METABOLISM OF GLUCOSE-1-^{14}C AND GLUCOSE-6-^{14}C BY TESTIS AND LIVER TISSUE FROM 5-THIO-D-GLUCOSE TREATED MICE

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ABSTRACT

Catherine A. Newton. METABOLISM OF GLUCOSE-1-^{14}C AND GLUCOSE-6-^{14}C BY TESTIS AND LIVER TISSUE FROM 5-THIO-D-GLUCOSE TREATED MICE. (Under the direction of Dr. Everett C. Simpson). Department of Biology, April, 1979.

Male Dub:ICR mice were treated with 5-thio-D-glucose at a rate of 35mg/kg/day for either 7, 14, 21, 28, or 42 days. At the end of each treatment period, radiorespirametric studies with glucose-1-^{14}C and glucose-6-^{14}C were done on testis and liver tissue from treated and control mice. C-1/C-6 ratio was calculated to determine the relative activity of pentose phosphate pathway to glycolysis-tricarboxylic acid pathway.

The liver tissue showed no significant difference in $^{14}CO_2$ percent recovery with one exception. A lower recovery from glucose-6-^{14}C during the 14-day period was observed in the treated. There was no difference in C-1/C-6 ratio for the liver.

By 42 days of treatment, the percent recovery from glucose-1-^{14}C was significantly higher in the treated testes. The C-1/C-6 ratio in the same period was also significantly higher, indicating increased pentose phosphate activity in the treated. There was no observable effect of the treatment on the $^{14}CO_2$ activity derived from glucose-6-^{14}C in the testis tissue.

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INTRODUCTION

The compound, 5-thio-D-glucose, is an analogue of D-glucose and is known to competitively inhibit D-glucose transport (Hoffman and Whistler, 1968). Recently, it has been shown to have antispermatogenic properties (Zysk et al., 1975; Homm et al., 1977; Lobl and Porteus, 1978; Fick, 1979). Varying degrees of success with sterility (from complete reversible sterility to permanent sterility) have been obtained. The mechanism of its action on the testis, at this time, is only speculative. One aspect of the testis in which a glucose analogue would have a direct bearing is the importance of glucose metabolism to spermatogenesis.

This study was designed with two objectives- to examine the effect of 5-thio-D-glucose treatment on glucose metabolism in the testis and in a nontesticular tissue. Liver tissue was selected as the nontesticular tissue because it is dependent on glucose and it has the same major route of glucose oxidation (glycolysis-tricarboxylic acid cycle and pentose phosphate pathway) as the testis. The activity of these pathways as well as their relative activity to one another were examined for both tissues using radiorespirametric studies and labeled forms of glucose.

REVIEW OF LITERATURE

In recent years an increased interest in birth control has stimulated research to find a pharmaceutical agent for male contraception. Before any substance can be considered clinically usable for male sterility, two prerequisites must be met. First, the effect of the substance must be reversible, and second, the chemical must not interfere with libido. Presently, there exist two approaches to chemically induced sterility- to interrupt spermatogenesis or to interfere with post-testicular maturation of spermatozoa (Gomes, 1970; Jackson, 1973; deKretser, 1976; Gomes, 1977).

The latter approach is possible since spermatozoa are both immotile and incapable of fertilization when they leave the rete testis. Spermatozoa acquire motility and the ability to fertilize, during passage through epididymis and vas deferens in route out of the body. The two major compounds currently being investigated in post-testicular maturation are cyproterone acetate, which prevents acquisition of motility (Morse et al., 1973; Koch et al., 1976) and α-chlorohydrin, whose mechanism of action is uncertain (Coppola, 1969; Ericsson and Baker, 1970; Kirton et al., 1970; Vickery et al., 1974). At first α-chlorohydrin appeared to interfere with sperm maturation. However, Vickery et al. (1974) showed sperm from rats treated with α-chlorohydrin were capable of fertilization when incubated with ova in vitro. Thus, the mode of action of the compound is still in doubt. Both of these compounds is correct dosage, are reversible and have no apparent effect on libido. High dosages of cyproterone acetate decrease testosterone

levels and, therefore, interfere with libido. A major advantage of this approach is its rapid onset of sterility (7-9 days) and recovery (7-11 days). Interruption of spermatogenesis to reach sterility requires 3 to 5 weeks and 6 to 12 weeks to recover, varying with the compound used and with the species being studied.

The disturbance of spermatogenesis can be either indirect by acting on the hormone (mainly follicle stimulating hormone, FSH) which controls spermatogenesis or direct by chemical agents acting on spermatogenesis. Several steroid compounds which suppress the secretion of LH are currently being investigated. Varying degrees of success have been achieved with testosterone propionate (Heller et al., 1950; Reddy and Rao, 1972), testosterone oenathate (Heller et al., 1970; Mauss et al., 1974), Danazol (Sherins et al., 1971; Skoglund and Paulsen, 1973, 6-medroxyprogesterone acetate (Macleod and Tietz, 1964; Patanelli, 1975), and testosterone undecanoate (Hirschhauser et al., 1975; Nieschlag et al., 1975). Some nonsteroids affecting FSH which are being studied are reserpine, 5-hydroxytryptaime, and methallibure (Jackson, 1973).

Most chemicals which are presently known to act directly on spermatogenesis are generally too toxic and/or cause unwanted side effects and, therefore, are impractical. Nitrofurans (Hollinger and Davis, 1966; Jackson, 1966; Hershburger, 1969), dichloracetyl amine (Heller et al., 1961), chlorambucil (vanThiel et al., 1972), cyclophosphamide (Inskeep et al., 1971; Fairley et al., 1972; Buchanan et al., 1975), and alkylating agents (Jackson, 1970) are examples of these types of chemicals. Other methods with direct effects on spermatogenesis currently being investigated involve various aspects of

testicular metabolism. At the present, an effect on glucose metabolism seems the most promising.

A requirement for glucose by the testis is indicated by the effects of low blood glucose levels or by conditions where glucose is unable to be properly utilized by the tissues. Spermatogenic degeneration and damage to seminiferous tubules (sloughing, nuclear pyknosis, and multinucleated cells) result from insulin-induced hypoglycemia in adult rats (Mancine et al., 1960) or from alloxan diabetes in rats (Deb and Chattergee, 1963). This type of damage, to a lesser degree, has been observed in diabetic impotent men studied by Fearman et al. (1972) and in untreated diabetes mellitus cases examined by Warren and LaCompte (1952). Additional observations that have been made in diabetic males are increased incidences of impotence (Schoffling et al., 1963), decreased sperm count (Babbott et al., 1958), and poor sperm motility (Kebanow and MacLeod, 1960).

The degree of dependence of the testis on carbohydrates as an energy source and on exogenous sources for these carbohydrates has been estimated by studies involving respiratory quotients (RQ) and oxygen uptake rates with or without exogenous substrates. The RQ's determined for the testis fall between total carbohydrate combustion (1.0) and total lipid combustion (0.7) in all species studied, thus, indicating that both serve as energy sources. Since the RQ's are closer to 1.0 than 0.7, the predominant source appears to be carbohydrates (Free, 1970). The rate of O_2 uptake decreases in the absence of glucose in rat testis by as much as 50% (Paul et al., 1952) while the rate in the liver can be maintained for over 3 hours (Dickens and Greville, 1933).

