EFFECT OF 5-THIO-D-GLUCOSE ON REPRODUCTION IN MALE MICE: HISTOLOGICAL, HISTOCHEMICAL AND FERTILITY STUDIES

by

Kenneth Burgwin Fick

APPROVED BY:

SUPERVISOR OF THESIS Everett C. Simpson
Everett C. Simpson

THESIS COMMITTEE

Hubert W. Burden
Hubert W. Burden

Charles E. Bland
Charles E. Bland

Clifford B. Knight
Clifford B. Knight

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Joseph G. Boyette
Joseph G. Boyette

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DEDICATED TO MY PARENTS

WITHOUT THEIR "PERSUASION" I WOULD NEVER

HAVE FINISHED THE FIRST GRADE

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ABSTRACT

Kenneth Burgwin Fick. EFFECT OF 5-THIO-D-GLUCOSE ON REPRODUCTION IN MALE MICE: HISTOLOGICAL, HISTOCHEMICAL AND FERTILITY STUDIES. (Under the direction of Dr. Everett C. Simpson) Department of Biology, November, 1978.

Sexually mature male ICR strain mice were fed 5-thio-D-glucose daily by gavage and the effects of this treatment on testis and sex accessory gland histology, androgen biosynthesis, and breeding performance were determined. Histological observations of the testes showed multinucleated cells in peripheral seminiferous tubules as early as four weeks. Testes taken from animals treated for six weeks showed multinucleated cells in seminiferous tubules located throughout the entire organ. Histological examination of sex accessory glands from experimental animals were identical to controls. Androgen biosynthesis within the testis of treated animals was unaffected by the drug. Breeding performance was impaired in animals receiving the drug for six weeks, and normal fecundity was not regained until nine weeks following the end of drug administration.

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INTRODUCTION

Fertility control in mammals is a problem which has been widely investigated in past years. Much of the initial research in this area has been done with the female; but, in recent years, there has been increased emphasis on a method which safely and effectively inhibits or controls spermatogenesis. Techniques such as thermal inhibition, hormonal manipulation, and inhibition through use of various mineral elements and alkylating agents have been employed. Each of these methods brings about the desired control or inhibition of spermatogenesis, yet they produce undersirable side effects such as reduction of libido, acquisition of feminizing characteristics, impedance of normal cellular metabolism, and permanent sterility. Such side effects contraindicate the use of these techniques as safe and effective methods of controlling spermatogenesis (Johnson et al., 1970C).

Recently, investigators have tried altering the supply of carbohydrate, more specifically glucose, to the testis as a means of inhibiting spermatogenesis. According to Cavazos and Melampy (1954A), carbohydrate reserves are relatively low within the testicular tissue of animal species other than some reptiles and amphibians. Thus, the mammalian testis is probably dependent upon a constant supply of exogenous substrate. This statement is supported by Johnson et al. (1970B) in that glucose is considered to be an essential requirement for proper functioning of the testis. Many other investigators have additional supportive evidence that glucose is utilized by the testes of various mammals and has an important effect on normal testicular physiology. In this regard, Annison et al. (1963) reported that the testis and epididymis of unconscious rams

oxidize substantial quantities of glucose. Their findings indicate that approximately 33 percent of the glucose taken from the blood by these tissues was directly oxidized. Glucose is also reported to be a major "fuel" in the metabolism of the testes of conscious rams (Setchell and Waites, 1964; Setchell and Hinks, 1967). Similar findings have been reported in other mammals. Himwich and Nahum (1929) determined from the respiratory quotient of dog testes *in vitro* that glucose may be a major fuel of the testes. Bloom (1955) stated that rabbit testicular tissue incorporates large quantities of glucose and oxidizes it to carbon dioxide. Blackshaw (1962) working with mice, reported that glucose is metabolized by the testes under both aerobic and anaerobic conditions. He also stated that respiratory activity of the testes is stimulated by added glucose whereas fructose under the same conditions is much less utilized. According to Dickens and Greville (1933), Tepperman *et al.* (1949), and Tepperman *et al.* (1950), normal rat testes *in vitro* exhibit a marked dependence on an added supply of glucose for maintenance of oxygen consumption. Such oxygen consumption is low in the absence of added substrate and is increased in the presence of glucose (Dickens and Greville, 1933; Elliot *et al.*, 1937; Paul *et al.*, 1953; Free and Van Demark, 1969).

Glucose has been reported also to have a marked effect on the biosynthesis of proteins in the testis of rats. Davis and Morris (1963) found that addition of glucose to the incubation medium of rat testis *in vitro,* caused an increased incorporation of radioactively labeled lysine into the tissue by approximately 12 times that of other tissues. Furthermore, Davis and Firlit (1965) reported that very little protein labeling

occurred in the later cell stages of spermatogenesis in the absence of exogenous glucose. Upon addition of glucose, radioactive labeled lysine was incorporated into proteins in all of the successive cell stages of the seminiferous epithelium. Means and Hall (1968) reported also that glucose causes increased labeled lysine incorporation in testes of rats. This effect is only slight until about 28 days of age after which it becomes more evident as the animal matures. This finding is in agreement with that of Joshi and Macleod (1961) who reported that there is little or no oxidation of glucose in testes of rats until about 25 days of age, after which there is an incremental increase in the oxidative utilization of glucose until maturity. It seems, therefore, that glucose oxidation and lysine incorporation into proteins in the testes of rats parallel each other. The importance of lysine is suggested by Shettles (1960) who reports that this substance is essential for normal spermatogenesis in the rat. Any decrease from normal intracellular glucose levels in the testis occurring in conjunction with hypoglycemia is depicted by a decrease in testicular protein biosynthesis and is accompanied by atrophy of the seminiferous epithelium with a resulting impairment of spermatogenesis (Hollinger and Davis, 1968). Mann (1960) has suggested also that proteins and amino acids are a general requirement for normal testis function, and that lysine ranks high in general importance as a nutritional factor for reproduction in the male.

Alteration of the glucose supply to the testes can cause marked effects. Mancini et al. (1960) have stated that both low and high blood glucose levels cause disturbances of testicular function in mammals. Their observations along with those of VanDemark et al. (1968) suggested

that testes of adult rats made hypoglycemic showed injuries in the seminiferous epithelium such as nuclear pyknosis, intracellular vacuoles and formation of multinucleated giant cells. This finding is in agreement with that of Siperstein (1921) who demonstrated that acute inanition in rats caused degeneration of cells of the seminiferous epithelium which occurred inversely to spermatogenesis. Such degeneration in rats has been shown to result from a decrease in energy intake of 15 to 30 percent (Reid, 1949). Testicular atrophy and reduced fertility, due to under nutrition, has been reported also in mice (McClure, 1966) and rams (Setchell et al., 1965). Glucose shortage is implicated, as administration of this substance will restore normal fertility.

Hyperglycemia also has detrimental effects on testicular morphology and physiology. Work on rats (Soularc et al., 1948, as cited by Hunt and Bailey, 1961), mice (Lidell et al., 1966) and men(Klebanow and MacLeod, 1960; Schoffling et al., 1963) resulted in similar findings. In general, hyperglycemia causes degenerative changes in both the seminiferous epithelium and the spermatogenic process which is evidenced by reduction in size of the testes, atrophy of seminiferous tubules and frequent reduction in numbers of spermatogenic cells.

In summary, several methods of controlling spermatogenesis have been employed, each proving unsatisfactory. More recently, control via glucose supply to the testis has been attempted. Glucose is known to be an essential substrate for testis metabolism as oxidation of this substance provides energy for many testicular metabolic processes in mammals. Furthermore, when the exogenous supply of glucose to the testis is altered, normal physiology and morphology are not maintained. This is evidenced

by atrophy of seminiferous tubules, reduction in numbers of spermatogenic cells, decreased endocrine activity and reduced fertility.

Recently, a new glucose compound has been marketed for research purposes which shows promise as a reversible inhibitor of spermatogenesis. The drug, 5-Thio-D-Glucose, is a close chemical analog of biologically important D-Glucose, yet is competitively inhibits active transport of D-Glucose across cell membranes. Some D-Glucose does cross the membranes by passive diffusion, however (Whistler and Lake, 1972). When given in large dosages the drug causes a short-lived pseudodiabetogenic effect. At lower dosages, normal blood sugar levels are maintained (Whistler and Pitts, unpublished, as cited by Zysk et al., 1975). Initial investigation with this drug has shown reduced spermatogenic activity and fertility in mice (Zysk et al., 1975). Such results indicate the need for further investigation on the use of this drug. The present study, therefore, was undertaken to answer the following questions. (1) What effect does length of treatment have on spermatogenesis as indicated by testicular histology? (2) Are androgen secretions affected, as determined by histological study of the seminal vesicles and ventral prostates? (3) Does treatment with the drug cause detectable changes in androgen biosynthesis as determined by testis enzyme histochemistry? (4) What effect does length of treatment have on normal fertility as determined by breeding performance?

MATERIALS AND METHODS

Animals

Adult albino male mice (32-38 gm) of the ICR strain were used in this investigation. Adult female mice of the same strain were employed to check male fertility. The animals were housed in a room with a light regime of 12 hours of light (7:00 A.M.-7:00 P.M.) and 12 hours of darkness (7:00 P.M.-7:00 A.M.) and with a constantly maintained temperature of 22-25°C. All animals were marked for identification purposes and males and females were housed separately in plastic cages each containing up to 20 animals. All animals had free access to water and received Wayne Laboratory Feed ad lib.

Experimental Design

Three experimental groups were set up and designated as T-2, T-4, and T-6. Each group received by daily gavage 33mg. of 5-thio-D-glucose/Kg. of body weight while on treatment. This is the minimum dosage of drug that effectively inhibits spermatogensis in mice (Maugh, 1974). The T-2 group was removed from treatment at the end of two weeks. The T-4 and T-6 groups were removed at four and six weeks, respectively. Of six animals taken off treatment at the end of each time interval, four were allowed to breed and two were sacrificed in order to remove the testes, seminal vesicles, and ventral prostates for later histological studies. This same procedure was carried out simultaneously with control animals. In this manner, experimental animals could be compared with control animals at each time interval.

