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ANDROGEN INSENSITIVITY IN MALE PSEUDOHERMAPHRODITE RATS

APPROVED BY

DISSERTATION COMMITTEE

To my parents

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ANDROGEN INSENSITIVITY IN MALE PSEUDOHERMAPHRODITE RATS

CHAPTER 1

INTRODUCTION

During the last decade, rapid advances in genetics of sex have encouraged hope that fresh light soon will be shed on ways either to treat successfully or to eliminate hereditary intersexuality in man. Many people so affected often have no visible or easily detectable physiologic symptoms. Thus, many years may pass before they become aware of their affliction. For example, an individual with testicular feminization may not be aware of any abnormality until puberty. At that time, the patient (genetically male, but psychologically and phenotypically female) approaches the physician because of amenorrhea. Some such cases can be detected earlier, since more than half of those reported have hernias or inguinal gonads (Morris and Mahesh, 1963).

Certain anomalous conditions associated with reproductive organs have arisen in a line of King-Holtzman (Stanley and Gumbreck, 1964) hybrid rats maintained in their

laboratory. One of these is pseudohermaphroditism in the male with accompanying sterility. Such animals lack an entire reproductive tract with the exception of the testes. There are many similarities between this phenomenon in rats and testicular feminization in man (Allison, et al. 1965). Thus, because it is sometimes impossible to perform physiologic and genetic experiments using man as the subject, these animals appear to be a useful tool for the study of testicular feminization.

Somatic tissues normally sensitive to androgen are insensitive to patients suffering from testicular feminization (Morris and Mahesh, 1963). This dissertation represents the results of an attempt to determine whether the same is true of the male pseudohermaphrodite rat.

Historical Review: Determination of Embryonic Sex

An exact interpretation of sexual abnormalities in mammals, including man, cannot be arrived at without a knowledge of how sex is determined. A clear understanding of the genetics, genital organogenesis, and the endocrinology involved gives invaluable clues to the etiology of pathologic development.

Genetic Sex Determination

It is possible to distinguish between genetic

and phenotypic sex. Witschi and Opitz (1963) point out that genetic sex is concerned with the distribution to each body cell of genes that influence the ontogenetic course of sex differentiation, while phenotypic sex determination includes the entire ontogenetic processes of male-female differentiation. The latter involves action of sex-specific gene substances, environmental factors, and processes of induction which lead to the development of primary and secondary sex characteristics.

Inheritance in mammals, including man, follows the Mendelian pattern. In some amphibians it has been proved that maleness is heterozygous in relation to a sex-determining hereditary factor (the male produces gynosperms and androsperms) and that femaleness is homozygous. The production of approximately 50% male and 50% female offspring is thereby explained. It also has been shown that in amphibians, sex differentiation occuring during embryogenesis is initiated by localized inductors. Germ cells, regardless of genetic constitution, become eggs under the influence of cortical inductors, or become sperm under the influence of medullary inductors (Witschi, et al. 1957).

In all mammals so far examined the heterozygous condition XY always has been found in the male sex. Until quite recently it was thought that genes were not carried on the Y chromosome. Ford (1960) reveals that the gene for maleness in man may be located on the Y chromosome.

Welshons and Russell (1959) have presented evidence that the same condition holds for mice. Therefore, sex determination in these species should simply depend upon whether a masculinizing Y chromosome is present.

While studying the structural features of nerve cells in cats, Barr (1959) discovered a characteristic mass of chromatin in the nuclei of such cells from genetic females. In normal males this mass is seen in less than 10 per cent of the nuclei. This same chromidial structure occurs in man and, differential counts of this mass in cells from mucosal smears have revealed that some exhibit a genetic sex differing from the sex shown by their phenotype. Varying degrees of intersexuality exist in others. This may be easily understood if one keeps in mind that primordia of the sex glands in early embryonic life are identical in genetically male or female embryos, and thus are sexually bipotential. They develop in a male or in a female direction depending upon physiologic conditions.

Phenotypic Sex Determination

The first sexual primordium to appear is that of the gonads. This structure initially contains everything necessary to differentiate into either an ovary or a testis.

The components of the gonads (the germ cells, the cortex and the medulla) are derived from three different sources. The two latter components are closely related, as

both are derived from the mesonephric region, the cortex from the nephrostomic field, and the medulla from the deeper mesonephric blastema. Much evidence indicates that the germ cells arise extragonadally, apparently from the endoderm.

The origin of the germ cells (gonocytes). Witschi (1948) believes that in all vertebrates, primordial germ cells originate in endoderm and migrate in a characteristic way toward the mesonephric border to the site of the gonad primordia. He also believes that they are the sole source of definitive germ cells in the adult.

Living primordial germ cells of the mouse have been followed from the yolk sac to the genital ridges and their ameboid movements recorded cinematographically (Bishop, 1962 & Blandau, et al. 1963). In reptiles and birds, the gonocytes first become segregated in the area pellucida, migrate into the blood islands of the area vasculosa, and then are carried to the genital ridge by the peripheral circulation (Witschi, 1948). It is generally believed that the gonocytes are unable to differentiate into somatic elements, and those that fail to reach the gonadal primordia degenerate and disappear.

In the human embryo of 12-13 somites, the germ cells are easily identifiable for the first time (Witschi, 1956). They are located in the yolk sac epithelium in a fairly circumscribed area just dorsal the allantoic rudiment. A week later, they have transferred to the mesoderm of the

primitive gonadal folds at the inner edges of the mesonephric bodies. Some of the cells are passively transferred to the ventral and lateral walls of the hindgut. These cells then move to the top of the hindgut, penetrate its basement membrane, enter the mesentery and go around the coelomic angles toward the mesonephric ridges. Other germ cells leave the endoderm of the yolk sac and ascend directly through the mesenchyme toward the mesonephric borders. Histologically, they are easily identifiable by their amoeba-like pseudopodia. The means by which the gonia are guided in their migratory movement toward the mesonephric border still remains to be elucidated. According to Bouin (1900) and others, their migration is a consequence of their own ameboid movements, possibly aided by their capacity for histiolytic destruction of cells and membranes that block their way. It is not unlikely that a chemotropic mechanism is involved (Witschi, 1948). It also must be borne in mind that despite the seemingly long and tortuous path they follow, the germ cells are never more than 0.5 mm away from their destination at the medial edge of the mesonephric border.

There is some question whether the germ cells are necessary to the establishment of the gonadal primordia. Sterile testes were obtained from frog eggs whose germinal plasm had been destroyed by ultraviolet rays (Bounoure, 1950). Chick embryos were submitted to X-rays at early stages. When these embryos become adult, they contain sterile ovaries

(Salzgeber, 1950). Nevertheless, it remains difficult to ascertain whether germ cells participate in the differentiation of the very earliest gonadal primordia. Witschi (1951) defends the idea that a true cortex cannot differentiate in the complete absence of primordial germ cells. His opinion is based partly on observations made on sex glands of frogs and toads obtained from overripe eggs.

Gonadal sex differentiation.

The undifferentiated gonad. In spite of the fact that sex chromatin enables us to type the cells of the undifferentiated gonad of the rat as male or female, sex in man cannot be recognized from histologic preparations before the embryo has reached stage 32 (7 week human embryo) in its development (Witschi, 1962). The genital ridges first appear as bilateral thickenings along the mesial edges of the mesonephric kidneys. The gonocytes accumulate in the mid-portions of the germinal ridges, and only these areas contribute to the gonads; the sterile cranial region becomes the suspensory ligament, and the caudal portion contributes to the utero-ovarian ligament (Arey, 1954). All undifferentiated gonads have a compact cortex which is solidly attached to the surface epithelium. The base of the medullary cords consists of loosely arranged blastemal cells resting on the mesonephric corpuscles. Distally, near the cortex, these cords are more closely packed. At about stage 33, either the medullary or the cortical element of the gonad must become

dominant and move ahead in further differentiation through growth and multiplication of germ cells and mesenchymal cells. Concomitantly, the other system must regress. Witschi (1962) observed that gonadal sex differentiation in man becomes apparent during the last stages of metamorphosis, stage 34 (17 mm human embryo). During this stage of rapid growth, the medulla and the medullary cords become more distinct.

Testicular differentiation. A characteristic feature of beginning testicular differentiation is the appearance of a distinct mesenchymal membrane, the albuginea, which separates the thin peritoneal epithelium from the deeper layer of the cortex. The albuginea subsequently thickens and fills with fibrous connective tissue. It becomes vascularized by invasion of large blood vessels which arrange themselves in a network beneath the surface peritoneal epithelium. This arrangement is characteristic of the vascular system of the developing testis (Witschi, 1962). Along with vascularization, the cortical cells that have become separated by the albuginea organize into cords which are the primitive seminiferous tubules. Strands of tissue from the medulla simultaneously help to form the proximal part of the seminiferous tubules and the ductules which connect them to the rete. Some of these medullary strands also furnish sheath cells for the walls of the seminiferous tubules. All of the germ cells, regardless of their previous location in cortex or

medulla, now are contained in the tubules and are spermatogonia. Somatic elements within the cords become the sustentacular cells of Sertoli. The interstitial cells of Leydig arise from intertubular elements. The surface eipthelium, with its germ cells, typically disappears early, thus removing all potentiality for cortical development. Cortical (secondary) cords may start to form in the testis of man and certain other species, but such proliferations normally are abortive.

Ovarian differentiation. Ovarian differentiation may be said to be a continuation of the development of the undifferentiated stage. It consists primarily of cortical development. A widening of the space between the cortex and medulla is the first indication of ovarian differentiation (Witschi, 1962). At this time, stage 31-32, the medullary cords have not begun to regress. They still contain numerous germ cells. By stage 35 (60 mm human embryo), the medulla is still prominent and often contains hundreds of germ cells. Some of these cells persist in the hilar area of the ovary until about the 6th month (human female fetus) and a few may be found even postnatally. Ultimately they degenerate. The number of gonia in the cortex greatly increases (from one million to 5 or 6 million) during stage 35, and they become arranged in cords (the cortical cords or tubes of Pflüger). Some of the germ cells in these cords have already begun to mature, and ovocytes in various stages of synapsis may be

seen. It is important to note that the somatic elements within the medullary and cortical cords differentiate into Sertoli cells and follicle cells, respectively, and that these constituents of the testis and ovary are homologous.