A similar drop, not as sharp however, is observable in rabbit testis (Ewing and Vandemark, 1963). These O_2 uptake studies indicate that the testis is dependent on an exogenous supply of glucose. Free (1970) calculated that approximately 80% of the O_2 consumed by the rat testis is utilized for glucose oxidation which would correspond to the decrease in O_2 uptake in the absence of glucose. Furthermore, an increased rate of O_2 uptake in the presence of glucose has been observed in rat (Elliott et al., 1937), rabbit (Ewing, 1967), and chicken (Ewing et al., 1964) suggesting that glucose is being oxidized. Free and Vandemark (1969) used radiorespirametric studies, where tissues are incubated with labeled glucose and $^{14}CO_2$ liberated is measured, to substantiate glucose oxidation within the testis. Additional evidence indicating glucose as an exogenous energy source for the testis has been provided by Means and Hall (1968b). Through in vitro tissue cultures, they observed that ATP levels declined in rat testis in the absence of glucose and that these levels could be maintained, even increased, in the presence of glucose.

A number of studies on the incorporation of lysine and other amino acids into testicular tissue indicates a relationship between glucose and protein synthesis in certain testicular cells. Davis and Morris (1963) with the addition of 0.009M glucose to the incubation medium increased the incorporation of lysine-U-^{14}C into proteins of rat testis tissue by 600%. Similar addition of glucose to sections of other tissues (thymus, spleen, kidney, brain, heart, liver, seminar vesicles) caused either no change or up to a 50% increase in lysine incorporation. When this type of study was done on testicular tissue lacking spermatids

such as the cryptorchid testis (Davis et al., 1964; Free et al., 1969) or immature testis (Means and Hall, 1968a), glucose displayed no stimulatory effect on amino acid incorporation into proteins. Therefore, it appears that this glucose enhancement on protein synthesis is confined mostly to the more mature germinal cells of the testis. Audioradiography studies by Davis and Firlit (1965) in the presence and the absence of glucose further substantiate this conclusion. They found that in the presence of glucose most of the radioactivity was incorporated into the spermatids and pachytene spermatocytes. Means and Hall (1968) found a correlation between lysine incorporation into testicular proteins and the levels of ATP and glucose. When glucose was added to the media at the time when ATP levels began to drop, both the concentration of ATP and the rate of protein synthesis increased. This indicates that the effect of glucose on protein synthesis is related to glucose enhancement of ATP production.

As a result of the series of investigations by Davis and associates and the work of others on glucose metabolism, protein synthesis, and cryptorchidism, Davis (1969) suggested that a possible clinically useful male contraceptive could be a glucose analogue (such as 5-thio-D-glucose). A glucose analogue could competitively inhibit the normal glucose functions thereby causing sterility. Davis theorized that the effects of an analogue would be completely reversible, since it is protein synthesis in spermatids that is dependent on glucose and not the earlier stages of spermatogenesis. Thus, reversibility of sterility would be imminent as the more immature cells, ones responsible for spermatogenic renewal, would not be affected by the glucose

deprivation.

The antispermatogenic properties of a glucose analogue, 5-thio-D-glucose (5-TDG), were first shown by Zysk et al. (1975). Swiss albino mice were fed 5-TDG at rates of 20-100 mg/kg/day for 7 weeks. The initial degeneration of the spermatogenic cells was noted in 1-2 weeks. After 4-6 weeks of treatment, sterility was achieved in mice receiving 30-100 mg/kg/day. This infertile condition, without any apparent impairment of libido, was maintained throughout the treatment period. Normal sperm development and fertility returned 5-8 weeks after treatment was discontinued. Normal litters were sired by males upon recovery. A testicular weight drop was observed with all dosages as would be expected due to the decrease in numbers of testicular cells. Also, a decrease in body weight was observed at high dosages. This decrease was assumed to be due to the diabetogenic action of 5-TDG which occurs when dosages are in excess of 50mg/kg (Hoffman and Whistler, 1968). Fick (1979) observed similar results with 5-TDG using a daily dosage of 33 mg/kg in Dub:ICR mice.

The antispermatogenic effect of 5-TDG received immediate attention as a promising nonhormonal male contraceptive (Maugh, 1974; Ricketts, 1974; deKretser, 1976). However, additional fertility-sterility studies in rats and mice contradicted the earlier results (Homm et al., 1977; Lobl and Porteus, 1978). Homm et al. (1977) treated male Wister rats with 5-TDG in 50, 25, 12.5 mg/kg daily dosages. Sterility was achieved in 8 weeks for 50 and 25 mg/kg groups and in 14 weeks for 12.5 mg/kg group. Although histological examination of the testis showed a similar condition as in the mice examined by Zysk et al. (1975)

(i.e. Sertoli cells and spermatogonia present), the sterility was permanent. One year after the drug was discontinued, fertility had not returned.

Lobl and Porteus (1978) subjected male CF_1 mice to a dosage of 50 mg/kg/day of 5-TDG for seven weeks. Contrary to the work of Zysk et al. (1975), only 33% of the mice recovered and were fertile 10 weeks after the drug was discontinued. These fertile mice on examination had reduced testicular weight and numerous aspermatogenic tubules, indicating some degree of permanent damage from the treatment. Although these recent fertility-sterility studies are disappointing, they do not discount the ongoing research studies with 5-TDG and its antispermatogenic capacity. Even if 5-TDG does not prove to be useful as a male contraceptive, the information about the testis obtained from these studies will add to a more complete understanding of testicular functions and, possibly, another approach to male contraception.

One major issue raised after the initial sterility study with 5-TDG was its possible side effects to the brain (Kakat, 1974). Since glucose is one of the major metabolites in the brain (Kakat, 1974) and 5-TDG interfers with D-glucose transport (Whistler and Lake, 1972), it is feasible that 5-TDG could affect functions within the brain. Bushway et al. (1977) conducted maze performance tests on Sprague-Dawley rats treated with 0, 50, or 100 mg/kg/day of 5-TDG for 14 days. They found no significant difference between the treated and the control groups in terms of the ability of the rats to rerun the maze or to solve problems of Hebb-William maze after treatment. Even though 5-TDG could be affecting regions of the brain other than learning and memory,

this initial study is encouraging.

The action of 5-TDG on spermatogenesis and the testis is uncertain. The compound, which has a sulfur atom substituted for the oxygen atom in the pyranose ring, is considered the closest existing analogue of D-glucose. Due to the similarities of its physical and chemical properties to D-glucose, 5-TDG is capable of competitive inhibition of both active and facilitated diffusion transport of D-glucose as well as D-xylose. The diabetogenic action of 5-TDG is due in part to this interference in cellular transport processes of D-glucose (Whistler and Lake, 1972). Little or no metabolism of 5-TDG appears to occur within tissue, since 97% is excreted into the urine within 24 hours (Hoffman and Whistler, 1968). However, 5-TDG has been demonstrated to serve as a substrate, although a poor substrate, for yeast hexokinase (Hoffman and Whistler, 1968) and rabbit skeletal muscle phosphoglucomutase (Chen and Whistler, 1975). In addition, 5-TDG can interfere with glycogen utilization by non-competitive inhibition of phosphorylase _a_ and _b_, and by inactivation of phosphorylase _a_ through conversion to the _b_ form (Chen and Whistler, 1977). Whether one or a combination of these factors, or as yet some undiscovered factor, are responsible for the antispermatogenic ability of 5-TDG is the basis of several current investigations.

One mode of action for 5-TDG could be through its diabetogenic action, since diabetic condition in males attributes to fertility problems, as previously mentioned. However, when male Sprague-Dawley rats were given 50 mg/kg/day of 5-TDG and 0.5 unit/day of insulin (the dosage that counteracts the diabetogenic effect), spermatogenesis was

still impaired (Lobl and Porteus, 1978). This study also found that after 30 days of treatment with 5-TDG alone, the diabetogenic effect was not present indicating that the rats were refractory to the effect. This study implies that some action other than interference with D-glucose transport is responsible for antispermatogenic effect.

