible that ACTH stimulates adenyl cyclase in the nuclear membrane of adrenal cortical cells in such a way as to cause an increase in cyclic 3',5'-adenosine monomphosphate (cAMP) concentration. The cAMP in turn stimulates the synthesis of a specific enzyme which increases the side-chain cleavage of cholesterol.

There is some evidence that LH is also capable of stimulating the adrenal cortex to release increased amounts of androgen, including 17-Ks. The action of HCG seems in many ways to be both immunologically and clinically identical to that of LH (Wide and Gemzell, 1962; Lostroh et al., 1963; Gross and Taymor, 1962; Taymor et al., 1963; Gross and Lewis, 1964). The results are not uniform because of the variability of the amounts administered. Moracci (1953), Borell (1954), Cerasuolo (1954), Decio (1955), Hibbit (1958), and Pauerstein and Solomon (1966) found that in man, HCG given after castration will cause an increase in total 17-Ks. However, one patient with virilizing male pseudohermaphroditism failed to exhibit this response to HCG after he underwent gonadectomy. Plate (1952) indicated that HCG injected into surgically castrated females causes a rise in the excretion of all urinary 17-Ks

fractions. Patients with testicular feminization exhibit a rise in total 17-Ks during administration of HCG. Of these, androsterone and etiocholanolone are most responsive (David et al., 1965).

The other gonadotropin, FSH, appears to have no effect on the 17-Ks output of the adrenal (David et al., 1965; Hulka and Solomon, 1966).

In man, large amounts of urinary 17-Ks are excreted by most patients with hyperplasia of the adrenal cortex (Crooke and Callow, 1939; Friedgood and Whidden, 1939; Talbot et al., 1940; Fraser et al., 1941; Patterson et al., 1942; Engstrom et al., 1944). Also, the testes of patients with testicular feminization contain abnormally large numbers of interstitial cells and, in addition, these individuals excrete levels of urinary 17-Ks that average higher than those for normal males, but lower than for normal females. In the light of these observations and since there are many physiologic and anatomic homologies between testicular feminization in man and male pseudohermaphroditism in the rat (Allison, 1965), it is not surprising that the average 24-hour output of urinary 17-Ks from the Ps rat is about three times higher than normal (Tables 1, 2 and 3 and Figs. 1 and 2).

Reported normal values for the average amount of urinary 17-Ks excreted by the rat vary considerably (Table 7). We have found that the average quantity produced by a normal male rat in a 24-hour period is 28.6 μg. This is close to the 30 μg recorded by Danford and Danford (1951), Langecker (1952), and Kullander (1960). According to Taceva and Pospisil (1967), the mean 24-hour output from a normal rat is 54 to 71 μg. Easley (1968)

TABLE 7

REPORTED URINARY 17-KETOSTEROIDS
PRODUCED BY NORMAL RATS

Author(s) & Year	Animal	Method	U-17Ks µg/24 hrs.
Kowalewski and Bastenie, 1950	Female rats, 210-295 gm	Warren's modification of Callow's method, (1938)	330
Kowalewski et al., 1951	Female rats, 160-200 gm	Warren's modification of Callow's method (1938)	190
Danford and Danford, 1951	Male rats, 34 days or 120 gm	Engstrom and Mason (1943)	30
del Greco et al., 1952	Female rats, 150-250 gm	Drekter et al. (1952)	198
Langerker, 1952	Male and female rats, 120-200 gm	Drekter et al. (1947)	30
Bucchus and Heiffer, 1953	Female rats, 200 gm	Drekter et al. (1952)	137
Kullander, 1960	Female rats, 6 months - 2 years	Jensen's modification of Callow et al. (1938)	30
Taceva and Pospisil, 1967	Female rats, 208 ± 3 gm	Rappaport et al. (1960)	54-71
Easley and Stanley, 1968	Male and female rats, 6 months	Modification of Drekter et al. (1952)	18 (M) 34 (F)
Chung and Allison, 1971	Male rats, 35-42 days	Modification of Drekter et al. by Klendoshoj (1953)	28.6

reported 18 μg for the male and 34 μg for the female. Although such amounts fail to agree with ours, they do not deviate by nearly as much as the values of 330 μg reported by Kowalewski and Bastenie (1950), of 190 μg reported by Kowalewski et al. (1951) and the 198 μg by del Greco (1952). There also is lack of agreement among these workers. The method used, the age of the rat or environmental conditions may account for the variability in these reports (Mason and Engstrom, 1950; Kullander, 1960).