By late stage 35, all gonia have transformed into ovocytes. Following this, the fertile part of the cortex becomes separated from the surface epithelium by the development of a mesenchymal albuginea. However, the ovarian albuginea, unlike that of the testis, is not the route of vascularization. In the ovary, blood vessels spread through partially open spaces between the medullary nodes and the cortex.

An extensive medullary remnant persists in the hilar region of the ovary. It consists of a rete corresponding to that of the testis and a more central complex of cords and nodules, which may be homologous to the straight tubules. Undifferentiated germ cells are found in both parts of the rete, and a few actually may transform into spermatogonia and persist, as mentioned above, into postnatal stages. These ventually degenerate.

Theca cells for the ovocytes will be supplied by strands of cells which grow out from the medulla between the cortical cords. These eventually invest individual auxocytes and their granulosa envelopes.

The sex-determining mechanism which is established at fertilization directs and controls all of the late

ontogenetic processes (postgenetic differentiation) involved in the formation of testes or ovaries and corresponding accessory genitalia. According to Witschi (1948), the primordial germ cells are dependent upon the cortical and medullary components of the early gonad for the specific influences that direct their differentiation into sperms or eggs. The cortex and medulla are, of course, genetically determined, and they appear to exert contrasting influences upon the early germ cells. The basic problem is how the sex-determining genes act upon the genital primordia to condition their differentiation into male or female organs.

The accessory sex structure. In conjunction with the morphogenesis of accessory sex structure in man, the following studies should be mentioned: Felix (1912), Spaulding (1921), Wilson (1926), Stieve (1930), Vilas (1933), Koff (1933), Witschi (1948), Gillman (1948), Gruenwald (1943), Tonutti (1960), and Watzka (1963). Some interesting cytological and histochemical descriptions have been given by McKay, et al. (1953), Rossi, et al. (1957), Jirasek (1962), Jirasek and Raboch (1963), Baillie, et al. (1965), and Mancini, et al. (1965).

Modifications of Postgenetic Sex Development

Hormonal theory. As early as 1903 Bouin and Ancel had already suggested that an internal secretion of the testis was essential for the development of male sex characteristics.

Later, Lillie (1917) postulated a hormonal condition as the causative factor in the production of the freemartin, a sterile intersex bovine female which often develops as a co-twin was responsible for the development of abnormal or intersex gonads and of varying degrees of intersexuality in the accessory sex organs of the female. After sex hormones became available for laboratory experimentation, numerous attempts were made to duplicate the freemartin effect. However, control of gonadal sex differentiation in eutherian mammals, through the administration of steroid hormones to pregnant females or directly to embryos, has not been achieved. Consequently, there is no experimental evidence supporting of a steroid theory for the freemartin effect. It will be seen, however, that usually the non-gonadal portions of the reproductive tract in mammals, particularly the urogenital sinus and derivatives, readily respond to the administration of sex steroids with the consequence that varying degrees of fetal intersexuality are brought about.

Cortico-medullary theory. The undifferentiated gonad has a fundamental bisexual constitution (cortex and medulla). Realization of one sex necessitates the suppression of the heterologous component. In the theory of cortico-medullary antagonism, it is assumed that each component produces a morphogenetic substance antagonistic to the other. Testicular differentiation results from the prevalence of the medullary morphogenetic substance (Witschi's medullarin).

This substance succeeds in inhibiting cortical differentiation and in masculinizing the now medullary germ cells. Similarly, ovarian differentiation results from the activity of a substance produced by the cortex called corticine.

Genetic sex factors are responsible for the prevalence of either the medulla or the cortex. This turning point in gonadal organogenesis constitutes their main effect and their mode of expression. It is not yet clear whether the cortical or medullary prevalence results from a genetic factor or from a difference in sensitivity of each component to the opposite substance. The observation of the freemartin afforded the first and classic observation favoring the concept of gonadal morphogenetic substances. Freemartins are genetic females (Moore, et al. 1955). Their ovaries have been extraordinarily inhibited by the testicular morphogenetic substance of a male co-twin. This is a result of blood exchange produced by the fusion of the embryonic annexes. In some cases after the cortex has been inhibited, a few sterile seminiferous tubules, develop representing a trend toward testicular organogenesis in the medulla.

A long series of brilliant and classic experiments in amphibians, carried out by Burns, Humphrey and Witschi, permitted a detailed study of the sex inductor substances (reviews in Witschi, 1939; Jost, 1948).

Experimental sex development. Some classical methods of endocrinology, developed mainly in amphibians and birds

(Witschi, 1939), have aided in elucidating the factors involved in sex differentiation (gonadal and non-gonadal parts of the genital tract). Techniques used in such studies also have resulted in the production of various intersexual states in mammals. Hormone administration, ablation of endocrine glands, parabiosis, gonad transplantation, organ culture, exposure of amphibian females to extreme temperature changes, and the production of overripe eggs have been among the most successful techniques. When applied to mammalian experimentation, they have yielded information which has added to our understanding of not only the normal course of sex differentiation, but also of the nature and cause of congenital intersexuality as well.

The early studies of Raynaud (1956, 1958) on the mouse and of Greene, et al. (1939) on the rat, reveal that estrogens injected into gestating females easily induce intersexual development in fetuses of both sexes.

It has been shown by Brunner and Witschi (1946) that androgens administered to the golden hamster any time after the 9th day of gestation abruptly stop the descent of the uterovaginal rudiment.

In the rabbit (Jost, 1947a, 1958), hypertrophy of the medullary area of the fetal ovary occurs when androgens or progesterone are administered during early stages of pregnancy.

Zander and Müller (1953), Hoffman, et al. (1955),

Wilkins, et al. (1958) and Wilkins (1964) have reported that in man, certain hormones (e.g. progesterone, methyltestosterone, ethinylestrodiol, and stilbestrol) used therapeutically during pregnancy will masculinize female fetuses.

Although it has been known for some time that fetal interstitial cells of both the testis and ovary can respond to gonadotropin (Aron, 1933; Cole et al. 1933), it has been difficult to determine whether the interstitial elements actually secrete during the period of somatic sexual differentiation, and, if so, whether they require stimulation from fetal pituitary gonadotropin or from another source (maternal or placental). From observations on the genital tracts of male rabbit fetuses, hypophysectomized at different times between days 19 and 24 and sacrificed on days 22-24, 26 or 28, Jost (1947b, 1951, 1953) concluded that the fetal hypophysis not only steadily maintains testicular interstitial elements, but also rapidly discharges secretion between days 22 and 24, a period which is critical in so far as the establishment of male secondary sex characteristics is concerned.

Histologic studies of the fetal hypophysis (Jost and Danysz, 1952), when correlated with the studies on the genital tract, lend confirmation to this idea.

Modification of gonadal differentiation in mammalian embryos has been achieved successfully through the technique of gonad transplants (MacIntyre, 1956; MacIntyre,

et al. 1960). Not only has the inhibitory action of testicular secretions on ovarian development been demonstrated, but, for the first time, evidence of ovarian inhibition of testicular differentiation has been obtained.

Heterosexual pairs of embryonic rat gonads (15½ days gestation age) can be transplanted to a subcapsular position in kidneys of castrated adult females. If they are allowed to remain in such a site for 3 weeks, the testis will grow and differentiate while the ovary will become severely inhibited or will be transformed into a testis-like organ containing structures resembling seminiferous tubules. Similar findings have been reported for the rabbit (Holyoke and Beber, 1958). It has been shown further (MacIntyre, et al. 1960) that the degree of suppression of cortical (ovarian) differentiation and concomitant testicular (medullary) development is dependent upon the distance between the two transplants. Ovaries placed 1-5 mm from testicular tissue are inhibited most severely, whereas those 8-10 mm away are not affected.

Thus, it appears that the mechanism whereby the gonads become sexually differentiated in mammals is essentially the same as that originally proposed for amphibians by Witschi in his cortical medullary theory.

Finally, it appears that so far only a few of the developmental processes and correlations leading to the differentiation of normal sex glands have been explored so far.

Nevertheless, all degrees of variation between true ovaries, true testes, underdeveloped, vestigial, or completely missing gonads may appear in animals regardless of their genetic sex. It is more than likely that further studies in this direction will afford important bases for the understanding of human gonadal abnormalities.

Male Pseudohermaphroditism in Man

Intersex in man has long been recognized, and the term hermaphroditism comes from Greek mythology. Herodotus and Pliny the Elder referred to hermaphrodites. There are several statues of male hermaphrodites to be found in the museums of Athens, Rome, Paris, and London (Jones and Scott, 1958).

Male pseudohermaphrodites are genetic males; but their secondary sex characters are either female or mixed male and female. In most extreme cases the testes fail to descend and are very small. In the absence of masculinizing stimulation the gonaducts and external genitalia differentiate in a neutral, i.e. female direction. Male pseudohermaphrodites are chromatin negative, the karyotype of 46/XY is that of a normal male. They would appear to be genetic males who present almost complete sex reversal. The hereditary factor is either an autosomal dominant sex limited to male or an X linked recessive (Armstrong, 1955, Jacobs, et al. 1959; Morris and Mahesh, 1963).

At puberty, patients exhibiting testicular feminization (complete male pseudohermaphroditism) develop breasts of normal female size but lack pubic or axillary hair. They do not menstruate. If they exhibit a vagina, it is usually shorter than normal. They lack an uterus, and have testes situated intra-abdominally or in the inguinal canals. Spermatogenesis in these patients resembles that seen in the cryptorchid. Sertoli cells are very well developed and filled with lipid (Jones and Scott, 1958). Cells of Leydig appear to be present in larger than normal numbers (French, et al. 1966).

Jost (1961) indicates that the degree of masculine development depends on the stage at which the foetal testes fail to function. If this happens early in development, the testes remain undescended and the individual so affected has a female body and female psychology as in the testicular feminization syndrome. If it happens later in development, a male body and male psychological sex appear with only some feminizing feature such as hypospadias.

The cause of the early breakdown of the testis is unknown. It is known, however, that testes in such cases are unable to use 17 a-hydroxyprogesterone for the formation of androgen and estrogen (Armstrong, 1955; Jacobs, et al. 1959).

Evans and Riley (1953) report that after puberty, although the homonal picture is variable in male pseudoherma-

phrodites, gonadotrophin and estrogen levels are normal or slightly increased. The fact that the estrogens disappear and the gonadotropin level rises after castration indicates that the testes produce estrogen. This could account for adult female development (Witschi and Mengert, 1942; Mishell, 1938).