Another study supporting this conclusion was done by Burton and Wells (1977). They conducted a study on 5-TDG effect on myo-inositol and glucose-6-phosphate levels in the testis. Myo-inositol, although its function is unclear, is synthesized in the testis at high rates from glucose (Middleton and Setchell, 1972). Male Spb HCR mice were treated with 5-TDG at 50 or 250 mg/kg/day rates for 7 days at which time the myo-inositol and glucose-6-phosphate levels were measured. They found elevated myo-inositol levels in both the 50 and 250 mg/kg groups indicating an increased synthesis from glucose. The glucose-6-phosphate levels were normal in the 50 mg/kg group, but were significantly elevated in the 250 mg/kg group. These increased levels of both compounds imply some mechanism other than reduced intracellular glucose is involved in 5-TDG antispermatogenic activity.

Additional research on the direct action of 5-TDG on spermatogenesis at the present is limited. Working with phenylalanine-U-^{14}C incorporation into testicular proteins, Nakamura and Hall (1976, 1977) observed varying results between whole testis sections and different testicular cell fractions. In the earlier study (1976), normal incorporation occurred in the whole testis from a rat treated with 5-TDG for 2 days, whether 5-TDG was present in the media or not. When the testicular cells were separated into fractions by centrifugal

elutriation, however, inhibition from 5-TDG was observed in the immature spermatid fraction and, to a lesser degree, in the mature spermatid and heterogenous cell fractions (both fractions were contaminated with immature spermatids). In the later study (1977), they used more purified and additional cell fractions. Inhibition of amino acid incorporation occurred in the spermatocyte fraction. However, when glucose was present in the media, it protected against this inhibition. Since exogenous glucose is present in the testis, it is doubtful this effect would occur *in vivo*. No inhibition of incorporation was observed in Sertoli or Leydig cell fractions. Their results correspond to earlier studies of glucose effect on protein synthesis (Davis *et al.*, 1964; Free *et al.*, 1969; Means and Hall, 1968; Davis and Firlit, 1965). These earlier studies showed the enhancing effect from glucose on amino acid incorporation into testicular proteins was specific for the spermatid stage of spermatogenesis.

Recent studies of 5-TDG indicate that the drug's effect is not confined to glucose transport. It would, therefore, be advantageous to examine 5-TDG treated testes for changes in glucose metabolism pathways. The major pathways within the testis are glycolysis-tricarboxylic acid (TCA) pathway, pentose phosphate pathway (PPP), and glucuronate-gulonate pathway, with the glycolysis-TCA predominating (Free *et al.*, 1969; Free, 1970). Each of these pathways, with their different functions, seem to concentrate their activity in different cell types. Experiments indicate that the glycolysis-TCA pathway, which is predominately involved in energy production, is most active in spermatids and spermatocytes (Free, 1970). Leydig cells and

spermatogonia appear to be the major sites for the PPP, which generates NADPH and D-ribose (Free, 1970). The glucuronate-gulonate, which is an alternate source of pentoses, apparently is most active in the interstitial cells (Free et al., 1969). Since 5-TDG has been shown to have no effect on the libido, therefore, no effect on the interstitial cells (Zysk et al., 1975; Homm et al., 1977; Lobl and Porteus, 1977; Fick, 1979) and since glucuronate-gulonate pathway is not as active as the other two pathways, it was excluded in this study. The present study attempts to ascertain the effect of 5-TDG on glucose metabolism in the testis throughout the period required to reach sterility by using radiorespirametric procedures. By using glucose-1-^{14}C (G-1-^{14}C) and glucose-6-^{14}C (G-6-^{14}C), the relative activity of PPP to glycolysis-TCA pathway was examined.

The possibility exist that tissues other than testis are being affected by the 5-TDG treatments. Therefore, another aspects of this study was to examine the effect of 5-TDG on a non-testicular tissue. Glucose is important to the function of most tissue in the body, especially the brain and the liver. Any compound entering the blood supply from the intestines, such as 5-TDG given orally, passes through the liver. Malfunctions of glucose metabolism are responsible for several liver disorders such as Kwashiorkor syndrome and liver phosphorylase deficiency (Banks et al., 1976). For these reasons and because glucose is oxidized in the liver by the same major pathways active in the testis (Altszuler and Finegold, 1974), liver was selected as the nontesticular tissue for the additional radiorespirametric studies,

MATERIALS AND METHODS

Animals

One hundred and thirty adult male mice of the Dub;ICR strain were used in this study. All mice were sexually mature (13 to 14 weeks old) upon initiation of the experiment. They were maintained at approximately 22°C with a 12-hour light regime throughout the study. Wayne Laboratory Rat Chow and water were provided ad libitium.

Treatment

The treated animals were administered a daily dosage of 5-thio-D-glucose (Pfanstiehl Laboratories, Waukegan, Illinois) at a dosage level of 37 mg per kg of body weight. The compound was dissolved in water and was given orally by intubation in 0.25ml, 0.275ml, or 0.3ml aliquots depending on the size of the mouse. The mice were lightly etherized to facilitate the intubation procedure. The control mice were treated in the same manner with the exception of receiving only water. The experiment was divided into five treatment periods of 7, 14, 21, 28, and 42 days.

Design of Experiment

The experiment was designed so that 5 incubation runs could be made weekly (i.e. five runs per treatment period). Each run involved 4 separate incubation tests- Glucose-1-^{14}C (G-1-^{14}C) treated, G-1-^{14}C control, Glucose-6-^{14}C (G-6-^{14}C) treated, and G-6-^{14}C control- for the liver and for the testes, resulting in a total of 8 tests. Two control and two treated mice were sacrificed 3 hours after the final daily treatment to initiate each run. Small sections of testes from

one of the treated and one of the control mice were incubated individually in flasks containing G-1-^{14}C, while testis sections from the other two mice were incubated with G-6-^{14}C. Liver sections from the same mice were incubated in similar, but separate flasks. Each run, therefore, had 8 flasks which correspond to the 8 incubation tests being done. By running the control and treated tissue simultaneously an attempt was made to eliminate factors due to incubation procedures. In addition, one control and one treated mouse was sacrificed every 7 days for histological studies of the testis and the liver.

Tissue Preparation and Incubation

The testes and one lobe of the liver were removed immediately after cervical dislocation of the mouse and placed in cold Krebs-Ringer Phosphate Buffer pH 7.4 (Appendix A). The tissues were cut into smaller portions and weighed on a Torsion Balance. Then, a small portion of each tissue of a known weight (100±20 mg), were cut into sections with scissors and placed into the appropriate incubation flask containing either Glucose-1-^{14}C (specific activity 55.56mCi/mmol) or Glucose-6-^{14}C (specific activity 52.8mCi/mmol) (New England Nuclear Laboratories).