Drug Administration

The drug dosage was determined by converting the combined gram weight of animals on treatment into kilograms and multiplying this by 33 mg. The resultant daily drug dosage to be administered was multiplied by seven to determine a weekly drug dosage. This dosage was readjusted at weekly intervals to compensate for body weight fluctuations or for animals that were removed from treatment. All animals received their daily dosage in 0.25 cc aqueous solution by gavage.

The drug was administered with a 0.5 cc syringe with an attached piece of polyethylene tubing. To facilitate administration of the drug, each animal was lightly etherized. The tubing was inserted into the animal's mouth, pushed down the esophagus into the stomach and the solution injected. Each animal was placed in a small cage until full consciousness was regained, whereupon, it was transferred back to the original cage. Control animals were handled similarly, except they received a water placebo.

Autopsy Procedure

At the termination of each time interval, two control and two experimental animals were sacrificed by an overdose of ether. They were incised ventrally and both testes, the seminal vesicles, and the ventral prostates were removed, stripped of fat, and put on ice until dissection was compleated. Tissues were blotted to remove excess moisture, weighed on a torsion balance and immediately transferred to their respective fixatives to prepare them for subsequent histological and histochemical examination.

Histological Procedure

One testis, the seminal vesicles, and the ventral prostates from control and experimental animals were fixed in 10% neutral buffered formalin solution. Following fixation, tissues were placed in an Auto-technicon for dehydration, clearing and infiltration before embedding in paraffin blocks. Blocks were sectioned on a rotary microtome at a thickness of six to eight microns. Selected sections were mounted on clean glass slides and stained with Delafield's Hematoxylin and Eosin Y. Tissues were examined microscopically and morphometric measurements were taken randomly from representative sections of each tissue. The diameter of the seminiferous tubules within the testis, and the heights of the secretory epithelial cells from the seminal vesicles and ventral prostates were measured with a calibrated occular micrometer and recorded. Photomicrographs of representative sections were taken with a Nikon AFM 35 mm camera attached to a microscope adapter. Kodak "Panatomic X" film was used (ASA 32).

Histochemical Procedure

The contralateral testis from control and experimental animals sacrificed at each time interval was removed, weighed and immediately prepared for histochemical analysis. The histochemical procedure was employed to investigate the presence and staining intensity, of certain oxidative enzymes that catalyze intermediate reactions within the androgen biosynthetic pathway. The testis was immersed in a plastic Beem capsule containing OCT compound and immediately frozen at -80^{o}C in a Dewar flask containing a mixture of acetone and solid carbon dioxide.

Sections approximately 10 microns thick were cut with a cryostat, placed on a clean glass slide, and allowed to air dry. Sections were incubated for three hours at 37°C in a medium consisting of 20 ml of 0.1 M Tris buffer-pH 7.4, 10 mg nitro-blue tetrazolium, 20 mg of NAD and 2 mg of steroid substrate dissolved in 0.5 ml N, N dimethyl formamide. In this investigation, two intermediate sites within the pathway were checked for the presence of oxidative enzymes. (1) Progesterone: This reaction indicates the presence of Δ^5-3 β hydroxysteroid dehydrogenase, requires the cofactor NAD as a hydrogen acceptor and pregnenolone as substrate. (2) Androstenedione: This reaction also indicates the presence of Δ^5-3 β hydroxysteroid dehydrogenase, requires the cofactor NAD and dehydroepiandrosterone as substrate.

Two types of control incubations were carried out with each tissue. In the first, the medium without the steroid substrate was used to verify the specificity of the reaction. The second was done to verify the presence of NADH diaphorase which is necessary for transferring hydrogen from the dehydrogenase reaction to the tetrazolium salt for formazan precipitation. The presence of this enzyme was verified by incubating sections 15 minutes in a medium containing buffer, NADH and nitro-blue tetrazolium. After incubation, all sections were fixed in phosphate buffered formalin for ten minutes, rinsed in distilled water, counterstained lightly with neutral red and mounted in glycerol (Blaha and Leavitt, 1970).

Enzyme activity was evaluated subjectively using a zero to four scale with zero being no color reaction and four being maximal color reaction.

Breeding Procedure

At each time interval, four control and four experimental animals were allowed to breed. Each male was placed in a small cage with a female and observed for approximately 15 minutes following introduction to determine if there were any observable changes in behavior or libido caused by the drug. Males remained with the females for seven days. At the end of this week-long period, the females were removed to another cage and the males were exposed to four new females for seven additional days. At termination of the second week, all females were housed together and observed daily for signs of pregnancy. If there was a high incidence of pregnancy and occurrence of normal litter sizes among females, then the males to which they were bred were considered to be fertile and breeding was terminated. If, however, there was a low incidence of pregnancy in females from the initial two-week breedings, the males were re-exposed to females at designated intervals until normal fecundity had returned.

Each female exposed to a male was sacrificed by an overdose of ether 19 to 20 days following introduction to the males. A mid-ventral laparatomy was done and the uteri were removed and inspected for developing fetuses. If fetuses were present, they were removed from the uterus, counted, and grossly examined for malformations. This method for counting the number of young was employed rather than allowing females to litter in order to eliminate possible inaccurate litter counts due to cannibalism.

RESULTS AND DISCUSSION

Sex Organ Weight Variation Studies

One parameter that may be used to demonstrate the influence of 5-thio-D-glucose on reproductive physiology is weight variations of the testes and sex accessory glands of treated animals. The weights of the seminal vesicles and ventral prostates from experimental animals varied greatly within and between groups (Appendix A). It appears that administration of the drug caused no observable trends in weight changes of these glands. Variation perhaps attributed to loss of glandular fluids during removal of these organs. Testicular weight variation in experimental animals follows a more definite trend (Appendix A). The greatest weight loss was observed in testes taken from animals treated for six weeks. Testicular weight loss accompanied by histological degeneration and reduced spermatogenic activity has been shown to occur as a result of cryptorchidism (Moore, 1922; Moore, 1924a, b), heat treatment (Fukui, 1923), and hypophysectomy (Hardberger, 1950).

Histological Studies of the Testis

Histological study is widely used as a parameter for investigation of the influence of various substances on spermatogenic activity. The temporal effect of 5-thio-D-glucose on spermatogenesis and sex accessory gland morphology were observed in the present investigation. The effect of drug on testicular histology and spermatogenesis in animals treated two weeks are presented in Plate I. Seminiferous tubules in testes taken from control animals (Figure 1) are very active in spermatogenesis and are characteristically round or oblong shaped. After two weeks treatment

with 5-thio-D-glucose no observable degenerative changes are seen that may be attributed to the drug (Figure 2). The seminiferous epithelium is active in spermatogenesis and the tubules are round or oblong. Higher magnification of control testis reveals the cell types occurring in the normal spermatogenic process (Figure 3). Spermatogonia are located adjacent to the basement membrane of the seminiferous tubules and are responsible for initiating spermatogenesis. Primary and secondary spermatocytes are positioned closer to the tubule lumen. Spermatids, located near the lumen of the seminiferous tubules, vary in shape from small round nucleated cells containing moderate cytoplasm to small oval or slightly elongate nucleated cells containing very little cytoplasm. These cells represent the final cell stage in the spermatogenic process preceding formation of spermatozoa. Spermatozoa have elongated or curved nuclei and no cytoplasm. They are located in or near the lumen of seminiferous tubules. Higher magnification of a section of the testis taken from an experimental animal (Figure 4) allows identification of all successive cell stages occurring in the normal spermatogenic process. This indicates that treatment with 5-thio-D-glucose for two weeks causes no observable degenerative effects on spermatogenesis.

The effects of 5-thio-D-glucose on spermatogenesis in animals treated for four weeks are presented in Plate II. Seminiferous tubules within testes taken from control animals (Figure 5) are highly active. Centrally located seminiferous tubules in a testis taken from an experimental animal (Figure 6) are unimpaired as tubules show good spermatogenic activity. Peripherally located tubules, however, contain large eosinophillic masses circumscribed by basophillic nuclei (Figures 7 and 8).

These masses are multinucleated cells and are characteristic of testicular degeneration caused by hypoglycemia (Mancini et al., 1960), inanition (Siperstein, 1921) and hypophysectomy (Ludwig, 1950). In addition, multinucleated cells have been shown to accompany testicular degeneration in mice administered 5-thio-D-glucose (Zysk et al., 1975). Higher magnification of a control testis (Figure 9) and seminiferous tubules in the center of an experimental testis (Figure 10) reveals unimpaired spermatogenic activity as all successive cell stages occurring during normal spermatogenesis can be identified. Higher magnification of peripherally located seminiferous tubules in testes taken from experimental animals (Figure 11) shows concentrically arranged multinucleated cells. Enlargement of these multinucleated cells reveals nuclei characteristic of those belonging to late spermatids or spermatozoa (Figure 12).

Initial testicular degeneration observed following four weeks treatment with the drug appears to be multinucleated masses comprised of spermatid or spermatozoa nuclei. Such degeneration of the most mature cells first has been previously suggested by Siperstein (1921) who stated that spermatogenic degeneration occurs in reverse to the normal spermatogenic process. Also Zysk et al. (1975) stated that the first multinucleated cells seen as a result of 5-thio-D-glucose administration were located in the spermatid zone. This investigator reserves judgment as to the involved cell types, since the nuclear shape indicates that some of the cells may be in a transitory phase from spermatids to spermatozoa. The photographs presented on Plate II show a definite degenerative effect in the spermatogenic process resulting from four weeks of treatment

with the drug. Spermatogenic degeneration at this time, however, is confined to seminiferous tubules located at the peripheral part of the testis. Perhaps such degeneration is a result of how well different parts of the testis are supplied with blood. In rodents, the internal spermatic artery passes down the epididymal margin and enters the testis near the cranial pole. The artery courses past the rete and divides into radiate arteries which enter into the parenchyma. Capillaries branch from these arteries and are classed into two main types. Intertubular capillaries course within the interstitium parallel to seminiferous tubules. Peritubular capillaries surround individual tubules (Johnson, 1970a).