Morris and Mahesh (1963) suggest that end organs in such individuals fail to respond to androgens or are androgen insensitive. Cases have been described in relatives. Spark (1964) reported a case of twin half cousins who suffered from this syndrome. Family incidence has also been reported by Grumbach and Barr (1958), Lubs, et al. (1959), Jacobs, et al. (1959), and others.

Armstrong (1955) reported that in patients with testicular feminization the total excretion of 17-ketosteroid is normal and estrogen excretion is within normal limits for a male. This suggests enhanced response of the target organs to estrogen. These patients require no treatment. However, since there is evidence that in a significant number, intra-abdominal testes become malignant, Morris and Mahesh (1963) are of the opinion that it is advisable to remove them after adolesence. Goldberg and Maxwell (1948) report that the breasts atrophy and menopausal symptoms develop if the testes are removed. Thus, such treatment should be followed by continued estrogen therapy.

Male Pseudohermaphroditism in Rats

Male pseudohermaphroditism in the rat was first described by D'Amour and Funk in 1941. The line was subsequently lost, but the anomaly was rediscovered by Stanley and Gumbreck in 1959 in the King-Holtzmann hybrid rats in their colony at the University of Oklahoma Medical Center. The King stock was originated in 1902 by Helen Dean King from Norway rats picked up on the streets of Philadelphia. This colony was dispersed in 1946. The King animals were obtained from a pet fancier in New England in 1954. The Holtzmann animals used in the cross had been inbred for about 45 generations at the time they were obtained from the Holtzmann Rat Company, Madison, Wisconsin.

King-Holtzmann animals were started by crossing King males with Holtzmann females. They have been inbred for about 22 generations. According to Stanley and Gumbreck (1964), the defect of pseudohermaphroditism is apparently transmitted through the mother to one-half her sons. The gene producing the defect is thought to be on the X chromosome, and is also transmitted to one-half of the female offspring, but it has no apparent effect in this sex. A sample of 210 litters from mother carriers comprising a total of 1364 animals contains 620 females, 372 males and 372 male pseudohermaphrodites. The ratio between normal females, normal males, and male pseudohermaphrodites is 2:1:1 which

is the classical ratio produced by a sex-linked gene.

The reproductive tract in these animals, except for a pair of small perineal testes, is absent; no scrotum is present, nipples appear along the milk line, and the vagina (seldom over 2 mm in depth) ends blindly.

Rats so affected are sterile. According to Allison, et al. (1965) the karyotype of these defective rats is characteristic of the male and the nuclei of their somatic cells are negative for sex chromatin bodies. Prior to 17 days in utero, the reproductive tract of these animals appear normal. It begins to disappear on the 18th day of intrauterine development and is absent by the end of day 19 (Allison, 1966). Between 10% to 50% of the older pseudohermaphrodites, develop Sertoli cell tumors in one or both of their testes. Some of these grow quite large, weighing up to 10 grams (Easley, 1968).

The body weight of adult male pseudohermaphrodite rats is intermediate between that of normal males and females of comparable age. Adrenal and pituitary glands of these animals are heavier than those of normal animals, whereas their testes and kidneys are smaller.

Testosterone administration to adult pseudohermaphrodites has no effect on their kidneys and the urinary papilla does not increase in size or become hyperemic (Stanley, et al. 1966).

Stanley, et al. (1967) report plasma steroid determinations in male pseudohermaphrodite rats. The titer of

estrone, estriol, and estrodiol-17β in these animals is approximately double, whereas dehydroepiandrosterone is about 6 times greater than in normal male littermates. Androsterone values are the same in both. Plasma androsterone and etiocholanolone are between 2 and 3 times greater in pseudohermaphrodites than in normal males.

Such studies in man and in rats accentuate the many similarities between male pseudohermaphroditism in rats and testicular feminization in man. The similarities are

1. Both show a single X and Y chromosome.
2. Somatic cells in both are chromatin negative.
3. The defect appears to be sex linked in both.
4. The reproductive tract, with the exception of a pair of small perineal testes, is missing in both.
5. The histological picture of the testes in testicular feminization closely resemblesthat occuring in the pseudohermaphrodite rat.
6. The somatic cells of both are insensitive to androgen.
7. Both have normal or slightly increased pituitary and plasma gonadotropins and abnormally high urinary 17-ketosteroids.

Working Hypothesis: Supporting Evidence for Methodological Approach

Sterility in the pseudohermaphrodite is rather

obviously reflected in the histologic picture of the testes. In most of the seminiferous tubules, the germinal layer is one or two cells thick. The cell types consist of spermatogonia, Sertoli cells, and usually some primary spermatocytes. In contrast to the germinal line, there appears to be a marked hyperplasia of the interstitial cells of Leydig. Turner has shown that LH functions to stimulate the cells of Leydig to produce androgen. Since this is true, LH levels in these animals may not be abnormally low. This has been confirmed by Easley (1968). He found that the gonadotropin content (FSH and LH) of the pituitary and plasma of male pseudohermaphrodite rats is greater than that of normal male or female rats. Perhaps the testes in pseudohermaphrodite rats possess normal spermatogenic potential but fail to produce mature sperm either because the gonads are unable to respond to circulating gonadotropins or because the testes never descend into a scrotum.

If testes from such rats could be transplanted to normal male castrates of the same species in such a manner that the transplant would grow, and if such a transplant does not produce late germinal elements, it would appear possible that lack of late stages of spermatogenesis in the testes of pseudohermaphrodite rats is a manifestation of an inhibitor or an altered enzymatic process in the testes. Alteration of such an enzymatic process could block spermatogenesis even with a normal FSH and LH supply.

As mentioned previously, a possible defect in the peripheral response to androgen has been suggested by many investigators as a cause for failure of the Mullerian and Wolffian derivatives to continue to develop in testicular feminization in man.

Somatic tissues of sterile male pseudohermaphrodite rats also appear to be insensitive to androgen (Stanley, et al. 1969, Chan, et al. 1969 and Bardin, et al. 1969). An attempt has been made to confirm this finding by determining the citrate content of urine from maternal carriers before and after injection with testosterone propionate. Since testosterone has an anabolic effect (Brown and Samuels, 1956; Kenyon, et al. 1940; Knowlton, et al. 1942; Landau, et al. 1950) and the citrate titer in urine reflects the rate of metabolism of the somatic cells, the urinary citrate level of a normal rat will decrease after injecting a sufficient amount of testosterone propionate (Shorr, et al. 1948; French, et al. 1966). This effect of testosterone appears to be the result of an increased renal tubular reabsorption of citrates. French, et al. (1966) found that patients with the complete form of testicular feminization fail to show this anabolic response when treated with large doses of testosterone. This supports the hypothesis of somatic tissue refractoriness and indicates that the defect involves the anabolic as well as the virilizing effects of the hormone.

CHAPTER II

MATERIALS AND METHODS

Management of the Experimental Animals

A constant environment was maintained for all animals used. They were fed with Purina laboratory chow consisting f 23% protein, 4.5% fat, 6% fiber and 9% ash. Animals employed were nursling rats about 4 to 5-day old and young adult rats between 5 and 6 months of age. The nursling rats weighed from 8 to 11 grams, whereas the young adults averaged 310 grams.

Method of Transplantation

Determination of Sex

Phenotypic sex in the nursling rats was determined by comparing anogenital distance (Jackson, 1912). The anogenital distance in a normal male rat of 4 to 5 days of age is almost twice as long as it is in female siblings. The male pseudohermaphrodite rat has the same anogenital distance as the normal female (Gumbreck, 1967, personal communication).

Each nursling rat was then opened in order to observe the gonads. Ovaries of females are smaller than testes from males of the same size and are in the abdominal cavity in an undescended position.

Anesthesia

Nursling donors were decapitated and their testes removed at once for transplantation. Adults were anesthetized by intraperitoneal injection of sodium pentobarbitol. Four milligrams per 100 grams of body weight proves sufficient to anesthetize them for somewhat over an hour.

Surgical instruments used were sterilized in 95 per cent alcohol and rinsed in sterile saline just before use.

Castration

The skin of each animal was cleansed with 95% ethyl alcohol in the region of the intended incision. With adult rats clippers first were used to remove hair from this area.

Castration was performed at the time transplantation was carried out. After the skin was prepared as described above, the gonads were forced into the abdominal cavity by light pressure on the scrotum. In male pseudohermaphrodite rats, this was accomplished by exerting pressure in the inguinal region. The testes were then removed through a small incision in the caudo-ventral abdominal wall. Testicular vessels were tied off before the testes were

removed. Following the operation, the peritoneum and muscle were sutured; the rest of the incision was closed with skin clips.

Transplantation

Donors were 4 to 5-day old nurslings. Hosts were 120-day old young adults. The following diagram summarizes the type of transplantations carried out.

	Nurslings		Castrated Young Adults
1.	Normal	to	Normal
2.	Normal	to	Pseudohermaphrodite
3.	Pseudohermaphrodite	to	Normal
4.	Pseudohermaphrodite	to	Pseudohermaphrodite

The following technique was used for inserting the transplants into the eyes. The hosts were thoroughly anesthetized with sodium pentobarbitol. After castration, one eye of such recipient was cleansed with a week solution of benzalkonium. Excesses of this solution were removed from the eye with sterile cotton. The eye to be operated was pushed out of the socket by gentle pressure above and below the eye ball. The conjunctiva was then grasped with a number 5 watch maker's tweezer, and an incision about 2 mm in length was made in the dorsal part of the cornea, parallel but 1 mm anterior to the corneoscleral junction. Testes of donors were then inserted into the anterior chamber. Because of the small size of testes from the nursling donors, it was

possible to insert the entire testis into the anterior chamber. None of the testes were scarified. No sutures were necessary. With aseptic precautions taken, the incidence of infection was low. During the operation, a small amount of aqueous humor was lost but it was rapidly replaced as healing began. It was found advantageous to keep the animal anesthetized for at least 30 minutes subsequent to the operation in order to permit the clotted aqueous humor to close the incision thoroughly.

Histologic Studies

At the appointed times outlined in the results, these animals were sacrificed. The transplanted testis from each animal was removed and placed in Bouin's fixative for over 18 hours. They were subsequently imbedded in paraffin, sectioned at six microns, and stained with Delafield's hematoxylin.