Each incubation flask contained 3.0ml of Krebs-Ringer Phosphate Buffer pH 7.4 plus 180µg(300 units) of penicillin G, 3mg of D-glucose, and 0.25µCi of glucose-^{14}C. The flask was constructed from a 50ml heavy walled erlenmeyer flask to which a section (2.5 cm) of 15mm pyrex glass tubing was fused as a sidearm. A scintillation vial was connected to this sidearm through a 15mm hole drilled in the vial cap. Between the cap and the top of the vial was placed an "O" ring

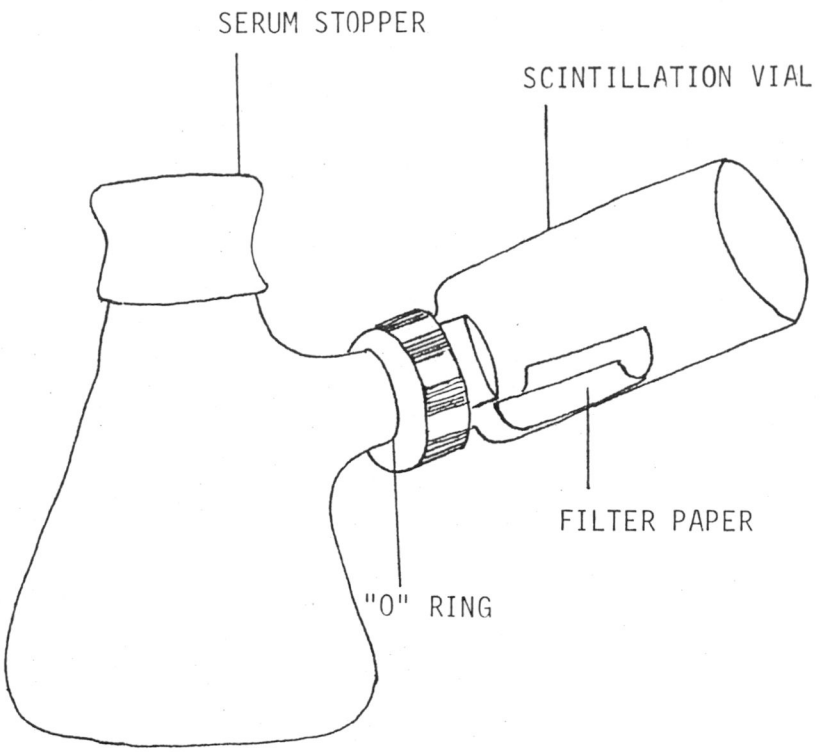


Figure 1. Schematic diagram of the incubation flask.

(9/16" x 3/4") to ensure an airtight seal. A serum stopper was used to cap the flask. (Figure 1). A section of Whatman No. 1 filter paper (1.5 cm^2) soaked with 0.2 ml of 10% KOH was placed within the vial to absorb the $^{14}CO_2$ (Buhler, 1972). The flask design allowed for easy removal and exchange of vials and the filter papers within vials at will without interrupting the ongoing tissue incubation.

The incubations were carried out in two shaker baths at 70 cycles per minute at the appropriate temperature for each tissue type (33°C for testes; 37°C for liver). After 5 hours of incubations, 0.2ml of 10N H_2SO_4 was added to each flask to terminate enzymatic processes and to facilitate the release of $^{14}CO_2$ from the tissues. The flask continued to shake for an additional hour to allow for equilibration. During the incubation period, filter papers were exchanged after the first, second, fifth, and sixth hour.

Counting

The filter papers were removed from the sidearm and placed on sheets of aluminum foil to dry. Once dried, they were placed into individual scintillation vials containing 15ml of fluid. This fluid was composed of toluene with 0.5% PPO (2,5 diphenyloxazole) as the primary fluor and 0.05% POPOP (1,4 bis-2-(4-Methyl-5-phenyloxazolyl-)-Benzene) as the secondary fluor. The scintillation vials were then placed in the dark at 5°C for 12 hours to ensure maximum counting efficiency.

The counts per minute (cpm) of the samples were determined with a Packard Instrument Co. Series 3000 Liquid Scintillation Counter at 0°C. The three channels were set identically (Gain 18; Window 0.5-10)

for optimimum counting efficiency of ^{14}C. The samples were counted for 20 minutes or 20,000 counts, whichever came first, to minimize the error from background counts (Wang et al., 1975).

Histology

Histological studies were done to examine the tissue for abnormalities associated with 5-thio-D-glucose treatment and to gauge the progress of degeneration of the spermatogenic cycle.

The tissues were fixed in Carnoy's fixative (Appendix A) for 2-3 hours. Tissues were dehydrated in a series of ethanol solutions and cleared in xylene (Appendix B). Paraffin infiltration was done in a 58°C oven using 4 changes of hot Tissuemat. The Timstation Tissue Center was employed for the paraffin embedding. All tissues were sectioned at 6-8 microns and stained with Hemotoxylin-Eosin (Appendix B).

Chromatography

A series of paper chromatography were run to check for the presence of 5-thio-D-glucose in the testis, liver, and urine at 3, 6, and 12 hours after treatment. Homogenates of testis and of liver were made using a hand homogenizer. All three samples as well as 5-thio-D-glucose and D-glucose standards were spotted on Whatman No. 1 Chromatography paper. The chromatograms were done in a desending glass chamber with 1-butanol-ethanol-water (40:11:19) as the developing solvent for 12 hours.

Detection of spots on the dried chromatograms were made by the silver nitrate reagent method of Trevelgan, Procter, and Harrison (1950). The chromatograms were first passed rapidly through a silver

nitrate reagent (prepared by diluting 0.1ml of saturated aqueous silver nitrate solution with 20ml of acetone and then adding water dropwise until the precipitant that formed redissolved). After the papers were dried, they were sprayed with a 0.5N solution of sodium hydroxide in aqueous ethanol (made by diluting saturated aqueous sodium hydroxide in aqueous ethanol). Once the reduction of the silver by the sugar was completed, the chromatograms were immersed in 6N ammonium hydroxide for 5 minutes and then placed under running water for 1 hour. The chromatograms were dried in an oven at 100°C. The spots formed were compared to the 5-thio-D-glucose standards by color and R_f values.

Analysis

For each sample, the percent yield of $^{14}CO_2$ was calculated using the following formula: (cpm of sample)/(cpm initially added to incubation flask). The mean of the five runs of each organ for the four groups (G-1-^{14}C treated and control; G-6-^{14}C treated and control) and for each hour sampled were determined as well as the mean for the total first 5 hours. The total 5 hours values of the treated were statistically compared to those of the controls using one-factor analysis of variance (ANOV) for each treatment period and each tissue.

The C-1/C-6 ratio (% yield of $^{14}CO_2$ from G-1-^{14}C to % yield of $^{14}CO_2$ from G-6-^{14}C) was used as an index of pentose phosphate pathway activity. The ratios were determined using the percent yield values from the first hour for all tissues, treated and control, for each run and for each treatment period. The ratios for each treatment

period were statistically analyzed by one-factor ANOV for both the testis and the liver.

RESULTS AND DISCUSSION

Chromatography Studies

Paper chromatography studies were done to determine the presence of and to estimate the duration of 5-TDG in the testis. The data (Table 1) indicated that 5-TDG was present in the testis after 3

Table 1. A summary of R_f values of samples ran at 3, 6, and 12 hours after 5-TDG treatment.

Time (in hours)	D-glucose	5-TDG	Testis	Liver*	Urine
3	0.24	0.30	0.30	—	0.30
6	0.23	0.29	NP**	—	0.29
12	0.25	0.35	NP	—	0.35

*Could not distinguish spots in the liver samples
**Not present

hours, but not after 6 hours. This information corresponds to an earlier report by Hoffman and Whistler (1968) on 5-TDG and hyperglycemia. They found that the diabetogenic effect of 5-TDG was absent by the fourth hour after the injection due to the loss of the drug into the urine. They also determined that 97% of 5-TDG was excreted with the urine by 24 hours after the injection. In the present study, 5-TDG was detectable in the urine during all times sampled (3, 6, 12 hours); although, the size of the spots (i.e. the quantity of 5-TDG) did diminish with time. Liver homogenates contained substances that formed a streaking spot, thus, preventing the identification of 5-TDG.

Histological Studies

Histological work was carried out to compare 5-TDG treated testicular cells from this study to cells of previous 5-TDG studies and to indicate the approximate time sterility was achieved. Treated and control testes were examined for each treatment period. No histological changes were observed in the controls throughout the experiment. (Figure 2) As expected, the treated germinal cells of the seminiferous tubules did undergo degeneration. The progression of this degeneration was similar to that reported by Zysk et al. (1975), Lobl and Porteus (1978), and Fick (1979).