The adrenal glands of the Ps rat are larger than those of the normal male and their testes exhibit interstitial cell hyperplasia. Castration and hypophysecomy cause the urinary 17-Ks output of Ps rats to drop to that of normal male animals with a similar operation. Thus, it appears that the secretory activity of the high number of interstitial cells and the large adrenal glands present in the Ps rat are due to hypophyseal action. At the same time that the urinary 17-Ks output drops in these OPs rats, their adrenal cortex atrophies to a thickness concomitant with that seen in ONM rats. Although the width of the adrenal cortex in OPs rats is restored to a normal or slightly greater than normal state following treatment with ACTH and/or LH, their urinary 17-Ks output is brought up to about one-half the value found in the urine of the unoperated Ps rat. So it appears that in the Ps rat the testicular contribution of urinary 17-Ks is greater than that provided by the adrenal glands. The large number of interstitial cells in the testes of Ps rats supports this conclusion.

Since it is commonly known that LH stimulates the interstitial cells of the testis to produce androgens, the presence of more than normal numbers of such

cells in Ps rats could be an indication of high LH activity (Easley, 1968). Sherins et al. (1971) found that the circulating LH in Ps rats was three times greater than in NM rats.

Most of the evidence cited in the preceding for man indicates that LH will stimulate the adrenal glands to produce 17-Ks. The observations of Diczfalusy et al. (1950) show that the same is true for the rat. They found that in castrated animals, HCG would stimulate the adrenal glands to produce urinary 17-Ks if the pituitary gland was intact. This is not in total agreement with the present findings in that LH by itself increased the urinary 17-Ks in hypophysectomized castrated NM and Ps rats. Easley (1966) maintained that LH is effective in stimulating production of urinary 17-Ks from the adrenal gland only if adrenal function is maintained by ACTH. Our results partially support this in that when LH and ACTH are given simultaneously to such animals, the urinary 17-Ks output increases to a level greater than when either of the hormones is given alone.

The Ps rat normally produces more urinary 17-Ks than does the NM rat and this substance rises to a higher level in the OPs rat following ACTH and/or LH than it does in the ONM rat. Under these circumstances, the higher titer of urinary 17-Ks is also accompanied by a greater hypertrophy of the adrenal cortex of the OPs rat than of the ONM rat. Thus, the adrenocortical cells in the Ps rat appear to be more sensitive to ACTH and LH than they are in the NM. In spite of the high output of urinary 17-Ks by the Ps rat, their titer of testosterone is lower than normal. Bardin et al. (1969), Schneider and Bardin (1970), and

Goldman (1970) found that the testes of these animals lack either 17β -hydroxysteroid or 3β -hydroxysteroid dehydrogenase. These enzymes are necessary in order that dehydroepiandrosterone can be converted to testosterone. Most of the tissues, including the hypothalamus in Ps rats, are insensitive to androgen (Bardin et al., 1970; Vanha-Perttula et al., 1970; Chan and Allison, 1971). Therefore, they lack a feedback inhibition of the LH releasing mechanism. This would explain the high titer of LH in Ps rats. Since LH will cause hypertrophy of their adrenals, the large adrenals in these Ps rats are probably the result of the high levels of circulating LH present. This, however, does not explain why the adrenal cortex of the Ps is abnormally sensitive to LH and/or ACTH.

CHAPTER V

SUMMARY

Thirty-five to 42 day-old male pseudohermaphrodite rats excrete three times more urinary 17-Ks in 24 hours than do normal rats the same age.

Administration of like amounts of ACTH and/or LH causes greater hypertrophy of the adrenal glands in hypophysectomized and castrated male pseudohermaphrodite rats age 35 to 42 days than it does to normal rats of like age that have undergone the same operation. Thus, the adrenal cortices of male pseudohermaphrodite rats appear to be hypersensitive to these hormones.

Luteinizing hormone as well as ACTH will stimulate the adrenal cortex of both normal male and male pseudohermaphrodite rats to produce androgens.

The rat adrenal cortex is not stimulated by FSH to either increase in size or to produce androgens.

The etiologies of and reasons for apparent hormonal manifestations of male pseudohermaphroditism in rats were discussed.

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APPENDIX

LEGEND FOR FIGURES

Legends used in Figures 4 through 35 are as follows:

NM	Normal male
Ps	Male pseudohermaphrodite
ONM	Operated normal male
OPs	Operated male pseudohermaphrodite

PLATE I

Figure 4. Paraffin section of adrenal gland from unoperated NM rat. The cortex shows well differentiated zona glomerulosa, zona fasciculata, and zona reticularis. Stained with Mallory's trichrome. X125.