According to Young (1961), following occlusion of the testicular artery, tubules become abnormal due to tissue ischemia. Damage is initially restricted to the central portion of the testis with damage spreading to all tubules except those near the epididymal border. It is likely that peripheral testicular tissues receive blood supply not only from interstitial capillaries but also a small amount from capillaries of the testicular tunics. Therefore, peripheral seminiferous tubules receive a slightly greater blood supply than do those in the center of the testis and any substance in the blood which inhibits spermatogenesis would have its greatest effect on peripheral tubules. In agreement, it was shown in the preceding photographs that initial spermatogenic degeneration results from 5-thio-D-glucose administration involved peripheral seminiferous tubules only.

The effects of six weeks of treatment with 5-thio-D-glucose are seen in Plates III and IV. Seminiferous tubules in testis taken from

control animals (Figure 13) show good spermatogenic activity. Seminiferous tubules located at the center (Figure 14) and periphery (Figures 15 and 16) of testes taken from experimental animals show marked degeneration. Some tubules contain multinucleated cells (Figure 14) while adjacent tubules may contain only eosinophillic masses without associated nuclei (Figure 15). Several seminiferous tubules show very extensive damage as the basement membrances are devoid of spermatogonia (Figure 16). Such extensive degeneration was an infrequent finding.

A higher magnification of a control testis (Figure 17) shows good spermatogenic activity. Seminiferous tubules located in the center of experimental animal testes appear contorted and irregularly shaped (Figure 18) or they contained eosinophillic masses (Figure 19) and multinucleated cells (Figure 20). Peripheral seminiferous tubules (Figure 21) are similarly damaged indicating that testicular degeneration following six weeks of drug administration is not confined to the peripheral portion of the testis only. Higher magnification of a seminiferous tubule containing multinucleated cells (Figure 22) shows that these cells are morphologically different from those observed following four weeks of treatment. Nuclei associated with these cells are eccentrically arranged and are rounded rather than elongated or curved. Such nuclei are characteristic of spermatocytes which indicates that degeneration had advanced one cell stage further than the damage observed following four weeks treatment. A layer of spermatogonia still exists within the tubule, suggesting that treatment has not devoided the animal of cells responsible for initiating the spermatogenic process. Degenerating

multinucleated cells (Figure 23) undergoing pyknosis and karyorrhexis, are believed to be an advanced stage of testicular degeneration progressing towards the formation of eosinophillic masses. Eosinophillic masses (Figure 24) are of similar density to the background material seen in multinucleated cells (Figures 20, 22, and 23). This investigator contends that these masses are the amorphous cytoplasmic material remaining following degeneration of the nuclei comprising multinucleated cells (Figures 23 and 24).

Degeneration seen in the previous figures is accompanied by atrophy of the testis. Observing Plates III and IV, it should be noted that the seminiferous tubules of experimental animal testes generally appear smaller in diameter than tubules from testes of control animals. To further study this, 50 morphometric measurements of seminiferous tubule diameter were taken from representative tissue sections of control and experimental animals at each time interval. The measurements were subjected to t-test analyses and the results appear in Table I. There was no significant difference between the diameter of control and experimental seminiferous tubules following two weeks of treatment. Following four weeks of drug administration, diameter of tubules within testes from experimental animals were significantly smaller than those of the controls. Following six weeks of treatment, tubules from testes of experimental animals were also significantly smaller than those of controls ($P < 0.01$).

In summary, it appears that 5-thio-D-glucose has a definite detrimental effect on spermatogenesis over a period of time. Following two weeks of treatment, the drug had no observable effect on spermatogenesis.

This was evidenced by the morphological similarity of experimental seminiferous tubules to those from control testes. Following four weeks of drug administration, the first observable detrimental effects on spermatogenesis were apparent. Many of the spermatids and spermatozoa had become incorporated into multinucleated cells. Degeneration was confined only to seminiferous tubules located at or near the peripheral portion of the testis. After six weeks of treatment with the drug, degeneration of spermatogenesis was apparent throughout the entire testis. Many seminiferous tubules contained multinucleated cells comprised of eccentrically arranged spermatocytes. Also, many tubules from testes of experimental animals were contorted and of smaller diameter than those from control testes.

Histological Studies of the Sex Accessory Glands

It has been well documented that certain accessory sex glands in male mammals, especially the prostate and seminal vesicles, are very sensitive to circulating androgens. Lindner and Mann (1960) have stated that basic knowledge of endocrine function of the mammalian testis has been learned through investigation of the chemical nature of androgens in the testis and through studies of specific changes caused in the seminal vesicles and prostate following administration of androgens. From this it can be concluded that accessory sex glands provide an excellent bioassay for circulating androgen levels, because the prostate and seminal vesicles are dependent upon androgens for maintenance of normal physiology and morphology.

The prostate is known to be dependent on circulating androgens for

maintenance of its normal histological characteristics (Brandes and Portella, 1960a). Normally the ventral prostate is composed of finger-like tubulo-alveoli glands. The secretory epithelium is generally one cell thick and the cells are classed as simple columnar. Height of the cells, however, may range from low to tall columnar depending upon the secretory state of the glands. When the gland is distended with fluid, the resting cells tend to be low, while actively secreting cells appear tall. Nuclei usually lie in the basal portion of each cell, and apical to each nucleus is a clear or light staining zone containing many Golgi bodies. These supranuclear Golgi function in secretion, and often secretion granules may be seen at the apical portion of each cell. Normal glands also have eosinophillic secretory material in the lumen (Moore, Price, and Gallagher, 1930; Korenchevsky and Dennison, 1935; Hornung, 1947; Brandes and Portella, 1960b; Franks and Barton, 1960).

Abnormal prostate morphology due to castration and insufficient diet has been well documented by several investigators (Moore, Price, and Gallagher, 1930; Moore and Samuels, 1931; Harsh, Overholser, and Wells, 1939; Grayhack and Scott, 1952; Ortiz, Price, Williams-Ashman, and Banks, 1956; Grayhack, 1958). The height of the secretory epithelium is low, with cells becoming more cubiodal in appearance. Also, the supranuclear Golgi zone disappears and secretory granules are not apparent. In addition, little to no eosinophillic secretory material is seen in the lumen of the gland and the atrophic gland is reduced in weight.

Ventral prostate tissue from control (Plate V, Figure 25) and experimental animals (Figure 26) appears very similar. Secretory epithelial cells are columnar with basally located nuclei. Also, most cells possess

a supranuclear light staining Golgi zone and eosinophillic secretory material is observed in the lumen of the gland. These characteristics suggest a functionally active prostate gland; thus, it appears that 5-thio-D-glucose causes no damage to ventral postate histology.

Seminal vesicle development and maintenance also is dependent upon secretion of testicular androgens (Moore, Hughes, and Gallagher, 1930; Freud, 1933). Normally, the secretory epithelium of the seminal vesicle is tall and narrow being classed as simple columnar, and nuclei are basally located. The cells contain secretory granules which are located apical to the clear staining supranuclear Golgi zone. In rats and mice as well as other mammals the seminal vesicles change histologically following castration. Epithelial cells become cuboidal, secretion granules disappear, and weight of the gland decreases primarily due to loss of secretory material (Denesly and Parkes, 1933; Melampy and Cavazos, 1953; Cavazos and Melampy, 1954b). The effects caused by castration, however, are reversed upon administration of androgens (Porter and Melampy, 1952; Fleischmann, 1957; Deane and Porter, 1960).

Seminal vesicle tissue from control (Figure 27) and experimental (Figure 28) animals appears very similar histologically. Experimental secretory epithelial cells like control, are columnar with basally located nuclei and possess a light staining supranuclear Golgi zone. In addition, eosinophillic secretory material is seen within the lumen of the gland. It appears that treatment with 5-thio-D-glucose causes no damaging effects on seminal vesicle histology.

Summarizing, 5-thio-D-glucose has no apparent degenerative effects on sex accessory gland histology. In contrast, statistical analyses of

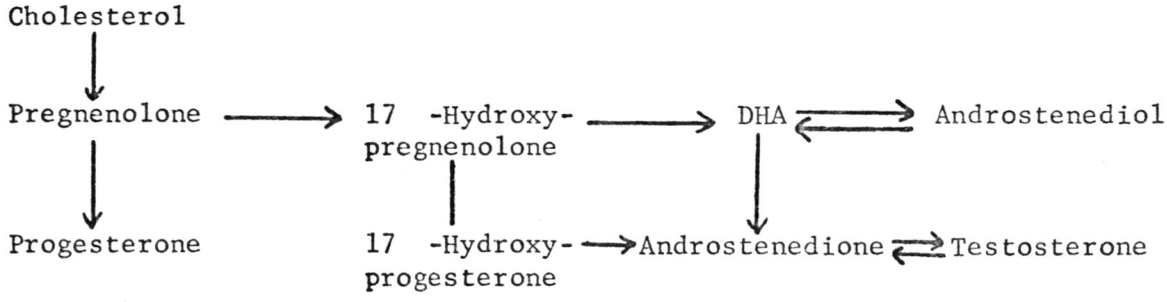
secretory epithelial cell height in control and experimental tissues revealed some significant differences (Table I). All pertinent significant differences were confined to increased height of the secretory epithelium of experimental tissues only. This indicates that androgen secretions were certainly not decreased, but perhaps slightly elevated as a result of 5-thio-D-glucose administration.