Determination of Citrate

Preparation of Experimental Animals

Urine for citric acid determination was collected by placing experimental and control rats in metabolism cages equipped for this purpose. Each cage housed a group of five animals of the same type. There were four groups of animals, namely, female carriers of the gene for male pseudoherma-

phroditism (maternal carriers), male pseudohermaphrodites, normal males and normal female siblings of the female carriers. The metabolism cages were cleansed thoroughly each day.

During an initial 10 day period, each rat was given a subcutaneous injection of 0.25 ml of pure sesame oil at 8:30 a.m. every morning. Urine pooled from each group of rats was collected and analyzed for citrate at the same time each morning. Subsequently, each rat was given a subcutaneous dose of 1 mg of testosterone propionate diluted in 0.25 ml of pure sesame oil at 8:30 a.m. every morning for another period of ten days. Urine was collected and analyzed in the same manner as before. After this, the dose was increased to 4 mg of testosterone and the citrate was analyzed daily for 10 more days.

Chemical Determination

The determination of citric acid by its conversion into pentabromoacetone (Stahre, 1895) has been greatly improved, notably by Pucher, Sherman and Vickery (1936), who introduced a sensitive colorimetric procedure. Recent modifications have further increased the reliability and sensitivity of the method. The method used was the modified method of Kraus (1965).

Reagents Used

1. Vanadic acid: 20 g of vanadium pentoxide are dissolved in 1 L of 50% sulphuric acid (V/V).

2. Brominating reagent: 40 g of potassium bromide and 11 g of potassium bromate are dissolved in 1 L of distilled water.

3. Ferrous sulfate, heptahydrate, 20% (W/V) solution: This solution can be kept only 10 minutes, and therefore it must be prepared immediately before use.

4. Color reagent: This contains 2 g of sodium sulfide (dihydrate) dissolved in 100 ml of the methanol-ethylene glycol mixture (35:65).

5. Citric acid stock solution, 10^{-3}M: Approximately 0.21 g of crystalline citric acid is dissolved in 1 L of water.

6. Chloroform, reagent grade.

7. Sodium sulfate, anhydrous.

Procedure

In order to make spectrophotometric determination of the amount of citrate in any solution such as urine, a standard curve must be constructed (Table 1 and Fig. 1). Each value in Table 1 is the average of 3 determinations. They are optical density readings taken on a Coleman Junior spectrophotometer of graded amounts of solutions each of

TABLE 1

AVERAGE DAILY MEASUREMENTS OF OPTICAL DENSITY OF DIFFERENT CONCENTRATIONS OF PURE CITRIC ACID AT 450μ

.3μM	.5μM	1μM	1.5μM	2μM	3μM	3.5μM	4μM
*.083	.121	.260	.379	.532	.780	.905	1.010
.088	.125	.271	.382	.529	.752	.930	1.013
.095	.123	.254	.380	.533	.777	.940	.980
.081	.129	.259	.375	.534	.782	.933	.950
.065	.124	.260	.379	.532	.784	.932	.990
.086	.121	.255	.379	.524	.755	.907	.976
.099	.120	.249	.377	.538	.775	.943	.895
.089	.120	.258	.383	.537	.783	.940	1.010
.081	.124	.260	.380	.533	.780	.937	.883
.071	.123	.256	.379	.533	.783	.920	.993
ave. .084	.123	.258	.379	.533	.777	.929	.970
s.d. ±.01	±.003	±.006	±.002	±.004	±.010	±.013	±.373

*Average of 3 optical density readings.

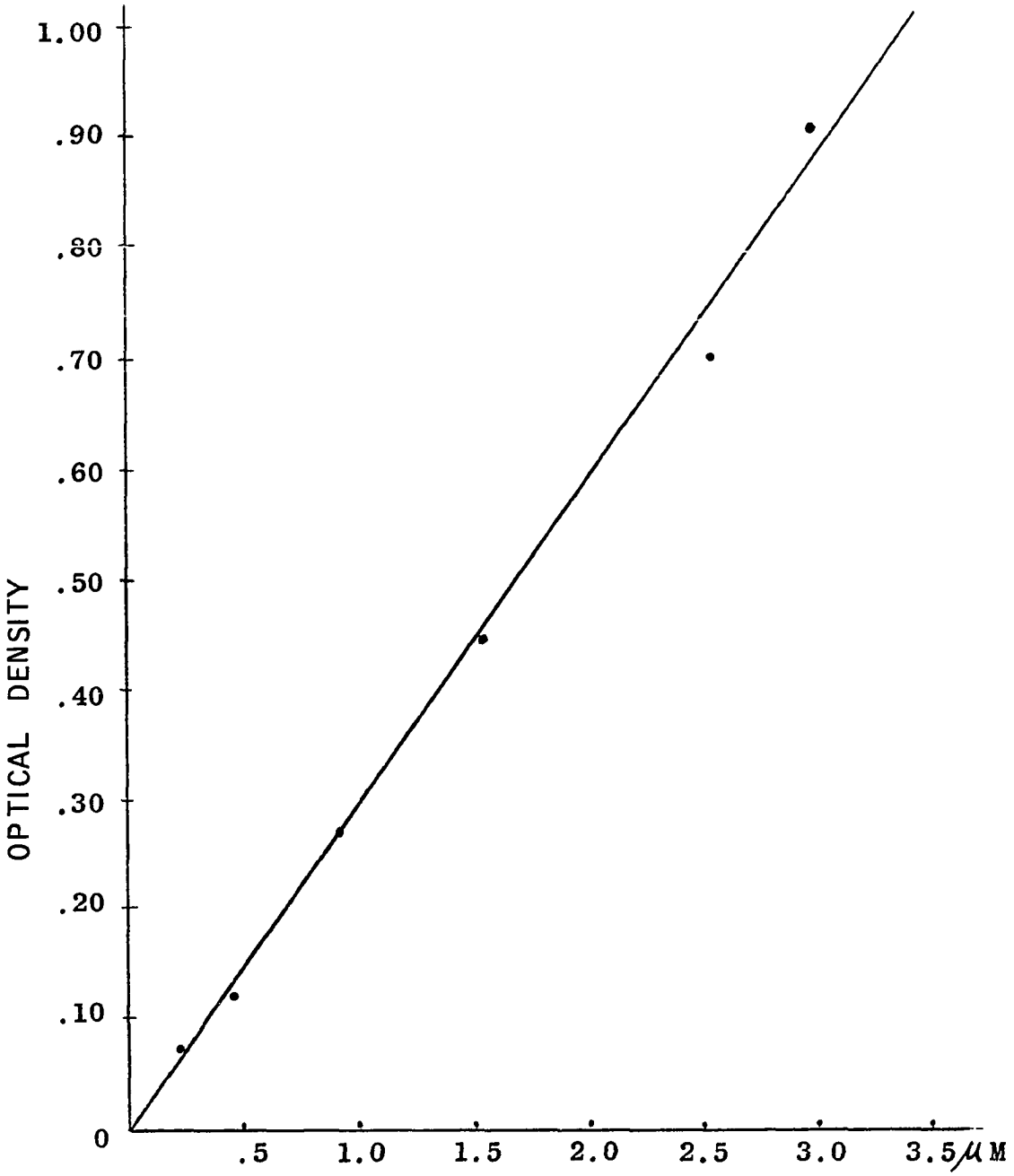

Figure 1. Standard curve obtained by plotting concentration of citrate against optical density at 450μ in the Coleman Junior spectrophotometer with 5 ml cuvettes.

which contained a known quantity of citric acid. The values obtained in this manner, represented in graphic form, make up the standard curve (Fig. 1). The citrate in each sample of urine was extracted and optical density readings made of such extracts. These readings were then fit into the standard curve in order to determine the amount of citrate in the urine of each rat studied.

In a test tube with ground glass stopper 2 ml of the sample which contained a specific dilution of citrate were mixed with 1 ml of vanadic acid and allowed to stand for 30 minutes at room temperature. Undue reduction of vanadic acid, occuring in the presence of high amounts of some glycides and hydroxy acid, is recognized by the color veering toward green. In such a case, additional amounts of vanadic acid (about 0.5 ml) were added until the color of the solution remained orange yellow during the whole period of standing. Subsequently, 1 ml of brominating reagent was added and the solution allowed to stand for another 30 minutes. After this, ferrous sulphate was added dropwise until the color turned emerald green and no bromine vapors were detectable. The solution was then extracted by shaking with 5 ml of chloroform for 1-2 minutes. After separation of the layers, the upper aqueous one was removed with a water suction apparatus and discarded. The chloroform was desiccated with sufficient amounts of anhydrous sodium sulfate and 3 ml were transferred into a dry test tube and

mixed with 2 ml of the color reagent. Three minutes after adding the color reagent, the optic density of this solution, compared to a blank, was measured in a Coleman Junior spectrophotometer at 450μ. The blank is prepared in the manner described above by using 2 ml water instead of stock citric acid solution.

The procedure employed for the estimation of urinary citrate is the same as that for determining the standard curve with the addition of a few steps. The added steps taken in the estimation of urinary citrate are

1. Initially, the urine is filtered.

2. The dilution of the urine is in 1 to 1 ratio with distilled water.

3. Since rat urine is highly pigmented (dark brown in color), it is impossible to get an orange yellow color during the standing period of oxidation by vanadic acid regardless of the amount of acid added. Therefore, in this experiment, the maximum amount of acid added was 1.5 ml.

4. Because of the turbidity of rat urine, centrifugal force is used to separate the aqueous layer from the chloroform layer. This was done after extraction with 5 ml of chloroform. If centrifuged at about 2,000 rpm for 3 minutes, the two layers separate clearly and the precipitates are forced to the bottom of the test tube.

CHAPTER III

EXPERIMENTAL RESULTS

Section One: Testicular Transplantation

Almost 100 per cent of the transplants were viable at the time of recovery (Table 2).

TABLE 2

Group	1	2	3	4
No. of transplants made	25	15	20	15
No. of transplants recovered	24	15	20	15
No. of days in host	75	75	75	75

Number of transplants recovered and the time that each recovered transplant was permitted to grow.

Each transplant recovered in all groups contained seminiferous tubules. The relative amount of germinal elements in the tubules varied from transplant to transplant

and from different areas of the same transplant. Therefore, the amount of the germinal elements present probably depends on which part of the transplant manages to obtain good vascularity while it is becoming established. It also may depend on how the transplant is oriented in the eye.