Figure 5. Paraffin section of adrenal gland from unoperated Ps rat. The cortex is wider than that of normal adrenal. Stained with Mallory's trichrome. X125.

Figure 6. Frozen section of adrenal gland from ONM rat. Note that the zona glomerulosa is wider than normal and that many degenerating highly vacuolated cells and abnormal amounts of connective tissue are present in the inner zones, particularly in the zona reticularis. The zona reticularis has almost completely disappeared. Stained with H and E. X125.

Figure 7. Frozen section of adrenal gland from OPs rat. Note a marked atrophy of the cortex, accompanied by wide zona glomerulosa. The cortex is markedly reduced in width. The whole picture is much like that in Fig. 6. Stained with H and E. X125.

PLATE I

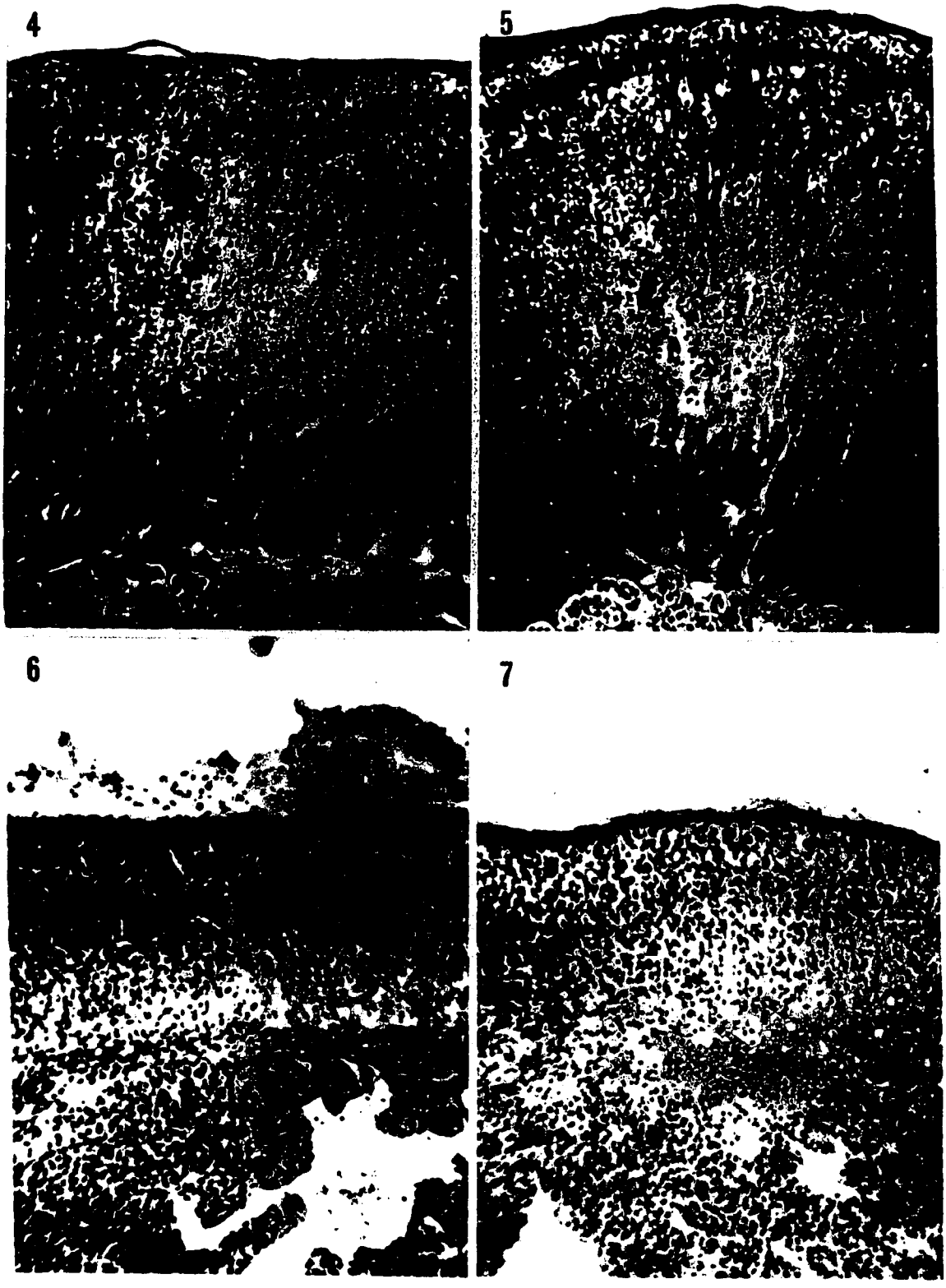

PLATE II

Figure 8. Frozen section of adrenal gland from unoperated NM rat. Lipid is evenly distributed throughout the cortex and occurs in fine droplets. Stained with Sudan Black B. X125.