Histochemical Studies

In addition to providing sex cells, the testis also synthesizes and secretes androgens. As stated above, androgens bring about the development of and subsequently maintain male accessory reproductive organs and secondary sexual characteristics. The testis secretes three main androgens; testosterone, androstenedione, and dehydroepiandrosterone (Dorfman and Shipley, 1956). Testosterone and androstenedione are considered to be the true testicular hormones of these three, and they are more potent than dehydroepiandrosterone. Interstitial cells of Leydig and seminiferous tubules are capable of converting progesterone into androgens *in vitro*, with the former being more active (Christensen and Mason, 1965). Hall *et al.* (1969) using similar preparations, have suggested that while Leydig cells are capable of converting cholesterol into androgens *in vitro*, seminiferous tubules cannot. Since cholesterol appears to be the major precursor of steroid hormones, it is indicated that Leydig cells are responsible for synthesizing androgens under physiological conditions and not the seminiferous tubules (Long, 1947; Hall, Nishizawa, and Eik-Nes, 1963; Hall and Koritz, 1965). Knowing that

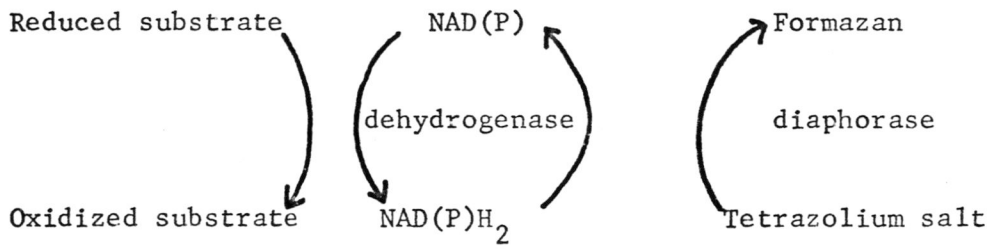
the androgens are primarily produced by the interstitial cells of Leydig, it is relatively simple to determine if androgen biosynthesis is proceeding normally through use of an enzyme histochemical technique. The function of this procedure is to demonstrate the presence of certain oxidative enzymes and the reaction of such enzymes to different physiological and pathological conditions. The normal distribution of enzymes in many tissues is well documented. The enzymology of the testis, however, is still largely unknown with the exception of enzymes involved in steroid synthesis. In the present study, enzyme histochemical techniques were employed to determine if administration of 5-thio-D-glucose had any observable effects on two selected androgen biosynthetic pathways within the testis. If the drug were to impede androgen biosynthesis, there would be a direct reduction in androgen secretion. Reduced circulating androgens, as mentioned previously, would cause discontinued maintenance of sex accessory organs, loss of secondary sexual characteristics and reduced libido.

Two sites within the androgen biosynthetic pathway were evaluated for the presence of enzymes necessary for catalysis of intermediate reactions. The following diagram illustrates the steps involved in the androgen biosynthetic pathway.



Reviewing briefly, the first site chosen in the pathway was the conversion of pregnenolone tc progesterone. The second site chosen was the conversion of dehydroepiandrosterone (DHA) to androstenedione. Both conversions require the enzyme Δ^5-3β-hydroxysteroid dehydrogenase.

The following diagram illustrates the two-step, histochemical reaction for demonstration of dehydrogenase enzymes.



In the first step, the substrate in the reduced form and the proper cofactor are added to the incubation medium containing the tissue slice and the tetrazolium salt (Nitroblue tetrazolium, NBT). If the necessary enzyme at each site is present, it will oxidize the substrate and donate the free electron (hydrogen) to the cofactor. In the second step, diaphorase, a unbiquitous enzyme in testicular tissue, transfers the electron from the cofactor to the tetrazolium salt. The salt, which is colorless in the oxidized state, becomes reduced and forms a dark red or blue insoluble formazan compound. This reaction, therefore, produces colored deposits wherever steroidogenic enzymes are present, thus, indicating the relative activity of the tissue in steroidogenesis.

Demonstration of the enzyme diaphorase in control (Plate VI, Figure 29) and experimental (Figure 30) tissues is indicated by dark deposits located primarily in interstitial cells between seminiferous tubules. This enzyme, as stated above, is necessary to complete the

second step of the histochemical reaction. The conversion of pregnenolone in control (Figure 31) and experimental tissues treated two weeks (Figure 32) shows no difference in Δ^5-3β-HSD activity. Control (Figure 33) and experimental tissues treated four weeks (Figure 34) also demonstrate similar enzymatic activity. Δ^5-3β-HSD activity in control (Figure 35) and experimental tissues treated six weeks (Figure 36) appears of similar intensity. Subjective evaluation of Δ^5-3β-HSD activity in this specific reaction (Table II) shows that seminiferous tubules in control and experimental tissues ranged in activity from 0 to $\pm$. Interstitial tissue activity in control and experimental tissues was rated ++.

The Δ^5-3β-HSD activity in the conversion of dehydroepiandrosterone is presented in Plate VII. Enzymatic activity in control (Figure 37) and experimental tissues treated two weeks (Figure 38) is similar. Following four weeks of treatment, control (Figure 39) and experimental (Figure 40) tissues show no difference in Δ^5-3β-HSD activity. Control (Figure 41) and experimental tissues treated six weeks (Figure 42) appear to have similar enzymatic activity also. Subjective evaluation of Δ^5-3β-HSD activity in this specific reaction (Table II) reveals that interstitial cells were rated at ++ while activity in seminiferous tubules ranges only from 0 to $\pm$.

In both of the above reactions, Δ^5-3β-HSD activity was greater in the interstitial cells of Leydig than in the seminiferous tubules. This histochemical evaluation is substantiated by the previous explanation that the Leydig cells--not seminiferous tubules--synthesize androgens under physiological conditions. Some reactivity was observed within the seminiferous tubules of each tissue, however. This is in agreement with

findings reported by Galil and Deane (1966) who stated that slight coloration of peripheral portions of seminiferous tubules often occurred suggesting utilization of exogenous substrates. Hall et al. (1969) have suggested that spermatogenic cells do have access to physiological amounts of exogenous progesterone from plasma or directly from Leydig cells. The tubules, therefore, require necessary enzymes to convert progesterone to testosterone. Testosterone is important in the spermatogenic process (Woods and Simpson, 1961) and, since progesterone can be converted to testosterone within the seminiferous tubules (Christensen and Mason, 1965), the above findings offer a plausible explanation for enzymatic activity within the tubules.

Observing Plates VI and VII the color intensity of the reaction and the relative amount of reactive tissue indicates that 5-thio-D-glucose does not have an inhibiting effect on androgen biosynthesis. An interesting finding, however, is that on each Plate at T-6, the relative amount of reactive deposits seen from experimental animal tissues appears to be greater than that from the controls. This observation is supported by Kuntz (1921) who stated that frequently degenerating testes are characterized by hyperplasia of the interstitial tissue. Such hyperplasia is considered to be a compensatory mechanism in an effort by the endocrine system to maintain spermatogenesis and testicular morphology. It can be concluded that 5-thio-D-glucose has no impeding effect on steroid synthesis by the testis. In contrast, it may stimulate an increase in steroid synthesis as is suggested by the apparent hyperplasia of the interstitium. Such an increase in androgens may account for the sex accessory gland hypertrophy previously discussed.

Fertility Studies

The final parameter investigated was the effect of length of treatment with the drug on breeding performance of the males. The results are included in Table III, with figures representing average litter sizes sired by control and experimental animals at each time interval. Based on pilot breedings done before this investigation, the average range of litter size for ICR mice was found to be 10 to 15 young.

Animals administered the drug for two weeks did not produce normal litter sizes the first week following treatment (Table III). The second week, however, litters sired by these animals averaged 10.5 young and breeding was discontinued. Following four weeks of drug administration, breeding performance suffered greater impairment. Average litter sizes were 9.5 and 8.0 young for the first and second weeks, respectively. The fourth week following treatment, however, fertility was back to normal as average litter size was 10.6 young. Following six weeks of 5-thio-D-glucose administration, breeding was greatly impaired. Experimental animals had sired no young at four weeks post-treatment, and average litter size was only 2.0 young at six weeks post-treatment. By nine weeks following treatment, males had returned to normal fertility as litter size averaged 13.5 young.

Following two weeks of treatment, fertility was slightly impaired and normal fecundity was not regained until two weeks post-treatment. Seminiferous tubules within testes taken from the two week experimental animals showed no apparent histological damage, however, it is assumed that the numbers of spermatozoa were reduced below normal levels. Following four weeks of treatment, fertility was impaired for a greater

length of time. Plate II offers histological evidence for this reduced fertility as many spermatid and spermatozoa multinucleated cells are seen. This indicates that spermatogenic damage has progressed up to the spermatocyte stage, thus, fewer spermatozoa are available for fertilization. Following six weeks of treatment, fertility was impaired completely and was not restored to normal until the seven to nine week post-treatment period. Plates III and IV show many spermatocyte multinucleated cells indicating that damage has progressed up to the spermatogonia stage.

According to Oakberg (1956a,b) the approximate length of time necessary to complete the entire spermatogenic process in the mouse is 34.5 days. He suggested that the process is divided into four cycles each lasting about 8.6 days. One cycle is required for production of primary spermatocytes from spermatogonia and three cycles are required for meiosis and spermiogenesis. These findings by Oakberg seem to correlate with the present data. Experimental animals of group T-2 showed slightly impaired fertility. It is assumed that only spermatozoa were damaged, thus, it should take at least 8.6 days for spermatids to mature into spermatozoa. Table III supports this contention since normal fertility was restored between 7 and 14 days. Animals of the T-4 group showed a greater impairment of fertility. Spermatid multinucleated cells were seen histologically on Plate III, thus, it should take about 17.2 days for spermatocytes to develop into spermatozoa. Normal fertility was regained between 14 and 28 days (Table III). Animals administered the drug for six weeks showed the greatest impairment of fertility. Spermatocyte multinucleated cells were observed histologically on Plates III and IV, thus, it whould take at least 34.5 days for spermatogonia to

to develop into spermatozoa. Normal fertility was not regained until between 42 and 63 days post-treatment (Table III). Perhaps the delay was due to the necessary buildup of sufficient numbers of spermatozoa for fertilization.