Histologic Picture in Normal Rat Testes

Spermatogonia, interspersed occasionally with Sertoli cells, are present just inside the basement membrane throughout all seminiferous tubules in normal rats age 75 days post partum (Figs. 3, 4, 5 and 6, Appendix). Many of the spermatogonia are undergoing mitosis. Other products of spermatogenesis such as primary spermatocytes, secondary spermatocytes, spermatids, and mature sperm appear in waves along the tubules. This is evidenced by the observation that along any given segment of a tubule, most or all of the cells central to the spermatogonia and Sertoli cells are of one kind. For example, in one section of a tubule most or all the cells present may be primary spermatocytes (Fig. 5, Appendix), whereas another section nearby may exhibit spermatids in a late stage of development (Fig. 6, Appendix). Mature spermatozoa are numerous in the tubules (Fig. 3, Appendix). Whenever a section of a seminiferous tubule exhibits a relatively large amount of mature sperm, there are usually several primary spermatocytes in the same segment. These spermatocytes are undergoing mitosis. Thus

it seems that a new generation of cells is being provided in order that the next wave of mature sperm may be produced.

Testes from 75 day-old Male Pseudohermaphroditic Rat

There are from 1 to 3 layers of cells present in the seminiferous tubules of 75 day old male pseudohermaphrodite rats. These cells are mostly spermatogonia and Sertoli cells. There are more spermatogonia than Sertoli cells (Figs. 7, 8 and 9, Appendix). An occasional primary spermatocyte is seen along the innermost layer of cells. These usually project into the lumen, which is relatively large and devoid of tissue elements. Some primary spermatocytes appear to be in early prophase, others exhibit necrotic nuclei. The interstitial cells are more abundant around the tubules at the periphery of the testis than at the center. They appear to be more numerous than in testes from normal rats.

Transplanted Normal Testes Recovered from Adult Normal Hosts

Some seminiferous tubules in the transplant exhibit only one layer of cells inside the basement membrane. These cells, most of which are Sertoli cells, are separated by wide spaces. The central portion of the tubules contains some lightly staining substance interspersed with a sparse network of more heavily stained material. An occasional

spermatogonium can be seen among the Sertoli cells. Some of these spermatogonia exhibit necrotic nuclei. The interstitial spaces contain nests of interstitial cells of Leydig lying in loose connective tissue. In many regions, this connective tissue is infiltrated with varying numbers of lymphocytes.

In other regions of the transplant, some of the seminiferous tubules resemble those from a 75 day-old normal animal (Fig. 10, Appendix). Out of 10 representative slides from 10 different transplanted testes, 17% of the tubules show mature sperm. Cells representative of all stages of spermatogenesis can be found in these tubules. These regions exhibit abundant interstitial cells with practically no lymphocytic infiltration.

Transplanted Normal Testes Recovered from Adult Male Pseudohermaphrodite Rats

The histologic picture is similar to that seen in the normal to normal transplant. In the same transplant, one region contains widely spaced seminiferous tubules in which only one layer of cells is seen. In other tubules all stages of spermatogenesis can be found (Fig. 11, Appendix). All 15 transplants recovered contain seminiferous tubules which exhibit germinal elements through the spermatid stage. Tubules in 6 of the 15 contain mature sperm.

Transplanted Testes of Male Pseudohermaphrodite Rats Recovered from Normal Adult Hosts

Some seminiferous tubules in these transplants contain only one to two layers of cells, most of which are Sertoli cells. The lumen of these tubules contains some lightly staining substance interspersed with a sparse network of more heavily stained material. Other tubules exhibit from 2 to 5 layers of cells. These consist of Sertoli cells, spermatogonia, and primary spermatocytes. Some of the spermatogonia and primary spermatocytes are undergoing division. A few tubules contain varying amounts of germinal elements up to and including late spermatids. Some of the spermatids appear abnormal in that two or more spermatid nuclei are surrounded by one mass of cytoplasm (Fig. 12, Appendix). It appears that either the primary and or secondary spermatocytes divide, but do not separate. All transplants recovered exhibit abundant interstitial cells of Leydig.

Transplanted Testes of Male Pseudohermaphroditic Rats Recovered from Adult Male Pseudohermaphroditic Rats

The histologic picture resembles that seen in transplants of the pseudohermaphrodite to normal group. Not only are spermatogonia present in what appears to be an approximately normal ratio to Sertoli cells, but primary spermatocytes are fairly abundant. Some of the primary spermatocytes

and spermatogonia are undergoing division. Quite a few tubules in all 15 transplants recovered in this series exhibit late spermatids. Some of these spermatids appear ready to differentiate into spermatozoa. Some, however, are multinucleate. Cells of Leydig are numerous in these transplants (Fig. 13, Appendix).

Seminal Vesicles

By 75 days following castration, seminal vesicles in otherwise normal castrated rats appear as vestiges. Their average weight is 35 mg. (Table 3). Male pseudohermaphrodite rats never exhibit seminal vesicles.

Seventy-five days after a castrated normal adult rat has received a transplant of a testis from a newborn normal rat, the seminal vesicles of the host are seen to be about ½ normal size. The average weight of these seminal vesicles is 562 mg. By comparison, the average weight of the seminal vesicles in castrated normal hosts which have received a testis from a newborn male pseudohermaphrodite donor is 486 mg.

TABLE 3

SEMINAL VESICLE WEIGHTS IN MG FROM ADULTS OF ALL GROUPS STUDIED (FIFTEEN ANIMALS IN EACH GROUP)

Normal Males	Castrated Normal Males*	Castrated Normal Male hosts carrying normal nursling testes	Castrated Normal Male hosts carrying male pseudoherma-phrodite testes
602	41	680	400
714	35	610	312
721	30	620	450
761	37	450	460
1390	33	420	660
1037	25	413	447
967	33	750	468
754	32	886	450
1084	29	820	354
1488	35	410	410
1372	38	412	490
1237	60	375	502
1104	52	446	670
1660	36	580	432
886	37	560	780
ave. 1067	35	562	486
s.d. ±247	±19	±158	±124

*Seminal vesicles weighed 75 days after castration.

The information set forth in Table 4 is a summary of the degree of specialization reached by germinal elements in each type of transplants. It also indicates the effect each type of transplant had on the seminal vesicles of the host.

TABLE 4

Group	Stage of spermatogenesis reached	Stage of seminal vesicle
1 ♂ → ♂	Mature sperm	Sustained
2 ♂ → ♀	Mature sperm	No seminal vesicle
3 ♀ → ♂	Spermatid	Sustained
4 ♀ → ♀	Spermatid	No seminal vesicle

Summary of the results of transplantation.

Section Two: Effect of Urinary Citrate Following Administration of Testosterone

In rats, the average daily citrate level, measured over a 10-day period, in the urine of normal females, fertile females carrying the gene for pseudohermaphrodites, normal males, and pseudohermaphrodites is 3.31, 3.43, 2.58, and 3.36μM per ml respectively. The first 3 of these values are essentially the same. There is, however, a significant difference between the values obtained for any one of these 3 and that for normal males (Tables 5-9 and Fig. 2).

The average daily citrate level, after 1 mg of testosterone propionate is injected daily for 10 days into each of these animals, decreases less in the pseudohermaphrodites than in the other 3 groups. After 4 mg of testosterone daily for 10 more days, the urinary citrate levels in these same rats, drops significantly in all but the pseudohermaphrodites (Tables 5-9 and Fig. 2). The drop is greatest on the third day after injection of 4 mg of testosterone propionate.

TABLE 5

μ MOLES/ML OF CITRATE IN POOLED URINE FROM 5 MATERNAL CARRIERS

Measurements taken over a 10 day period prior to injection	Measurements taken during 10 day period of daily injection of 1 mg of testosterone	Measurements taken during 10 day period of daily injection of 4 mg of testosterone
3.50	2.85	2.75
4.00	2.83	2.55
2.80	3.30	0.91
3.12	3.04	1.77
3.64	2.78	0.75
3.65	3.10	1.87
3.81	2.00	2.35
3.35	2.20	2.70
3.49	2.50	2.80
2.89	2.60	2.50
ave. 3.43	2.72	2.1
s.d. ±0.29	±0.31	±0.58

TABLE 6

μ MOLES/ML OF CITRATE IN POOLED URINE FROM 5 NORMAL FEMALES

Measurements taken over a 10 day period prior to injection	Measurements taken during 10 day period of daily injection of 1 mg of testosterone	Measurements taken during 10 day period of daily injection of 4 mg of testosterone
3.61	2.20	2.17
4.01	2.30	2.77
3.15	2.76	1.00
2.90	2.58	2.05
3.51	2.33	1.92
3.43	2.35	2.30
3.51	2.90	2.55
3.00	2.20	2.17
3.13	1.85	2.30
2.89	2.20	2.35
ave. 3.31	2.35	2.16
s.d. $\pm$0.28	$\pm$0.24	$\pm$0.47

TABLE 7

μ MOLES/ML OF CITRATE IN POOLED URINE FROM 5 NORMAL MALES

Measurements taken over a 10 day period prior to injection	Measurements taken during 10 day period of daily injection of 1 mg of testosterone	Measurements taken during 10 day period of daily injection of 4 mg of testosterone
2.39	2.30	1.19
2.84	2.15	1.80
2.50	2.18	0.77
2.66	2.30	0.58
2.26	1.02	1.56
3.25	2.25	2.14
2.76	2.45	2.30
2.50	2.55	2.50
2.32	2.05	2.00
2.32	2.35	1.85
ave. 2.58	2.16	1.77
s.d. $\pm$0.18	$\pm$0.16	$\pm$0.50

TABLE 8

μ MOLES/ML OF CITRATE IN POOLED URINE FROM 5 MALE PSEUDOHERMAPHRODITE RATS

Measurements taken over a 10 day period prior to injection	Measurements taken during 10 day period of daily injection of 1 mg of testosterone	Measurements taken during 10 day period of daily injection of 4 mg of testosterone
3.40	3.10	3.10
3.85	3.00	3.10
3.00	3.38	2.70
3.00	3.30	2.80
3.62	3.33	2.95
3.57	2.90	3.05
3.75	3.23	3.30
3.00	3.00	3.10
3.45	3.25	3.15
3.00	3.30	3.05
ave. 3.36	3.12	3.03
s.d. $\pm$0.26	$\pm$0.17	$\pm$0.16