Figure 9. Frozen section of adrenal gland from unoperated Ps rat, exhibiting lipid droplets uniformly distributed throughout a wide cortex. Stained with Sudan Black B. X125.

Figure 10. Frozen section of adrenal gland from ONM rat. Note a marked increase in size of lipid droplets, particularly in the zona glomerulosa. A lipid-free zone is very apparent between the glomerulosa and fasciculata. Stained with Sudan Black B. X125.

Figure 11. Frozen section of adrenal gland from OPs rat. Lipid droplets are coarse and deeply stained. Stained with Sudan Black B. X125.

PLATE II

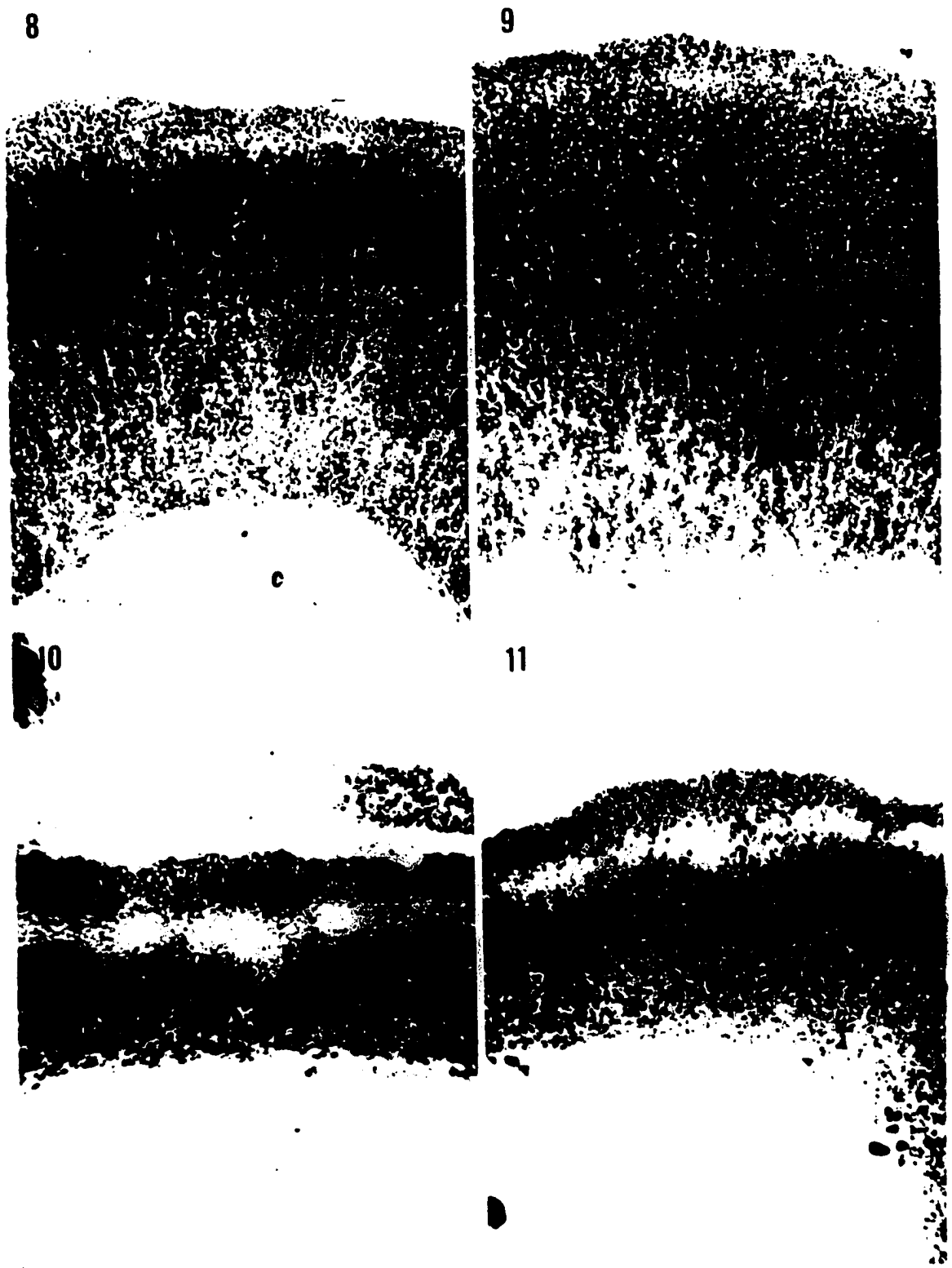

PLATE III

Figure 12. Paraffin section of adrenal gland from ONM rat treated with 50 μg of FSH, showing no regenerative changes in structure. Stained with Mallory's trichrome. X125.