After 14 days of treatment, spermatozoa and spermatids were normal in appearance and there were a few morphological changes. These changes were mainly vacuolated areas at or near the basement membrane of seminiferous tubules and enlarged spermatogenic cells. (Figure 3) Spermatozoa were still present in the testes after 21 days of treatment, but there were increasing numbers of abnormalities such as sloughing of germinal cells, debris from degenerating cells, and a few multinucleated cells. (Figures 4 and 5) These multinucleated masses are fusion of degenerating spermatogenic cells and have been observed in all 5-TDG treated testes (Zysk et al., 1975; Homm et al., 1977; Lobl and Porteus, 1978; Fick, 1979). By 28 days of treatment, the degeneration had increased substantially; although, spermatozoa and spermatids were still present. There were irregular shaped spermatids as well as more and larger multinucleated cells. The degeneration of cells appeared to progress through the tubules unevenly. Some tubules were extremely abnormal, while others appeared

normal. (Figure 6 and 7) The histological appearance of the testes indicated that sterility did occur by the end of the final treatment period (42 days). Although the degeneration was in different stages within the seminiferous tubules, the testes and the epididymis were devoid of spermatozoa. Spermatids were still, present, but many of them were irregularly formed. Some tubules contained only eosinophilic cellular debris and a few scattered cells. Other possessed many of the multinucleated cells. Most all of the tubules had normal appearing spermatogonia and Sertoli cells. (Figures 8, 9, 10 and 11) None of the testes examined showed any detectable changes in the interstitial cells. In all testes examined, cells undergoing division, as indicated by dark staining chromatin, were visible.

This progression of degeneration was similar to that observed by Zysk et al. (1975). They found a few large spermatogenic cells after 14 days of treatment. By 28 days, the testes showed increased degeneration, multinucleated cells, and cytoplasmic vacuoles. Although some tubules were devoid of spermatids, most have spermatogenesis actively occurring. The epididymis of the 28-day treated testes still contained spermatozoa. By 35 or 42 days of treatment, spermatozoa were loss in all testes and the testes showed severe spermatogenic degeneration.

At the end of the final treatment period, two treated mice were allowed to recovery for 15 weeks. At this time, the testes were histologically examined. Spermatozoa were numerous in the epididymis and the seminiferous tubules. Most tubules were normal in appearance however, a few tubules (<10%) were still in a degenerative state.

Since there were only Sertoli cells and a few spermatogonia in these tubules, the damage was apparently permanent. (Figures 12 and 13)

Two mice remaining after termination of histological and radiorespirametric studies were continued on 5-TDG for an additional 14 days. However, the treatment was reduced from the previous daily administration to twice a week. At the end of that time (42 days of daily treatments followed by the 14 days of twice weekly treatments) the testes were removed and examined histologically. The appearance of the testes were similar to the 42 days testes. (Figures 14 and 15) These results suggests an alternate treatment program to the continuous daily treatments. In this alternate program, the drug would be given daily until the testes were devoid of spermatozoa (28 to 42 days in the mice), at which time treatment would be reduced to once or twice a week to maintain the sterile condition. If this approach works, there would be a two-fold advantage. By limiting the deleterous effect to the spermatids and the spermatozoa, the recovery of spermatogenesis would be more rapid. Also, the permanent damage observed in some tubules of 5-TDG testes in this and other studies (Homm et al., 1977; Lobl and Porteus, 1978) might be eliminated.

Liver tissue was also histologically examined. No morphological changes in the treated livers were detected in any of the sections.

Figure 2. A section from a control testis showing normal spermogenic epithelium. BM, basement membrane; L, lumen; S, sertoli cell; SG spermatogonium; ST, spermatid; SZ, spermatozoan; PSC, primary spermatocytes. Magnification: 180X

Figure 3. A section from a testis after 14 days of 5-TDG treatment. The photomicrograph shows normal spermatogenesis still occurring. Magnification: 180X

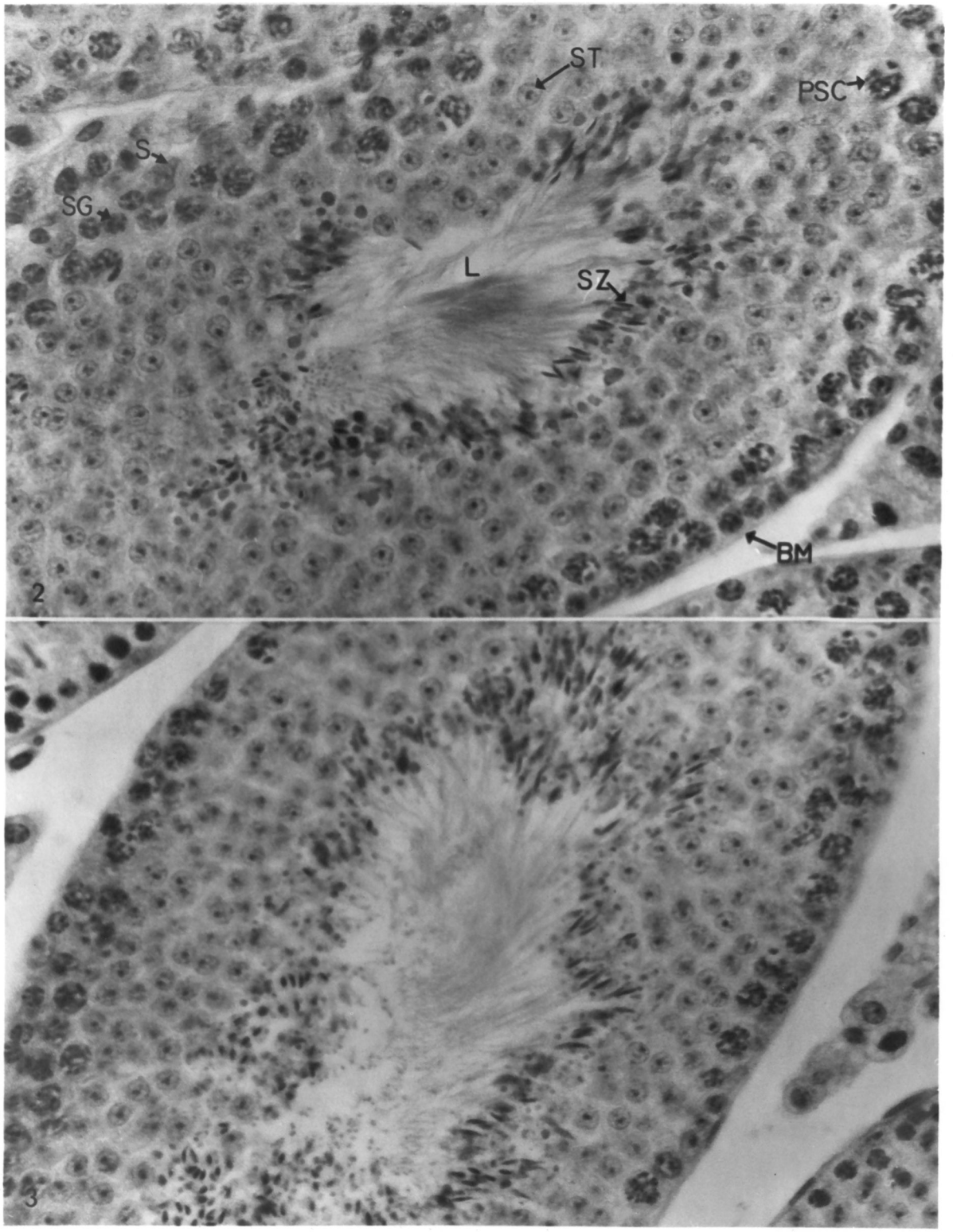
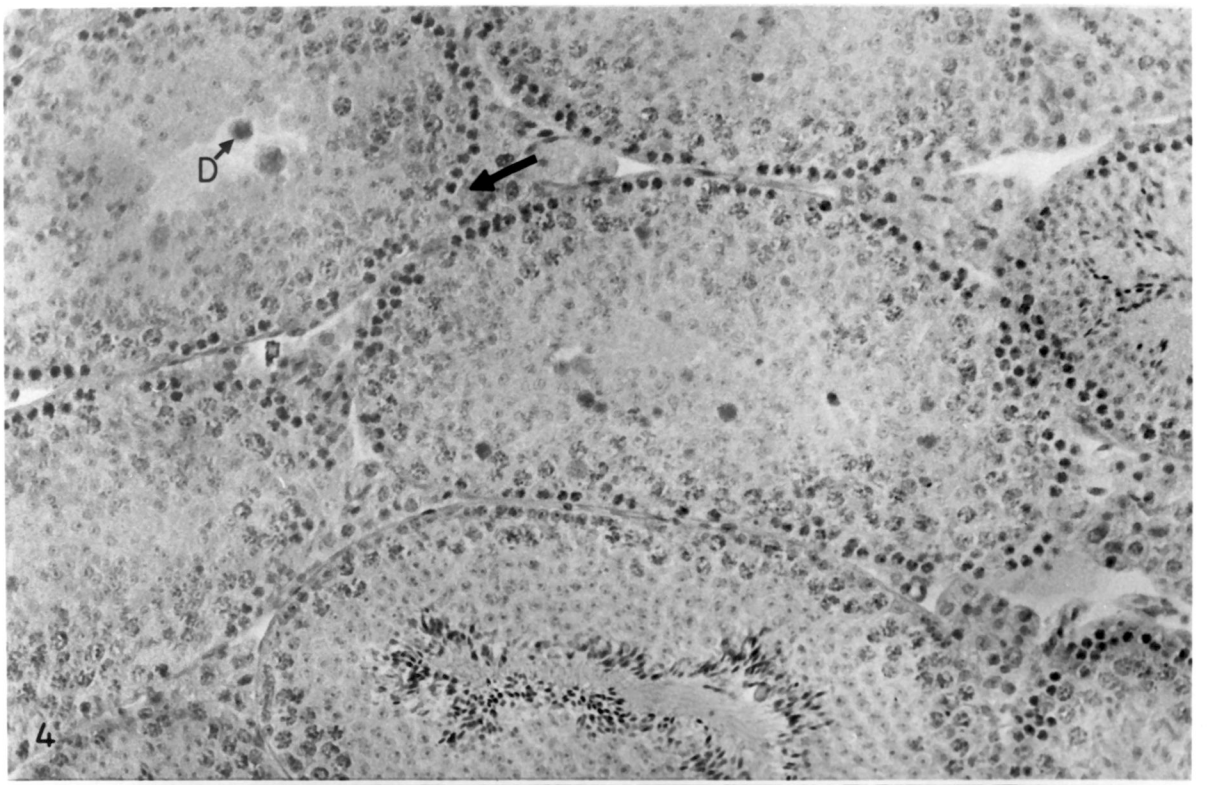