PLATE I. Cross sections of testicular tissue from animals treated two weeks with 5-Thio-D-Glucose and corresponding control tissues. Delafield's hematoxylin and Eosin Y.

Figure 1. Photograph taken from the center of a control testis. Seminiferous tubules (arrows) contain many spermatogenic cells between the basement membrane (bm) and lumen (L) of each tubule. Interstitial tissue (i) between tubules is also seen. 125X

Figure 2. Photograph taken from the center of an experimental testis. Seminiferous tubules (arrows) show good spermatogenic activity as indicated by the many cells located in the seminiferous epithelium (SE) of each tubule. Interstitial tissue (t) between tubules is abundant. 125X

Figure 3. Enlarged photograph of a testis taken from a control animal. Within seminiferous tubules (arrows) individual cell types occurring in the normal spermatogenic process can be identified. Cell types seen are spermatogonia (g), spermatocytes (c), spermatids (t), and spermatozoa (z). Interstitial tissue (i) is seen also. 312X

Figure 4. Enlarged photograph of a testis taken from an experimental animal. Individual cell types occurring in the spermatogenic process can be identified between the basement membrane (bm) and lumen (L) of the seminiferous tubules. Cell types seen are spermatogonia (g), spermatocytes (c), spermatids (t), and spermatozoa (z). Interstitial tissue (T) is present. 312X

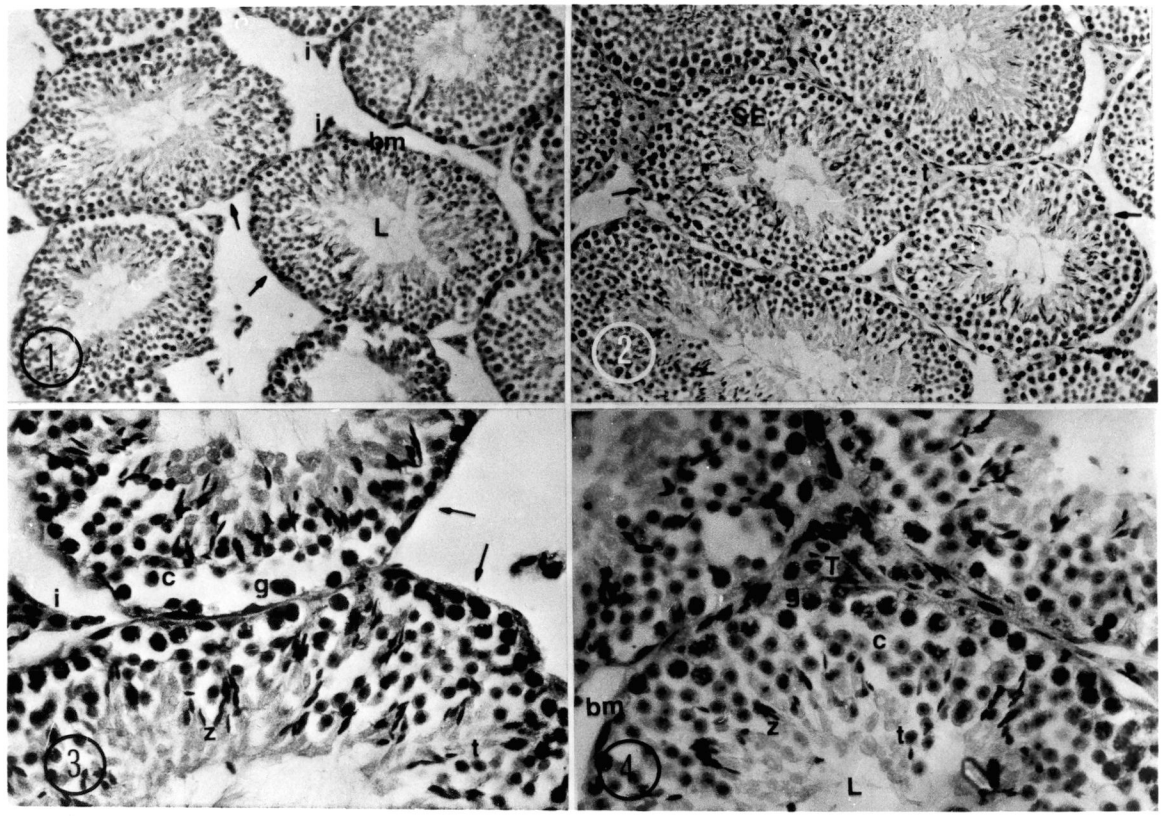

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PLATE II. Cross sections of testicular tissue from animals treated four weeks with 5-Thio-D-Glucose and corresponding control tissue. Delafield's hematoxylin and Eosin Y.

Figure 5. Control testis containing seminiferous tubules (arrows) highly active in spermatogenesis. Interstitial tissue (i) is present also. 125X

Figure 6. Seminiferous tubules (arrows) photographed at the center of a testis taken from an experimental animal. Tubules are highly active in spermatogenesis and appear very much like those seen in control tissues. 125X

Figure 7. Photograph from the periphery of a testis taken from an experimental animal. The indicated tubule (arrow) and the adjacent one show multinucleated cells (m) which are characteristic of testicular degeneration. 125X

Figure 8. Photograph taken from the periphery of a testis obtained from an experimental animal. Multinucleated cells (m) are seen in two seminiferous tubules located adjacent to the tunica (t). 125X

Figure 9. Enlarged photograph of seminiferous tubules within a testis taken from a control animal. Abundant spermatogenic cells indicates active spermatogenesis. 312X

Figure 10. Enlarged photograph of seminiferous tubules located in the center of a testis taken from an experimental animal. Individual cell types, spermatogonia (g), spermatocytes (c), spermatids (t), and spermatozoa (z) are distinguishable, indicating that spermatogenesis appears unimpaired. Interstitial tissue (i) is present. 312X

Figure 11. Seminiferous tubules located on the periphery of a testis taken from an experimental animal. Multinucleated cells (m) are comprised of nuclei concentrically arranged on the outer edge of an eosinophillic mass. 312X

Figure 12. Highly magnified photograph of seminiferous tubules located at the periphery of a testis taken from an experimental animal. Multinucleated cells (m) appear as eosinophillic masses with concentrically arranged nuclei. Nuclei are elongated and curved (arrows) and are characteristic of those belonging to late spermatids or spermatozoa. 500X

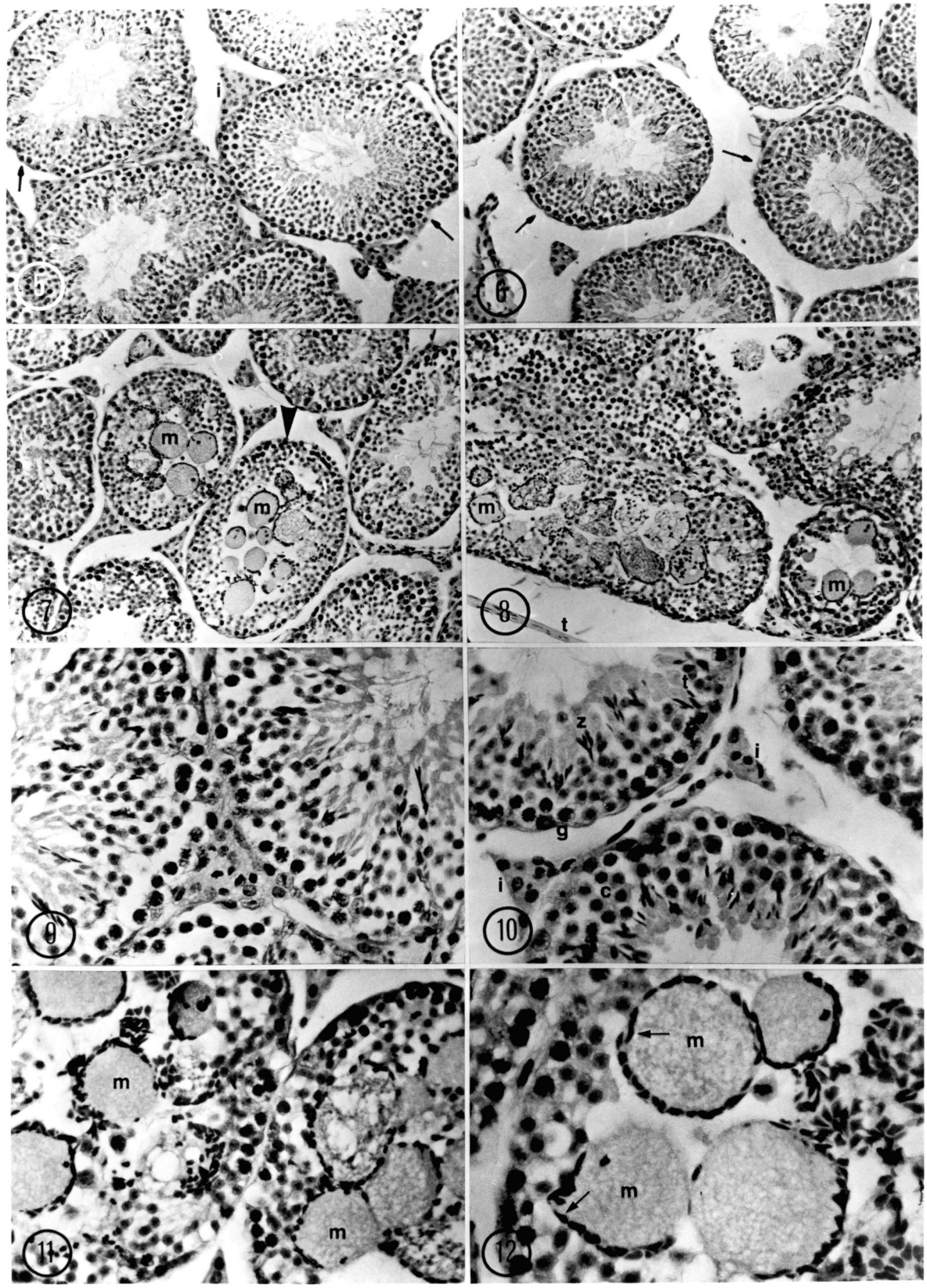
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PLATE III. Cross section of testicular tissue from animals treated six weeks with 5-Thio-D-Glucose and corresponding control tissue. Delafield's hematoxylin and Eosin Y.