Figure 13. Frozen section of adrenal gland from ONM rat treated with 50 μg of LH. The zona reticularis and the fasciculata are slightly wider than they are in the operated untreated animal. Stained with H and E. X125.

Figure 14. Paraffin section of adrenal gland from ONM rat treated with 500 μg of LH. The cortex appears narrower than normal, but the clear vacuoles are not seen. The fasciculata and reticularis are significantly wider than they are in ONM rats treated with 50 μg LH. Stained with Mallory's trichrome. X125.

Figure 15. Paraffin section of ONM rat treated with 2 USP units of ACTH. The adrenal cortex though extra wide is otherwise normal in structure. Stained with Mallory's trichrome. X125.

PLATE III

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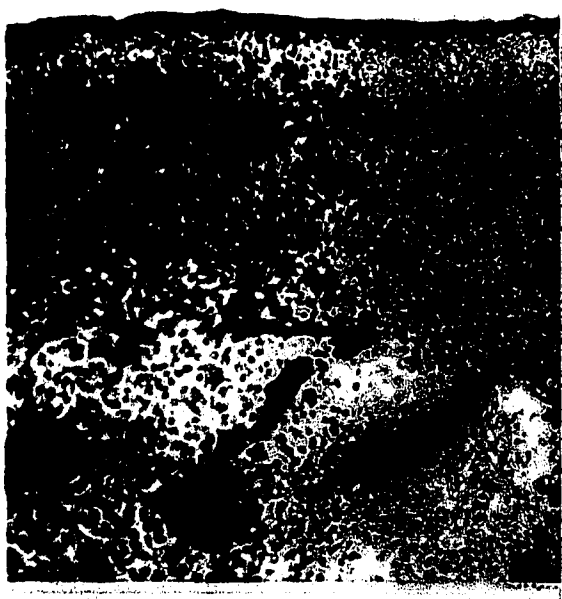

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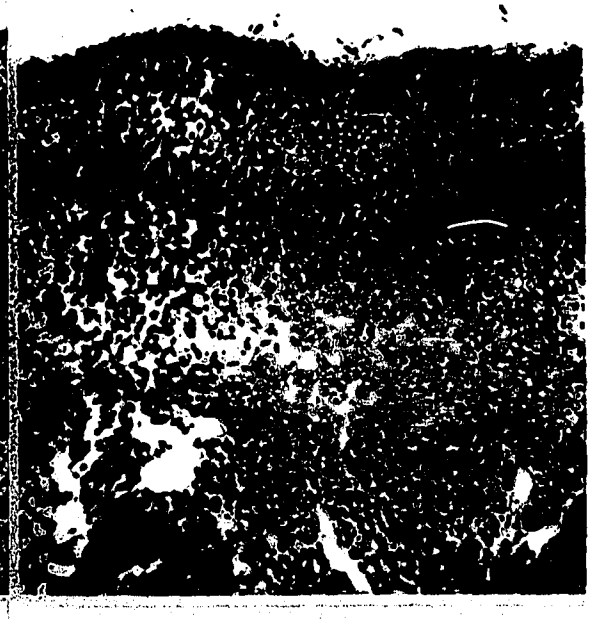