TABLE 9

μ MOLES/ML OF CITRATE IN POOLED URINE FROM 5 RATS OF EACH TYPE

	av. of measurements taken over a 10 day period prior to injection	av. of measurements taken during 10 day period of daily injection of 4 mg testosterone propionate	av. change
P ♀	3.43	2.1	1.33 ($p<0.001$)
N ♀	3.31	2.16	1.15 ($p<0.001$)
N ♂	2.58	1.77	0.81 ($p=0.0018$)
P ♂	3.36	3.03	0.33 ($p=0.150$)

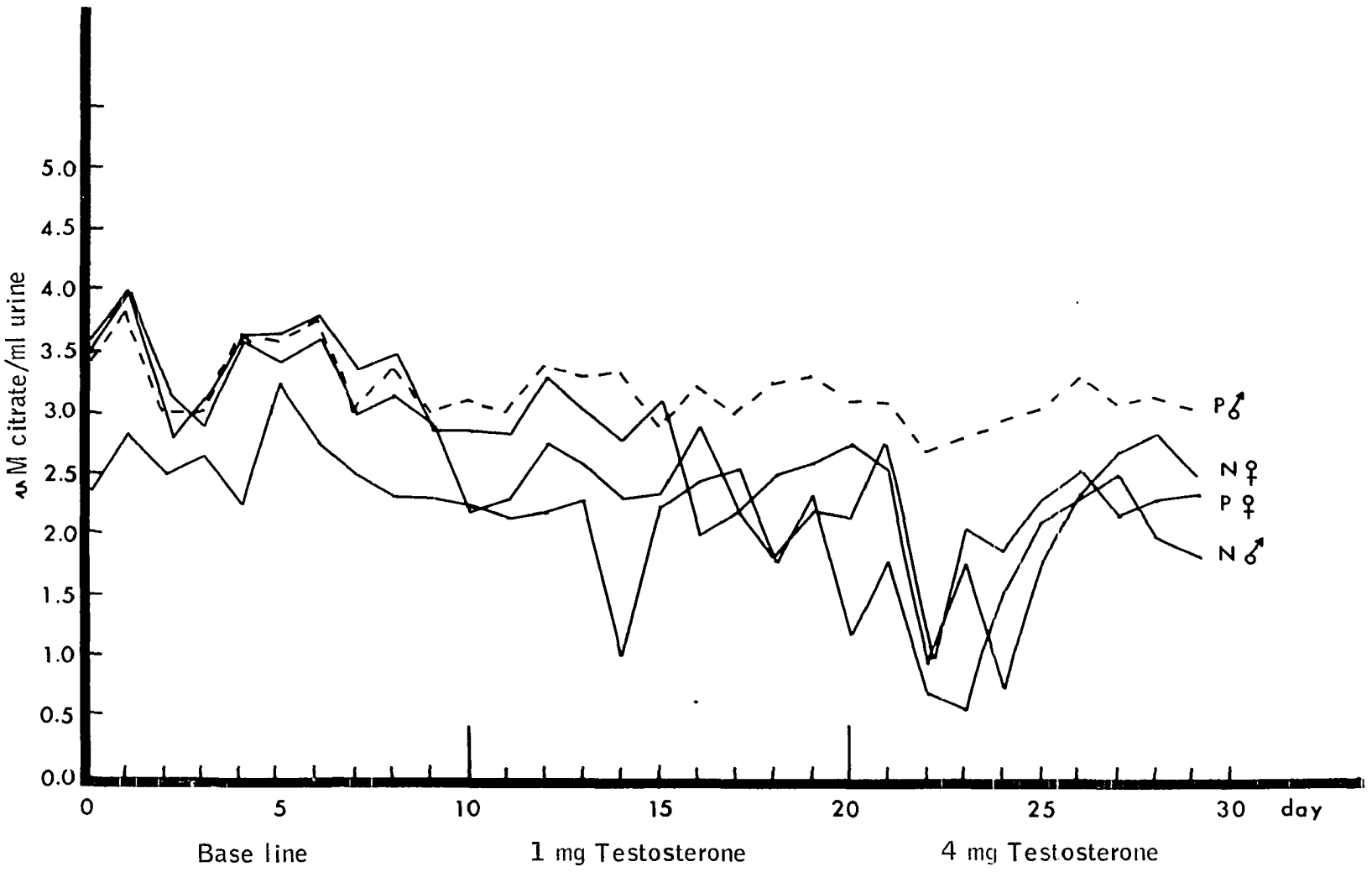

Figure 2. Summary of the results concerning the citrate determination.

An increase in circulating testosterone normally results in an increase in body weight. All animals except the male pseudohermaphrodites exhibit this increase in weight following the administration of testosterone propionate (Table 10).

Normally, seminiferous tubules of male pseudohermaphrodite rats exhibit no spermatogenic elements beyond the primary spermatocyte (Figs. 7, 8 and 9, Appendix). After these animals have been treated with testosterone propionate, spermatogenesis progresses through the spermatid stage. Many such spermatids are abnormal in that they contain more than one nucleus (Fig. 14, Appendix). The number of nuclei in the spermatids appears to be either 1, 2, or 4. Some of the secondary spermatocytes in these tubules contain two nucleoli (Figs. 15 and 16, Appendix).

TABLE 10

AVERAGE DAILY BODY WEIGHT IN GRAMS OF 4 GROUPS OF ANIMALS BEFORE AND AFTER INJECTION

Average body weight of five rats taken over a 10 day period prior to injection				Average body weight of five rats taken during 10 day period of daily injection of 4 mg of testosterone			
Maternal Carriers	Normal Females	Normal Males	Pseudo-hermaphrodites	Maternal Carriers	Normal Females	Normal Males	Pseudo-hermaphrodites
265	240	313	343	212	258	318	343
265	240	312	344	273	260	326	343
264	242	313	344	277	263	331	343
265	240	313	345	278	264	335	342
268	240	315	341	280	265	338	341
268	242	313	341	280	270	339	343
266	242	313	343	279	268	334	343
265	242	312	343	277	263	334	343
265	242	312	342	277	263	334	343
264	242	313	343	277	263	332	343
ave. 266	241	313	343	277	264	332	343
s.d. ±1.18	±0.78	±0.68	±.1				

CHAPTER IV

DISCUSSION

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

Appraisal of Methods

Testicular Transplantation

Testes transplanted from normal nursling rats into the anterior chamber of the eye of castrated young adult normal and male pseudohermaphrodite rats of the same strain can produce mature sperm. This tends to confirm the observation of Goodman (1934), Haterius, et al. (1935), and Turner (1938) that the anterior chamber of the eye is a very favorable site for the transplantation of immature testes. The technique is simple and the incidence of infection is low. Moreover, the anterior chamber, filled with fluid, presumably provides an environment in which the transplants are subjected to equalized pressure on all sides. It has been pointed out by Duke-Elder (1927a, b and c) that the intra-

ocular fluid is a dialyzate in equilibrium with the vascular fluid and that it is frequently renewed throughout the vascular tissues of the eye. Therefore, intra-ocular transplants are bathed by an isotonic fluid which provides them with the necessary anabolic materials and prevents the accumulation of catabolic products. The sufficiency of the aqueous humor in cases of non-vascularization is demonstrated by the work of Haterius, Schweizer and Charipper (1935) who observed that homoplastic transplants of the hypophysis of rabbits and guinea pigs into the anterior chamber of the eye retained their normal histological differentiation for as long as 4 months, even though no blood vessels penetrating the transplants were detected.

Results indicate that the temperature within the anterior chamber of the eye is low enough to permit complete differentiation of germinal elements in testicular transplants in addition to providing an excellent nutritive environment. Unlike some subcutaneous transplantation sites, the eye contains little fat, and evaporation of fluid from its surface aids in keeping the temperature down. Also, it is well insulated from the remainder of the body by the bones of the orbit. The scrotum, the eye and the subcutaneous connective tissue of the ear (Hill, 1941 and Chan, et al. 1969) seem to be locations in which temperature is maintained at a lower level than general body temperature.

Seminiferous tubules containing only one layer of

Sertoli-like cells are present in most of the transplants. Since degeneration of the germinal epithelium of the testis can be induced by many and varied stimuli, it is impossible to attribute this condition in the transplants to a particular factor.

Micro-determination of Citrate

Standard deviation values obtained in establishing the standard citric acid curve were low (between 0.5μM and 3.0μM, standard deviation was less than 2%). For this reason, results with the method used were highly reproducible. Before carrying out the procedure using rat urine, turbidities in the urine should be removed by filtration. The amount of vanadic acid to be added for complete oxidation is very critical. Precautions must be taken to insure the complete reduction of any free bromine which may adhere to the sides of the stopper. If insufficient ferrous sulfate is added, the excess of bromide may not be removed and this may give rise to high values. Centrifugal force sometimes is required for better separation after chloroform is added.

The color reagent of 2% solution of sodium sulfide is stable for about 30 minutes. The intensity of the yellow color will increase gradually with time. If exposed to strong light the color fades slowly.

Interpretation of Results

Testicular Transplantation

The male pseudohermaphrodite rat testes left in normal and pseudohermaphrodite hosts for 75 days exhibit spermatogenic elements including late spermatids. Presence of secondary spermatocytes and spermatids in these transplanted testes indicate that male pseudohermaphrodite rat testes are responsive to both normal amounts of gonadotropins produced by the hosts and the lower temperature provided by the anterior chamber of the eye. It is interesting to note that some of the spermatids in these transplants left for 75 days are not normal. Many are multinucleate. The multinucleate cells exhibit 2, or 4 nuclei surrounded by large, single, non-lobulated mass of cytoplasm (Figs. 12 and 13, Appendix). It may be postulated that somewhere in the spermatogenic progression, either the primary or secondary spermatocytes divide but do not separate. Secondary spermatocytes with 2 nuclei were found (Figs. 15 and 16, Appendix). Since spermatids in such transplants never become spermatozoa, it appears that male pseudohermaphrodite rat's testes will not produce sperm even in the presence of an optimum hormonal environment, proper temperature, and adequate nutrition environment.

Seminal vesicles of normal castrated host animals

carrying testes either from normal or pseudohermaphrodite donors are larger than they are in normal castrates. It appears from this that such transplants supply more androgens than normally are present in castrated hosts. Of course, androgens supplied by transplants from normal donors will not change the reproductive tract of pseudohermaphrodite hosts since they are completely degenerated before birth.