ST
PSC
S
SG
L
SZ
BM
2
3

Figure 4. A section from a testis treated with 5-TDG for 21 days. The beginning of degenerative products are present. Arrow indicates area magnified in Figure 5. D, degenerative product. Magnification: 180X

Figure 5. Area from Figure 4 at a higher magnification. Note the degenerative products in the lumen. Magnification: 450X



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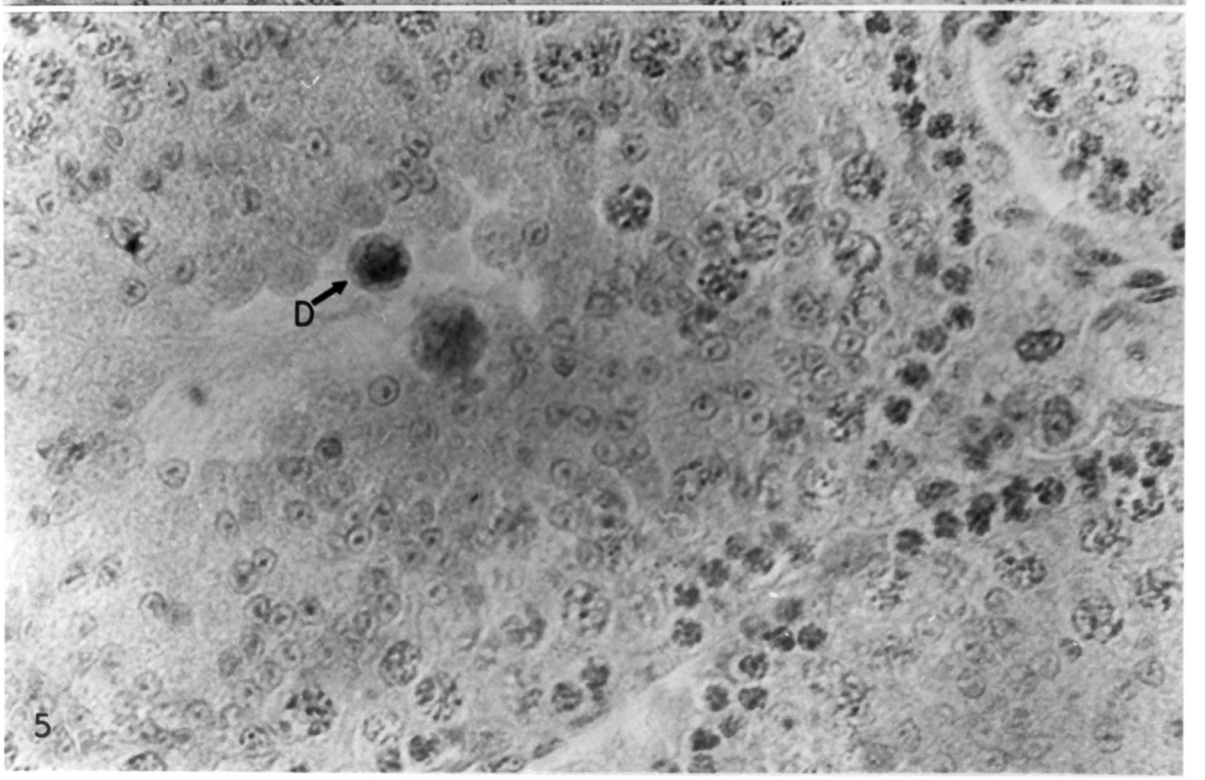