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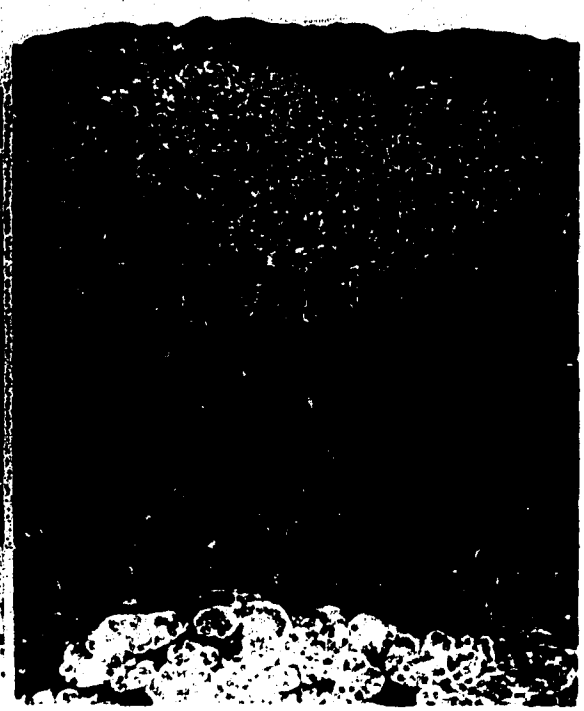

PLATE IV

Figure 16. Frozen section of adrenal gland from ONM rat treated with 50 μg of FSH. FSH has no effect on lipid depletion in the cortex. Stained with Sudan Black B. X125.

Figure 17. Frozen section of adrenal gland from ONM rat treated with 50 μg of LH. No significant change in lipid distribution is apparent. Stained with Sudan Black B. X125.

Figure 18. Frozen section of adrenal gland from ONM rat treated with 500 μg of LH. Lipid droplets are finer. Stained with Sudan Black B. X125.

Figure 19. Frozen section of adrenal gland from ONM rat treated with 2 USP units of ACTH. Fine lipid droplets are distributed throughout the cortex. Stained with Sudan Black B. X125.

PLATE IV

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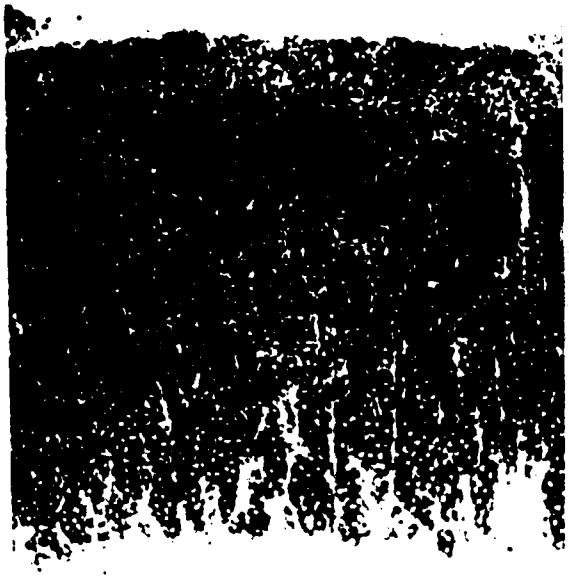

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PLATE V

Figure 20. Paraffin section of adrenal gland from OPs rat treated with 50 µg of FSH. There is no evidence of cortical repair. Stained with Mallory's trichrome. X125.

Figure 21. Paraffin section of adrenal gland from OPs rat treated with 50 µg of LH. Note the slight increase in width of cortex and reappearance of zona reticularis stained with Mallory's trichrome. X125.

Figure 22. Paraffin section of adrenal gland from OPs rat treated with 500 µg of LH. Cytoplasm of the cortical cells has been restored. The nuclei of the cells are no longer pycnotic. Stained with Mallory's trichrome. X125.

Figure 23. Paraffin section of adrenal gland from OPs rat treated with 2 USP units of ACTH. There is hypertrophy of the cortex. Stained with Mallory's trichrome. X125.

PLATE V

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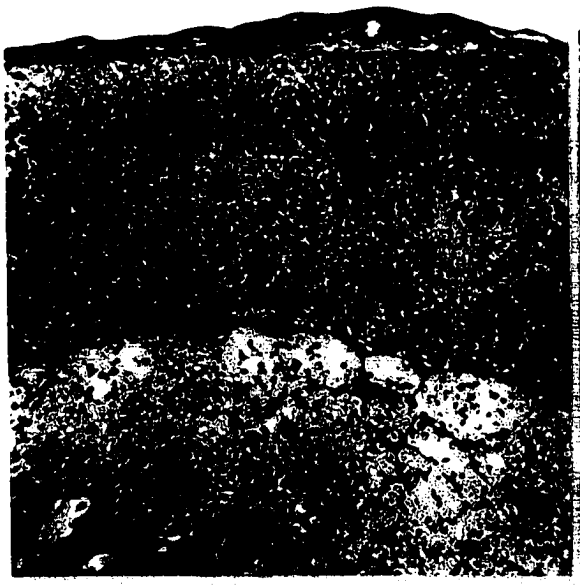