According to experimental results (Jost, 1948 and Witschi, 1939), the differentiation of the testis occurs before that of the ovary. It is postulated that the primordial testis secretes a substance that inhibits the differentiation of the ovary. In the absence of this hypothetical substance, ovarian development occurs. The genetic code for the production of such a substance could travel on the Y chromosome (Tepperman, 1966). Jost (1958) also indicates that the character of the internal and external genitalia is positively determined by the secretion or secretions of the testis. Thus, if the gonads are removed from the fetus of either sex, only female internal and external genital organs develop. Evidence given by Jost presents an explanation for the missing reproductive tract in the male pseudohermaphrodite. The male reproductive tract does not develop because the pseudohermaphrodite fetus is already insensitive to androgens secreted by the primordial testis. The female reproductive tract does not develop because the primordial testis inhibits development of the female anlage. Therefore, the result is

a complete absence of the reproductive tract of either male or female origin by the end of 19th day in utero.

Thus, some undetermined inherent abnormality must exist in the physiology of the developing and adult male pseudohermaphrodite rat which prevents the normal response of its somatic cells to testosterone. It is possible that in the male pseudohermaphrodite rat some enzyme or enzyme complex involved in testosterone-target organ activity is either missing or has been altered genetically. Alternatively, there may be a defect in their testes which prevents a normal response to gonadotropins.

Determination of Citrate

The results demonstrate that prior to injection of androgen, the daily citrate content of urine of normal females, maternal carriers, and male pseudohermaphrodite rats does not differ significantly. That of the normal male is significantly lower than that of the other three. After androgen is injected, the citrate drops in all but the male pseudohermaphrodite. The drop is greatest on the third day after beginning of daily injection of 4 mg of testosterone (Fig. 2). The sesame oil vehicle may release the hormone so slowly that its effect does not become sharply apparent until the third day. Thus, it appears that somatic tissues of pseudohermaphrodite rats are indeed insensitive to androgen. This conclusion has been confirmed recently by Bardin and

co-workers (1969). They found that testosterone did not increase hexobarbital metabolism or hepatic microsomal enzyme activity in pseudohermaphrodite rats although it maintained these activities in castrate rats at normal male levels. They also found that, in pseudohermaphrodites, hepatic steroid-4-reductase activity and adrenal weight were similar to those of normal females. In contrast to normal females, these features in pseudohermaphrodites were not decreased by testosterone. Finally, they were able to show that testosterone did not increase the weight of preputial glands in pseudohermaphrodites although there was a 50% increase of these glands in normal females (Stanley, et al. 1969)

The defect responsible for male pseudohermaphroditism in these animals appears to be effected by a mutant gene which is either a sex-linked recessive or male-limited autosomal dominant (Stanley and Gumbreck 1964). If it is a sex-linked recessive, it is not likely that androgen insensitivity exists in somatic tissues of maternal carriers of the gene, i.e. the male cannot transmit the defect and therefore, the mutant gene is on only one X chromosome. If somatic tissues of maternal carriers are insensitive to androgen, the gene is most likely dominant. It could be caused by a sex-limited gene; however, this is a rare phenomenon. According to Roberts (1959), it is impossible to distinguish between sex-linkage and complete sex limitation because the affected males do not reproduce, and so one cannot demonstrate

their inability to transmit the gene to their sons. Roberts indicates that sex linkage is so common and complete sex limitation is so rare that the alternative explanation cannot be disproved but, therefore, must be considered somewhat fanciful.

Normally, spermatogenesis in seminiferous tubules of the adult pseudohermaphrodite rat testes does not progress beyond the primary spermatocyte stage. After testosterone administration, testes from 10 to 180-day old male pseudohermaphrodite rats exhibit spermatogenic elements including late spermatids. Some of the spermatids are multinucleate (Figs. 14, 15 and 16, Appendix). However, following even large doses of testosterone, mature sperm are never found in these testes.

Bardin, et al. (1969) found that the testosterone level in the testicular vein of pseudohermaphrodite rats is lower than that in normal male rats of comparable age. It thus appears that the presence of spermatids in the testes of pseudohermaphrodite rats after androgen injection is due to a direct influence of testosterone on spermatogenesis. Temperature is not involved since the testes are still in the inguinal region. Clermont and Harvey (1965) have indicated that androgen has a direct supportive effect on the seminiferous tubules. The role of androgen in spermatogenic function of the testis remains obscure, but it may correlate in some manner with the protein anabolic action of testosterone.

Body weight increases in all androgen-treated rats except male pseudohermaphrodites. Thus, the maternal carriers respond to androgen whereas pseudohermaphrodite rats do not. This is further evidence that the defective gene is sex-linked recessive. The greatest increase in body weight is seen in normal males. It is possible that an increase in the amount of citrate and fructose in the seminal vesicles and prostate glands accounts for much of this (Shorr, et al. 1948 and Dorfman, 1959).

CHAPTER V

SUMMARY

Whole testes from normal nursling rats were transplanted to the anterior chamber of the eye of castrated young adult normal and pseudohermaphrodite male rats. These testes survive and produce all stages of spermatogenesis. Whole testes from nursling male pseudohermaphrodite rats also survive when transplanted to young normal and male pseudohermaphrodite hosts but do not produce germinal elements beyond the spermatid stage. Testes of male pseudohermaphrodite rats normally never exhibit spermatogenic stages later than primary spermatocytes.

Seminal vesicles in castrated normal young adults carrying testis transplanted from normal and male pseudohermaphrodite nursling donors remain significantly larger than in castrates not carrying transplants. Testes transplanted from normal nurslings into young adult castrated male pseudohermaphrodite rats have no effect on the secondary sex organs of the host.

In order to determine urinary citrate, normal

females, maternal carriers, normal males and male pseudohermaphrodites were given a daily injection of 1 mg of testosterone propionate subcutaneously for 10 days. This was followed by injections of 4 mg per day for 10 more days. Normally, the daily citrate content of urine of normal females, maternal carriers, and male pseudohermaphrodite rats is about the same. However, that of normal males is significantly lower than the other three. After androgen is injected, the citrate drops in all but the male pseudohermaphrodite.

The body weight is also increased in normal females, maternal carriers, and normal males when treated with androgen. But, the male pseudohermaphrodites maintain the same body weight throughout the test period.

Normally, spermatogenesis in testes of pseudohermaphrodite rats do not progress beyond the primary spermatocyte stage. Following treatment with testosterone propionate some tubules in these animals exhibit spermatids.

The preceding observations suggest the following conclusions concerning the male pseudohermaphrodite rat:

1. The defective gene is a sex-linked recessive.
2. Their tissues appear to be insensitive to androgen.
3. There are some disorders of androgen metabolism.
4. There could be formation of androgen inhibitors.
5. Pseudohermaphroditism in rats and testicular

feminization in man are similar in many details.

6. Temperature appears to be a factor in prevention of complete spermatogenesis in these animals; however, there are also indications that altered enzymatic factors prevent circulating androgens from having a normal effect on spermatogenesis.

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APPENDIX

LEGEND FOR FIGURES

The following figures are un-retouched photomicrographs taken with a camera using $2\frac{1}{4}$ by $3\frac{1}{4}$ inch contrast process panchromatic sheet film, with a Leitz microscope.

LIST OF ABBREVIATIONS

cl	cells of Leydig (interstitial cells)
ds	dividing spermatogonia
ps	primary spermatocytes
ss	secondary spermatocytes
sg	spermatogonia
st	spermatids
sz	spermatozoa
sc	Sertoli cells
uss	undivided secondary spermatocytes

Figure 3. Seminiferous tubules from normal adult rats. X100.

Figure 4. Seminiferous tubules from normal adult rats. Most cells inside the spermatogonia (sg) and Sertoli cells (sc) are secondary spermatocytes (ss). Mature sperm (sz) appear near the lumen of the tubules. X125.

Figure 5. A section of a tubule from normal adult rat showing primary spermatocytes (ps). X125.

Figure 6. A section of a tubule from normal adult rat showing spermatids (st) in a late stage development. X125.

PLATE I

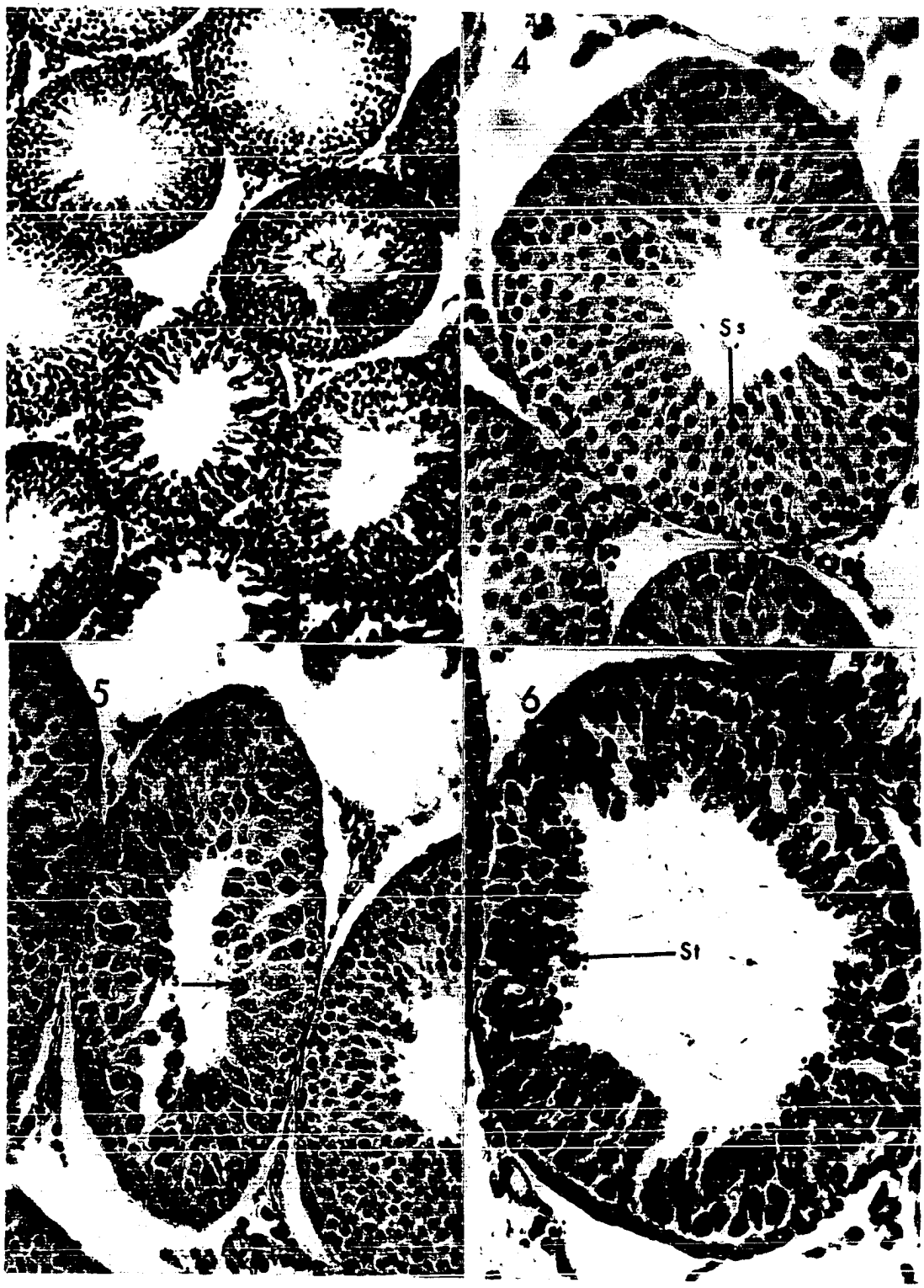

Figure 7. Testis from 180 day old male pseudohermaphrodite rat. X20.