Figure 13. Seminiferous tubules within a testis taken from a control animal. The seminiferous epithelium is highly active in spermatogenesis. 125X

Figure 14. Seminiferous tubules (arrows) photographed at the center of a testis taken from an experimental animal. Some tubules contain only eosinophillic masses (unshanked arrow) while other tubules (shanked arrow) contain multinucleated cells. Interstitial tissue (i) is abundant. 125X

Figure 15. Seminiferous tubules photographed at the periphery of a testis taken from an experimental animal. Eosinophillic masses (E) and multinucleated cells (M) are seen within these tubules. Interstitial tissue (i) is abundant between seminiferous tubules. 125X

Figure 16. Photograph taken at the periphery of a testis from an experimental animal. Several seminiferous tubules (T) are devoid of spermatogonia indicating severe damage. Interstitial tissue (arrows) comprises most of the area between tubules. 125X

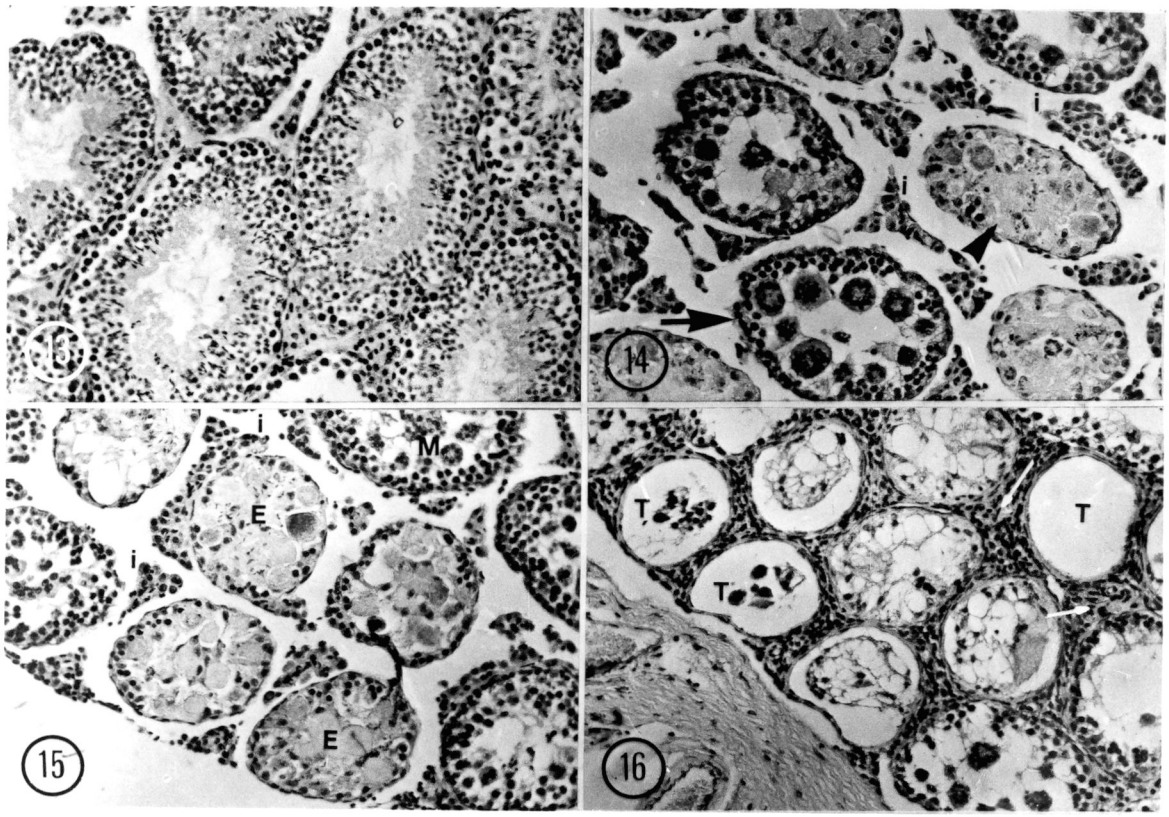
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PLATE IV. Cross section of testicular tissue from animals treated six weeks with 5-Thio-D-Glucose and corresponding control tissue. Delafield's hematoxylin and Eosin Y.

Figure 17. Photograph of seminiferous tubules in a testis taken from a control animal. Good spermatogenic activity is seen within the tubules. 312X

Figure 18. Seminiferous tubules located in the center of a testis taken from an experimental animal. Spermatogenesis is disrupted and tubules (arrows) are contorted and irregular shaped. 312X

Figure 19. Seminiferous tubules (arrows) at the center of a testis taken from an experimental animal. Tubules contain eosinophillic masses (E). Interstitial tissue (i) is present between tubules. 312X

Figure 20. A seminiferous tubule located at the center of a testis obtained from an experimental animal. Within the tubule are multinucleated cells (arrow). 312X

Figure 21. Photograph of seminiferous tubules located at the periphery of a testis taken from an experimental animal. One tubule contains multinucleated cells (M) while adjacent tubules contain only eosinophillic masses (E). 312X

Figure 22. Enlarged photograph of a seminiferous tubule within a testis taken from an experimental animal. Multinucleated cells (arrows) are comprised of eccentrically arranged spermatocytes. Spermatogonia (G) are still present in their normal location adjacent to the basement membrane of the tubule. 500X

Figure 23. Enlarged photograph of a seminiferous tubule within a testis taken from an experimental animal. Two degenerating multinucleated cells (arrows) are shown. The background material (e) is associated with these degenerating cells is of a similar density to the eosinophillic masses indicated in the previous figures. 500X

Figure 24. Seminiferous tubule within a testis taken from an experimental animal. Eosinophillic masses (E) are apparent. Remnants of a degenerated multinucleated cell (D) are seen also. 500X

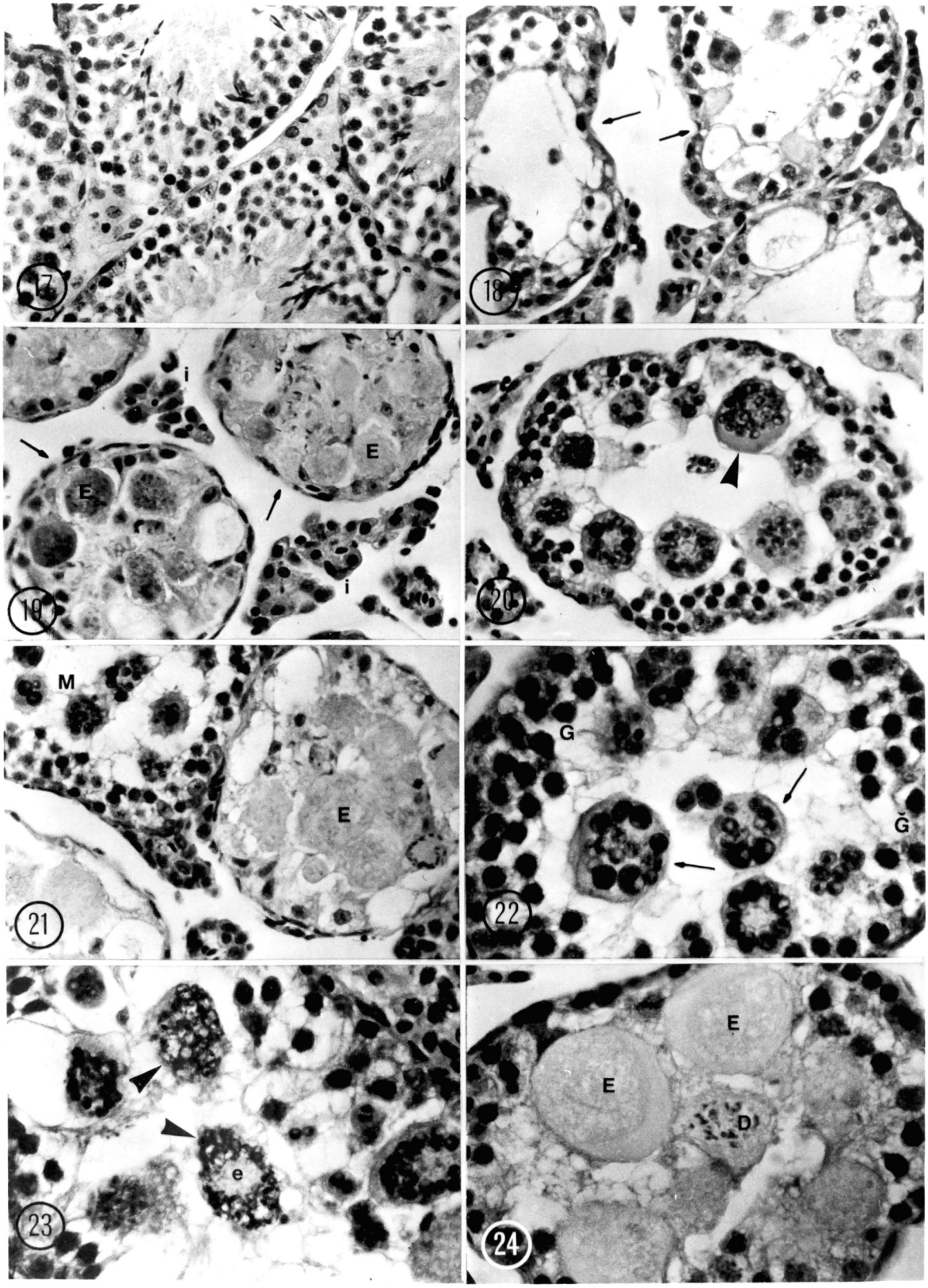
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PLATE V. Tissue sections of ventral prostate and seminal vesicle taken from animals treated six weeks with 5-Thio-D-Glucose and corresponding control tissues. Delafield's hematoxylin and Eosin Y.