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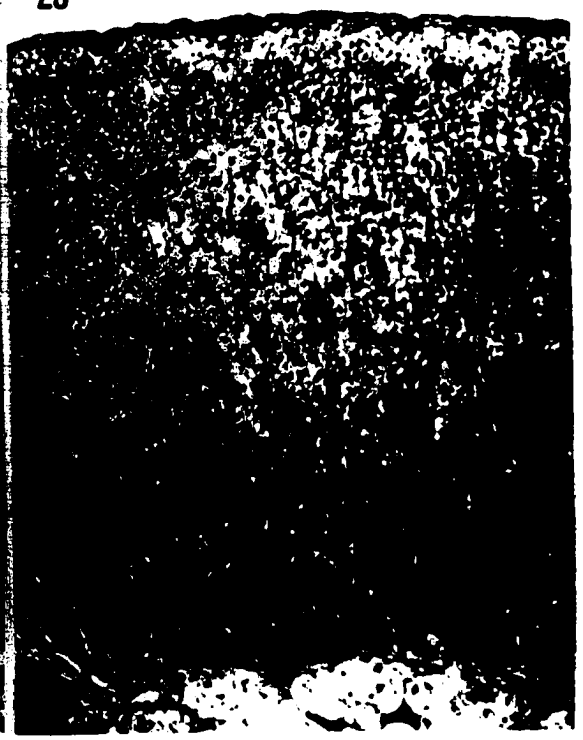

PLATE VI

Figure 24. Frozen section of adrenal gland from OPs rat treated with 50 µg of FSH. The lipid free zone between the zona glomerulosa and zona fasciculata is still present. Large lipid droplets are apparent in the zona fasciculata and reticularis. Stained with Sudan Black B. X125.

Figure 25. Frozen section of adrenal gland from OPs rat treated with 50 µg of LH, exhibiting a decrease in size of lipid droplets. Stained with Sudan Black B. X125.

Figure 26. Frozen section of adrenal gland from OPs rat treated with 500 µg of LH. Lipid distribution in cortex is almost uniform. Stained with Sudan Black B. X125.

Figure 27. Frozen section of adrenal gland from OPs rat treated with 2 USP units of ACTH. Note almost complete disappearance of lipid from zona reticularis except very fine granules.

PLATE VI

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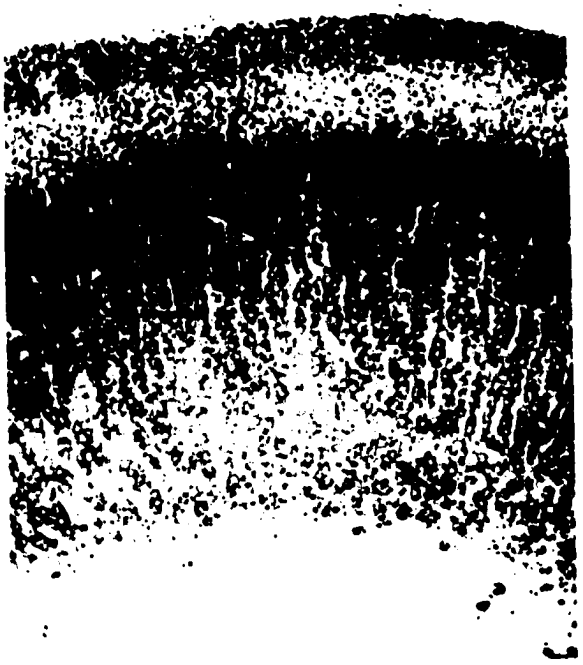

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PLATE VII

Figure 28. Paraffin section of adrenal gland from ONM rat treated with ACTH and LH together, showing a hypertrophy of adrenal cortex. Stained with Mallory's trichrome. X125.

Figure 29. Paraffin section of adrenal gland from ONM rat with simultaneous administration of all 3 hormones. The cortex shows a marked hypertrophy. Stained with Mallory's trichrome. X125.

Figure 30. Frozen section of adrenal gland from ONM rat treated with ACTH and LH together. The lipid droplets are very fine. Stained with Sudan Black B. X125.

Figure 31. Frozen section of adrenal gland from ONM rat with simultaneous administration of all 3 hormones. Very fine lipid droplets are uniformly distributed. Stained with Sudan Black B. X125.