Figure 8. Higher magnification of same testis as seen in Figure 5 showing tubules containing spermatogonia and Sertoli cells. X200.

Figure 9. Testis from 75 day old male pseudohermaphrodite rat showing spermatogonia, Sertoli cells, and primary spermatocytes. Interstitial cells appear more numerous than in testes from normal 75 day animals. X125.

PLATE II

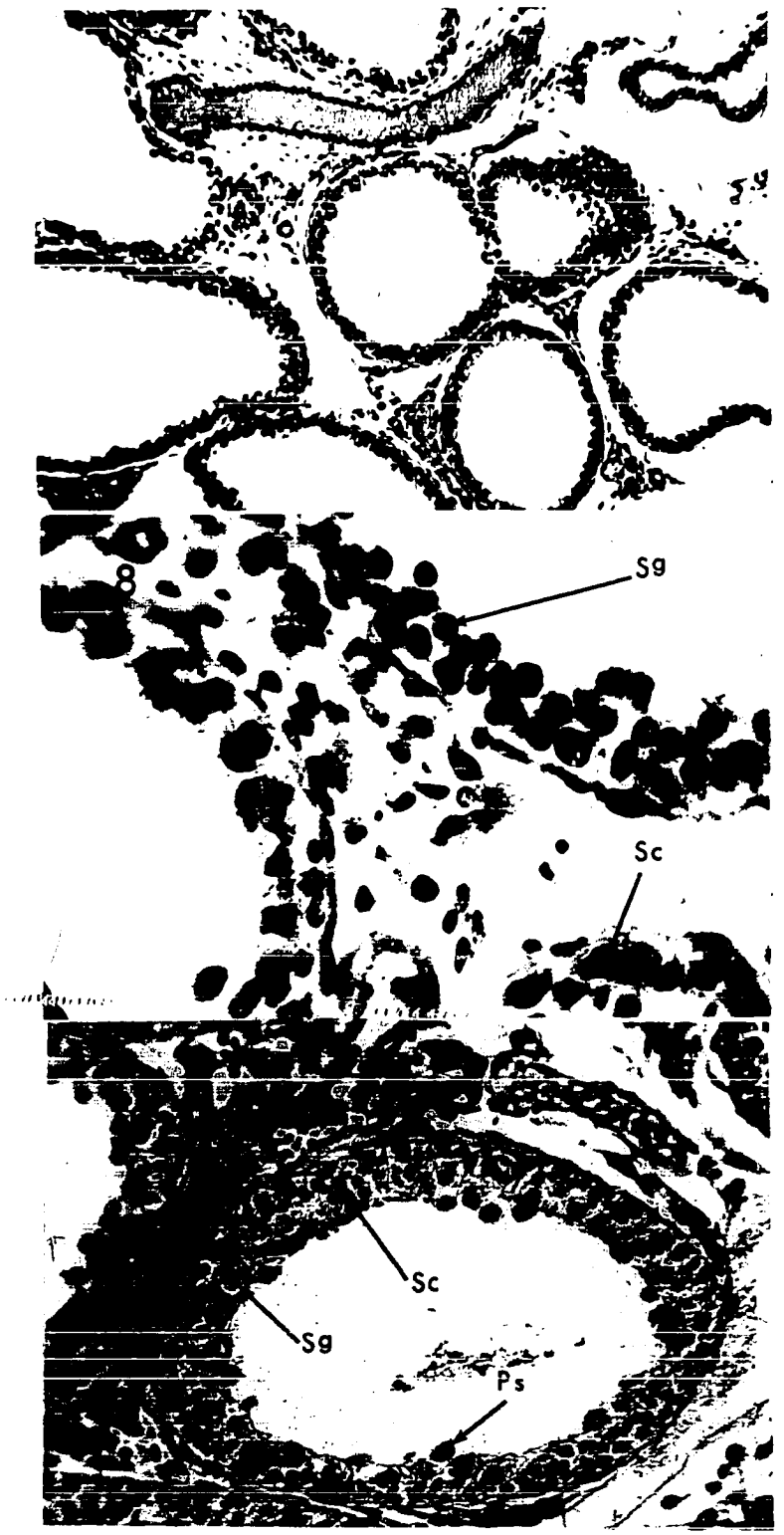

Figure 10. A 75 day transplanted testis from normal male to normal male rat showing complete spermatogenesis. X125.

Figure 11. A 75 day transplanted testis from normal male to male pseudohermaphrodite rat showing complete spermatogenesis. X125.

Figure 12. A 75 day transplanted testis from male pseudohermaphrodite to normal male rat showing spermatogenic stage up to late spermatids. X125.

Figure 13. A 75 day transplanted testis from male pseudohermaphrodite to male pseudohermaphrodite rat showing late spermatids. X125.

PLATE III

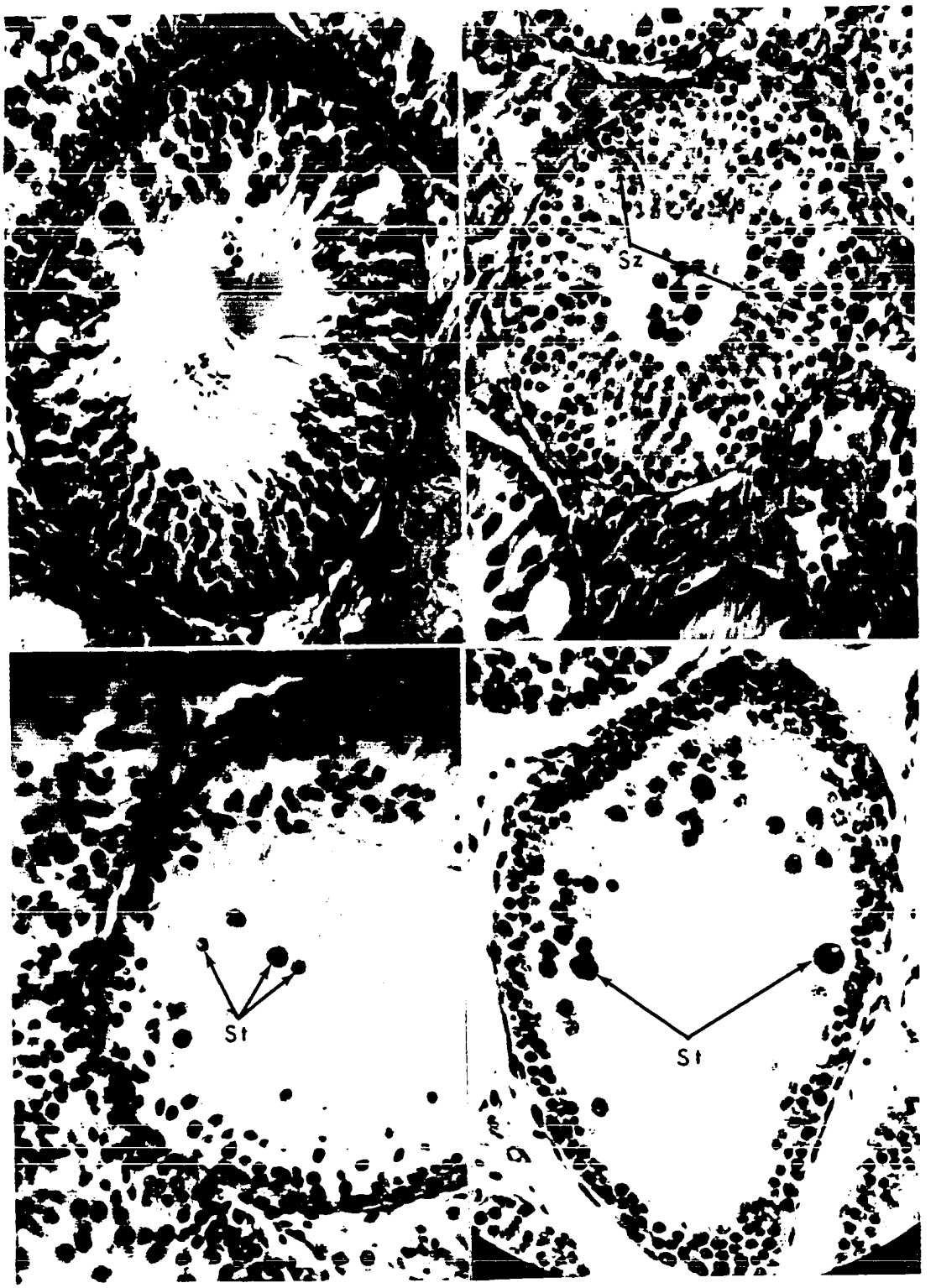

Figure 14. Testis from 180 day old male pseudohermaphrodite rat, after daily injection of 4 mg of testosterone propionate for a period of 10 days, showing late spermatids and undivided spermatids (ust). X125.

Figure 15. Same testis as seen in Figure 12 with higher magnification showing the undivided spermatids and an undivided secondary spermatocytes (uss) with 2 resting nuclei. X1250.

Figure 16. Same testis as seen in Figure 12 showing the secondary spermatocyte with double nuclei. X1250.

PLATE IV