Figure 25. Photograph of ventral prostate tissue taken from a control animal. Secretory epithelial cells (S) are columnar shaped with basally located nuclei and contain characteristic clear staining Golgi zones (arrow) in the apical portion of the cells. Secretory material (M) is seen within the lumen of the gland. 312X

Figure 26. Photograph of ventral prostate tissue taken from an experimental animal. Secretory epithelial cells appear normal as they are columnar shaped and show the Golgi zone (arrow) in the apical portion of the cells. The glandular lumen contains secretory material (M). 312X

Figure 27. Seminal vesicle tissue taken from a control animal. Secretory epithelial cells are columnar with nuclei (white arrow) located in the basal half of each cell. Cells show the Golgi zones (black arrow) apical to each nucleus and secretory material (M) is abundant within the lumen of the gland. 312X

Figure 28. Seminal vesicle tissue taken from an experimental animal. Secretory epithelial cells (S) are columnar shaped with basally located nuclei. Clear staining Golgi zones (arrow) are apparent and secretory material (M) is abundant within the lumen of the gland. 312X

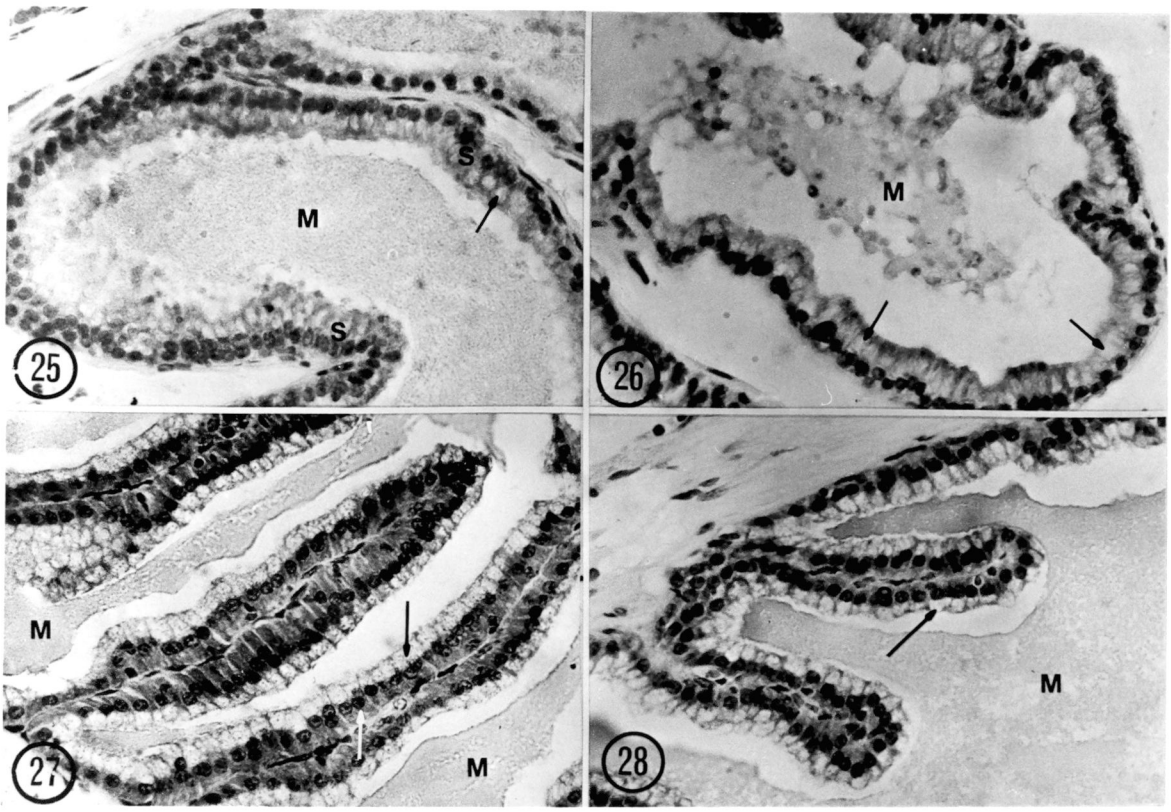
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PLATE VI. Histochemical demonstration of diaphorase and Δ^5-3-β-hydroxysteroid dehydrogenase (HSD) enzyme activity in testicular tissues from animals treated with 5-Thio-D-Glucose using pregnenolone as substrate. Corresponding control tissues are represented also. 80X

Figure 29. Diaphorase reaction in a testis taken from a control animal. Seminiferous tubules (T) demonstrate some enzymatic activity but the interstitum (arrows) shows very dark formazan deposits characteristic of greater activity.

Figure 30. Diaphorase reaction in a testis taken from an experimental animal. Seminiferous tubules (T) show some enzymatic activity but the most reactive tissue is the interstitum (arrow).

Figure 31. Δ^5-3-β-HSD activity in testicular tissue taken from a control animal maintained two weeks. The interstitum (arrow) shows greater enzymatic activity than do seminiferous tubules (T).

Figure 32. Δ^5-3-β-HSD activity in testicular tissue taken from an experimental animal treated two weeks. Interstitial tissue (arrow) shows greater enzymatic activity than seminiferous tubules (T) do.

Figure 33. Δ^5-3-β-HSD activity in testicular tissue taken from a control animal maintained four weeks. Interstitial tissue (arrow) shows greater enzymatic activity than do seminiferous tubules (T).

Figure 34. Δ^5-3-β-HSD activity in testicular tissue taken from an experimental animal treated four weeks. Formazan deposits are seen in the interstitum (arrow) indicating that enzymatic activity is present. Seminiferous tubules (T) show very little activity.

Figure 35. Δ^5-3-β-HSD activity in testicular tissue taken from a control animal maintained six weeks. Enzymatic activity is greater in interstitial tissue (arrow) than in seminiferous tubules (T).

Figure 36. Δ^5-3-β-HSD activity in testicular tissue taken from an experimental animal treated six weeks. Formazan deposits in the interstitum (arrow) appear larger than in corresponding control tissue. Little activity is seen in seminiferous tubules (T).

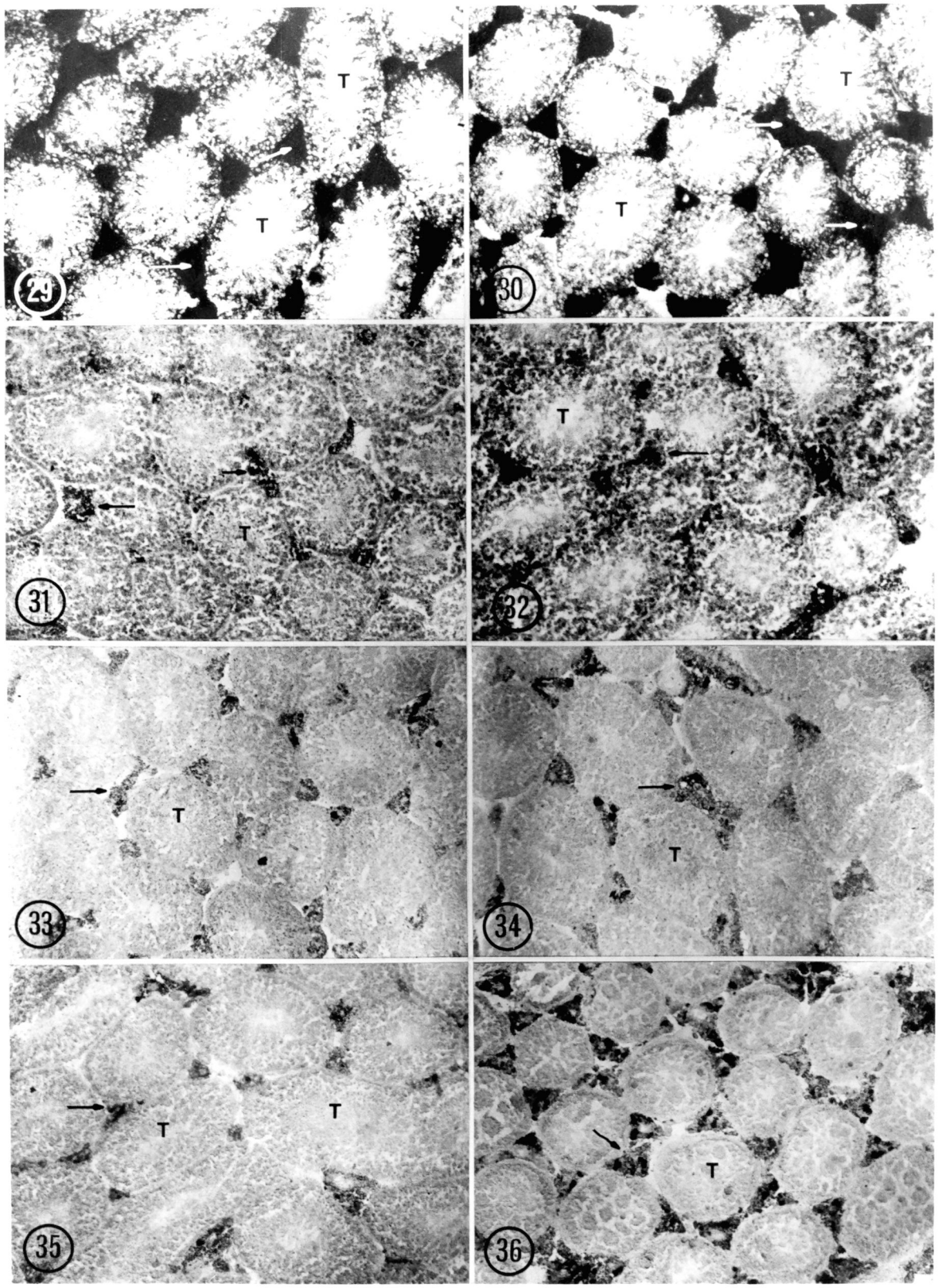
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Plate VII. Histochemical demonstration of Δ^5-3- β-hydroxysteroid dehydrogenase (HSD) enzyme activity in testicular tissue treated with 5-Thio-D-Glucose using dehydroepiandrosterone as substrate. Corresponding control tissues are represented also. 80X

Figure 37. Δ^5-3- β-HSD activity in testicular tissue taken from a control animal maintained two weeks. The interstitum (arrow) shows greater activity than seminiferous tubules (T) do.