PLATE VII

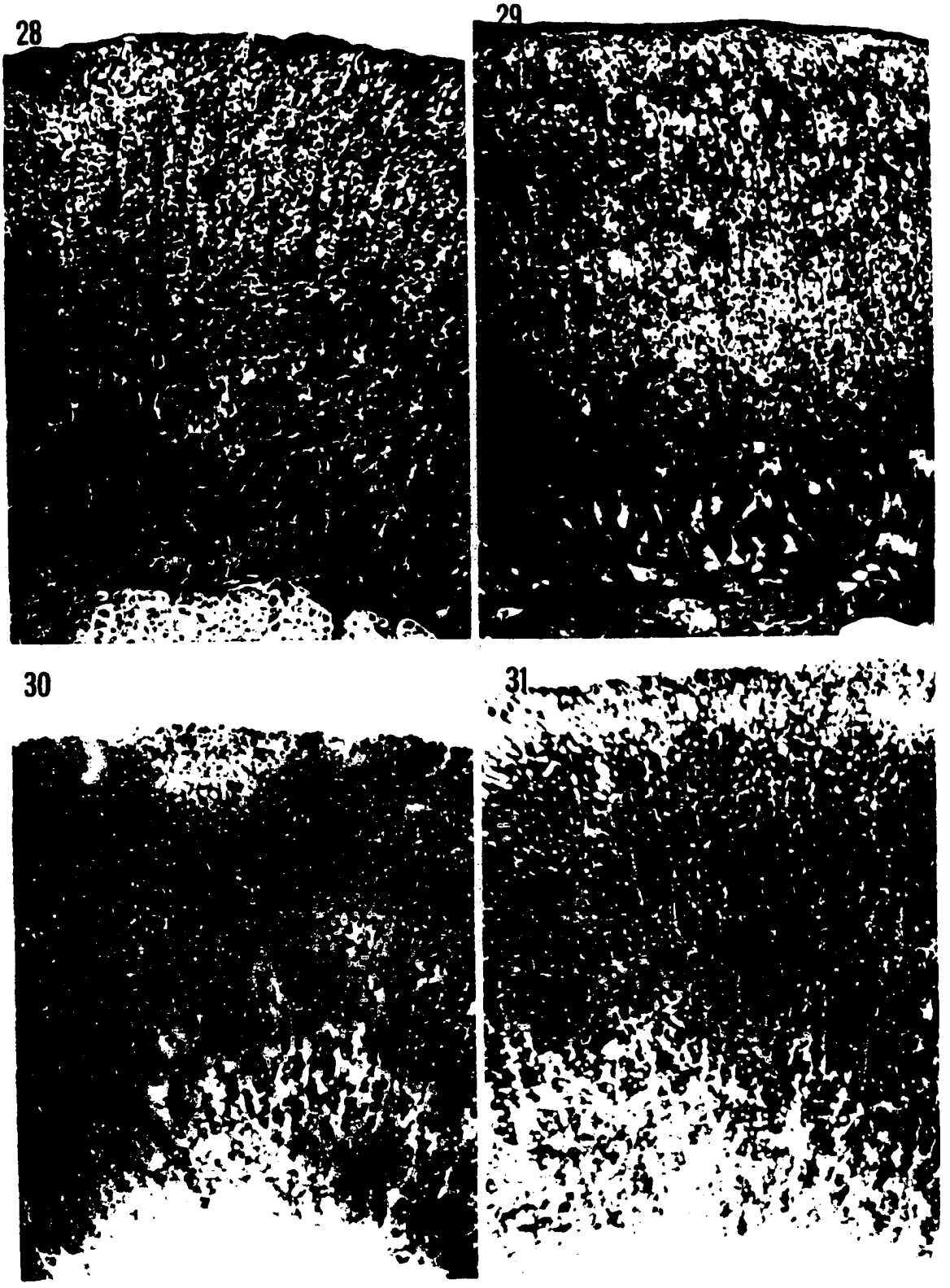

PLATE VIII

Figure 32. Paraffin section of adrenal gland from OPs rat treated with ACTH and LH together, showing hypertrophy of adrenal cortex. Stained with Mallory's trichrome. X125.

Figure 33. Paraffin section of adrenal gland from OPs rat with simultaneous administration of all 3 hormones. There is a marked enlargement of the adrenal cortex. Stained with H and E. X125.

Figure 34. Frozen section of adrenal gland from OPs rat treated with ACTH and LH together. Lipid droplets are uniformly distributed. Stained with Sudan Black B. X125.

Figure 35. Frozen section of adrenal gland from OPs rat that has simultaneously received all 3 hormones. There is hypertrophy of the entire cortex and marked lipid depletion of the zona reticularis and the inner part of the zona fasciculata.

PLATE VIII