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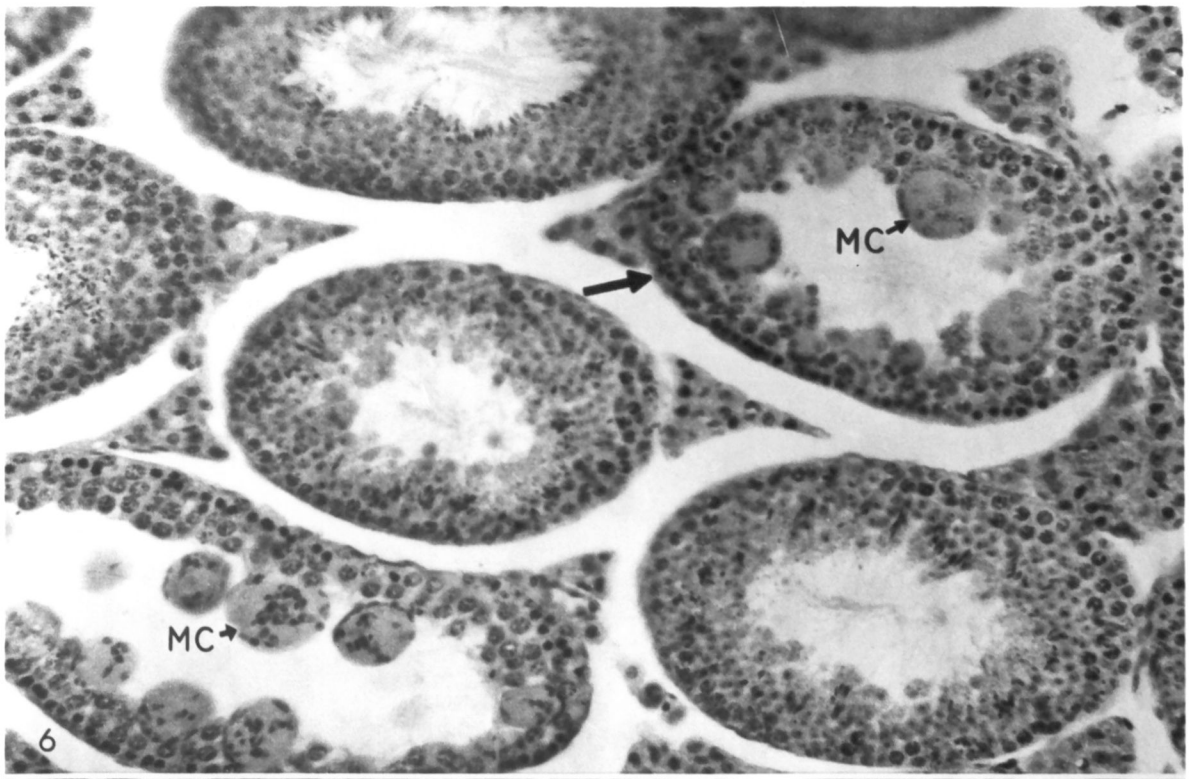
Figure 6. A section from a testis treated with 5-TDG for 28 days. Note the multinucleated cells (MC) is the tubules. Arrow indicates area magnified in Figure 7. Magnification: 180X

Figure 7. Area from Figure 6 at a higher magnification. Note multinucleated cells (MC) and spermatozoa tails in the lumen. Magnification: 450X


MC
MC
6

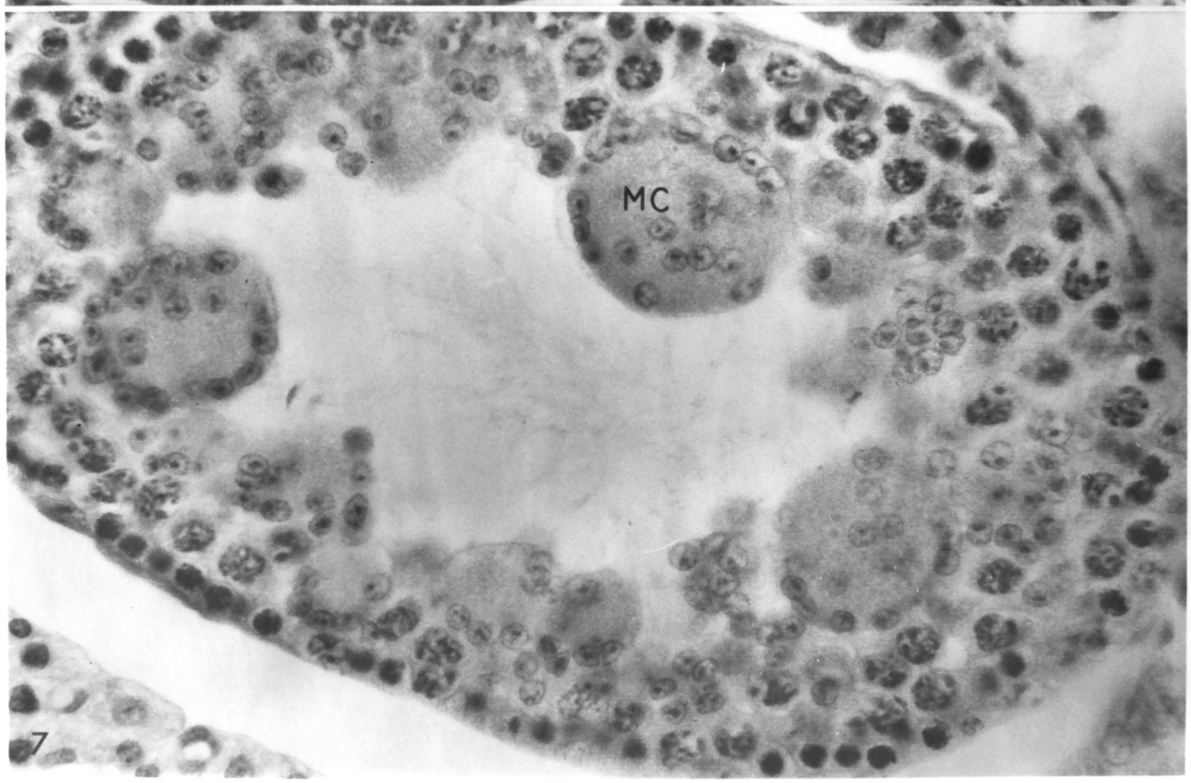
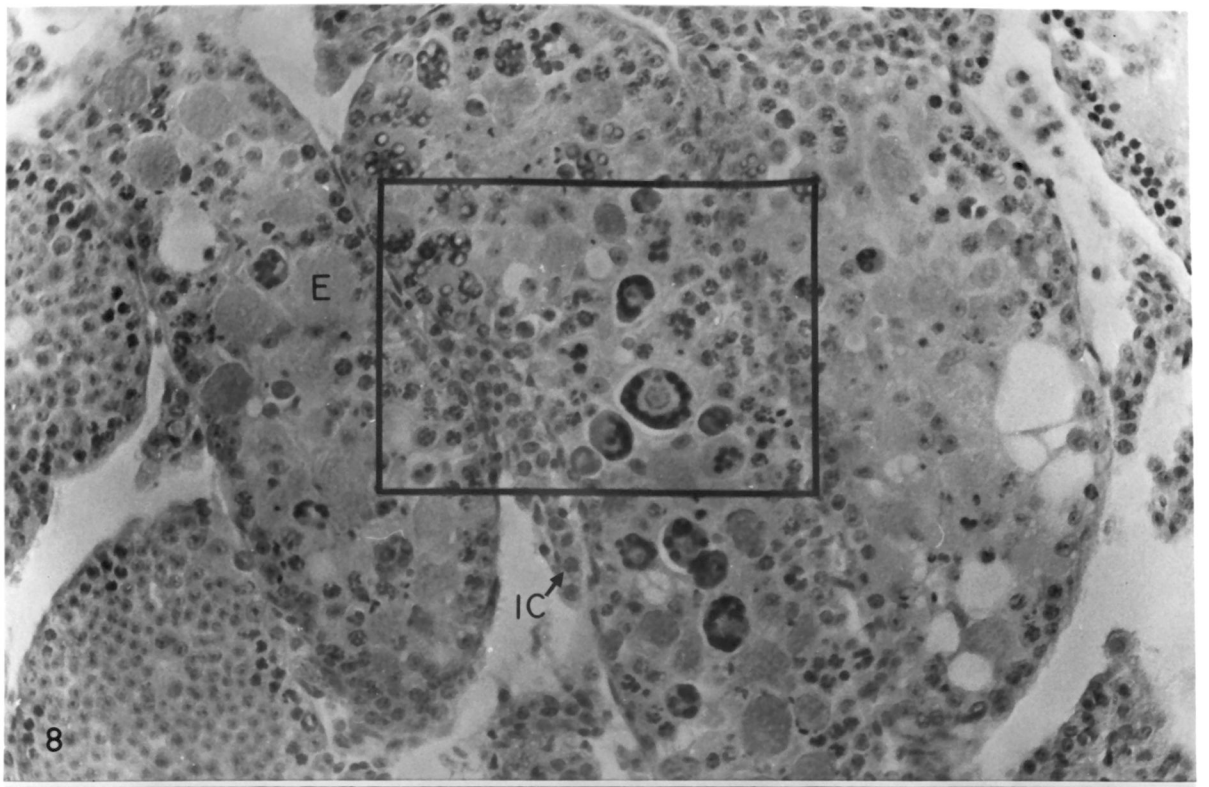