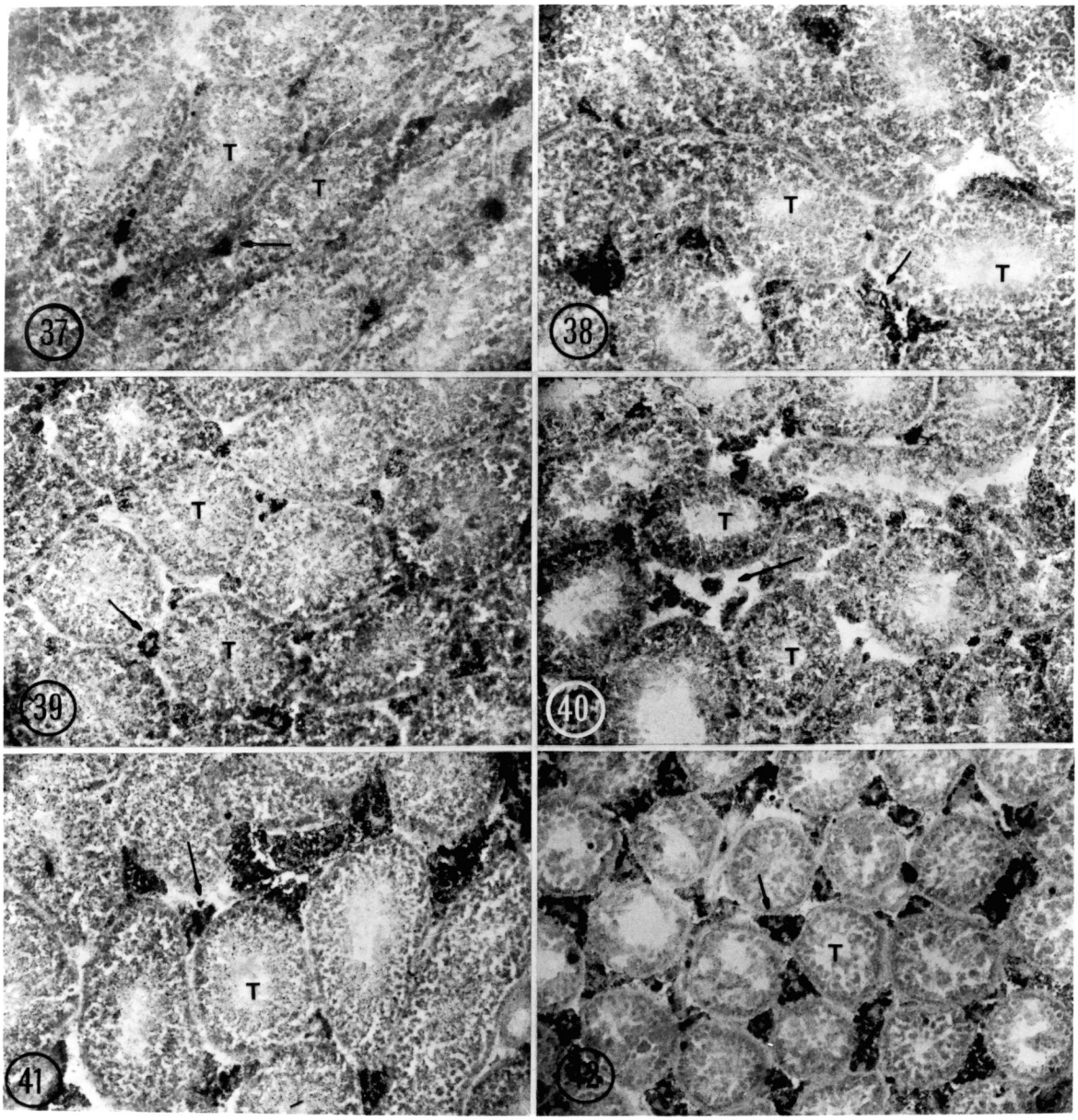
Figure 38. Δ^5-3- β-HSD activity in testicular tissue taken from an experimental animal treated two weeks. Activity is seen within the interstitial tissue (arrow) while seminiferous tubules (T) show little activity.

Figure 39. Δ^5-3- β-HSD activity in testicular tissue taken from a control animal maintained four weeks. Interstitial tissue (arrow) shows greater enzymatic activity than do seminiferous tubules (T).

Figure 40. Δ^5-3- β-HSD activity in testicular tissue taken from an experimental animal treated four weeks. Greater enzymatic is seen in interstitial tissue (arrow) than within seminiferous tubules (T).

Figure 41. Δ^5-3- β-HSD activity in testicular tissue taken from a control animal maintained six weeks. Enzymatic activity in the interstitium is apparent (arrow) while seminiferous tubules (T) show little activity.

Figure 42. Δ^5-3- β-HSD activity in testicular tissue taken from an experimental animal treated six weeks. The relative mass and color intensity of formazan deposits in interstitial tissue (arrow) appears greater than in corresponding control tissues. Seminiferous tubules (T) show little enzymatic activity.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Summary

Administration of 5-thio-D-glucose did interrupt the normal spermatogenic process and cause testicular atrophy in male mice receiving the drug from four to six weeks. In addition, the drug caused reduced fecundity to complete sterility in these mice, yet normal fertility was regained upon discontinued administration of this substance. The drug caused no apparent degenerative changes in androgen synthesis or utilization. It is concluded; therefore, that while 5-thio-D-glucose does effectively inhibit spermatogenesis and reduce fertility, it does not simultaneously affect androgen secretions or cause undesirable side effects like most of the previously employed spermatogenic inhibitors. The reversible effects of this drug along with the fact that it causes no decrease in libido or degenerative effects on male secondary sex characters make it unique.

Table I. Results of t-test analyses of morphometric measurements taken from testes, seminal vesicles and prostates at each time interval.

Time in Weeks	Tissue	Level of Significance
T-2	Testis	Not significant
	Prostate	Not significant
	Seminal Vesicle	.05 significance*
T-4	Testis	.01 significance**
	Prostate	.01 significance*
	Seminal Vesicle	Not significant
T-6	Testis	.01 significance**
	Prostate	.01 significance*
	Seminal Vesicle	Not significant

*Significant levels shown pertained to increased height of secretory epithelium from experimental tissues.

**Significant levels shown pertained to decreased diameter of seminiferous tubules from experimental tissues.

Table II. Subjective evaluation of Δ^5-3β-dydroxysteroid dehydrogenase activity at two intermediate sites of androgen biosynthesis within the testis. Control versus animals administered 5-thio-D-glucose for two, four, and six weeks. P(Pregnenolone), DHA(Dehydroepiandrosterone).

Group	Treatment Interval (Weeks)	Substrate	Seminiferous Tubule	Interstitium
Control	T-2	P	0	++
		DHA	0	++
	T-4	P	±	++
		DHA	±	++
	T-6	P	±	++
		DHA	±	++
Experimental	T-2	P	0	++
		DHA	0	++
	T-4	P	±	++
		DHA	±	++
	T-6	P	±	++
		DHA	±	++

Table III. Post treatment breeding results on mice administered 33 mg drug/kg body weight for two, four, and six weeks as compared to control animals. All animals were bred as designated intervals until normal fecundity was regained.

Group	Treatment period	Week bread following treatment*	Average No. Young**
Control (daily water gavage)	two weeks	1st	11.5
		2nd	12.5
	four weeks	1st	12.0
		2nd	13.0
	six weeks	1st	12.0
		2nd	15.0
Experimental (daily drug gavage)	two weeks	1st	8.6
		2nd	10.5
	four weeks	1st	9.5
		2nd	8.0
		4th	10.6
	six weeks	1st	0
		2nd	0
		4th	0
		6th	2.0
		9th	13.5

*First week began with day taken off treatment.
**Average number young for those females pregnant at day 19.

Appendix A. Weight in milligrams of the testes, seminal vesicles and prostates from control and experimental animals at each time interval.

Time	Group	Testis	Seminal Vesicle	Prostate
T-2	Control	A-95.4 / B-97.3	75.7	4.5
		A-95.4 / B-92.3	149.4	12.8
	Experimental	A-133.9 / B-134.7	175.5	16.1
		A-128.2 / B-134.4	157.2	13.3
T-4	Control	A-89.6 / B-86.9	118.9	11.9
		A-111.2 / B-110.8	173.6	10.0
	Experimental	A-106.9 / B-104.2	317.3	16.5
		A-136.1 / B-127.3	287.1	10.7
T-6	Control	A-112.2 / B-117.5	216.3	15.3
		A-85.9 / B-86.3	130.2	8.4
	Experimental	A-61.8 / B-66.9	324.8	11.4
		A-57.9 / B-54.4	170.8	11.6

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