MC
7

Figure 8. A section from a testis treated for 42 days with 5-TDG. Note the disrupted spermatogenic epithelium with multinucleated cells and eosinophilic masses (E) in the lumen. Interstitial cells (IC) appear normal. Black indicates the area magnified in Figure 9. Magnification: 180X

Figure 9. Area from Figure 8 under higher magnification. E, eosinophilic masses; IC, interstitial cells; MC, multinucleated cells. Magnification: 450X


E
IC
8

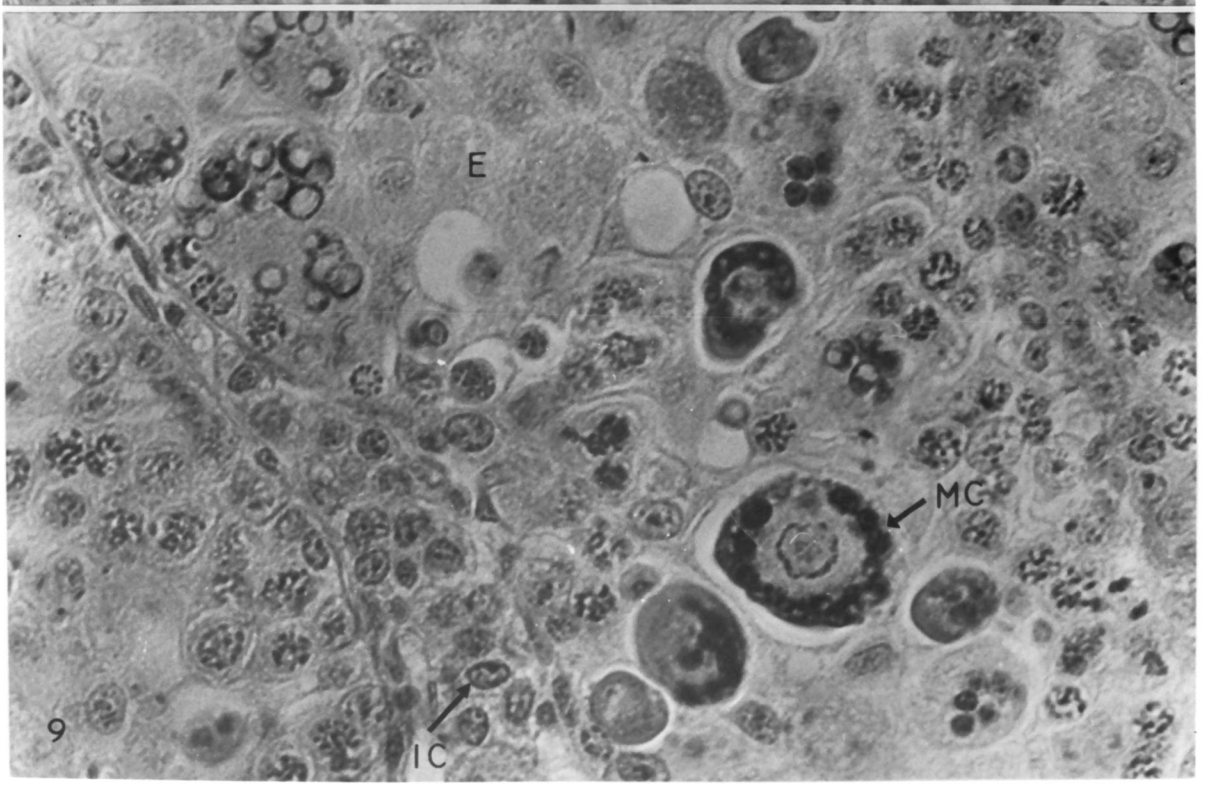
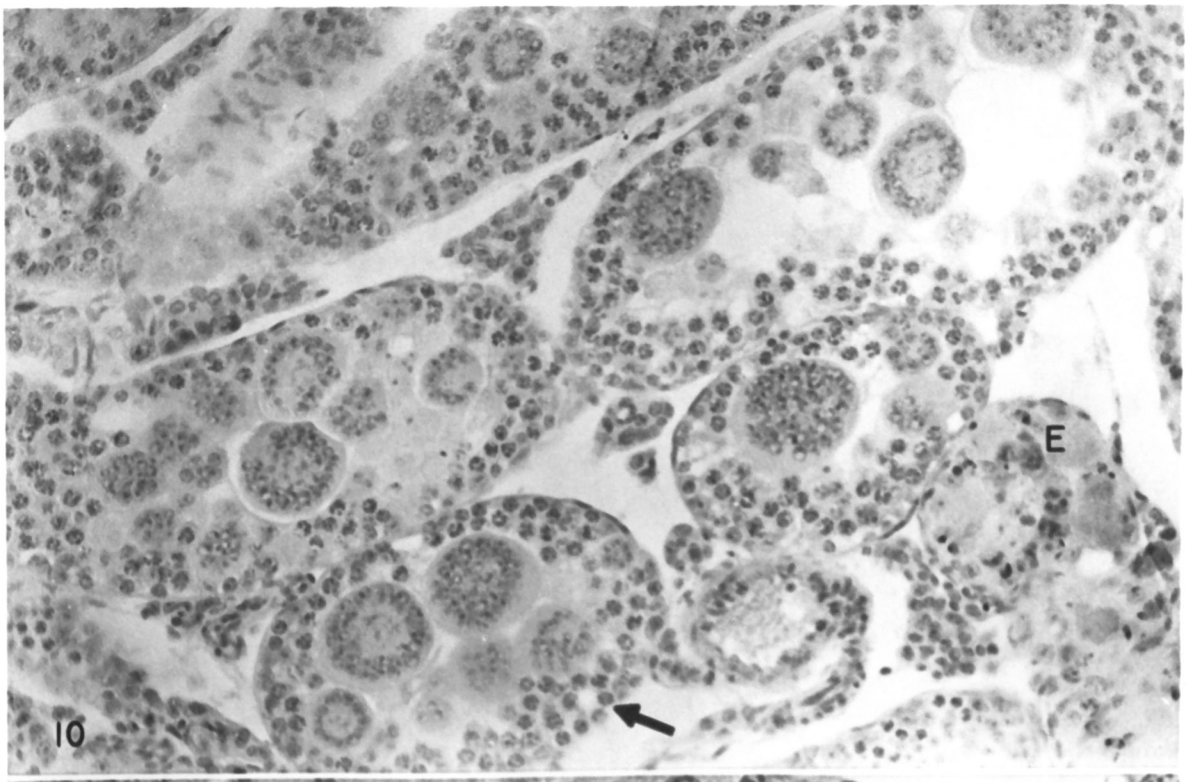

E
MC
IC
9

Figure 10. A section of the testis after 42 days of 5-TDG treatment, showing multinucleated cells. Arrow indicates tubule magnified in Figure 11. Magnification: 180X

Figure 11. Tubule from Figure 10 under higher magnification. Note multinucleated cells (MC) and primary spermatocytes still undergoing division. Magnification: 450X


E
10

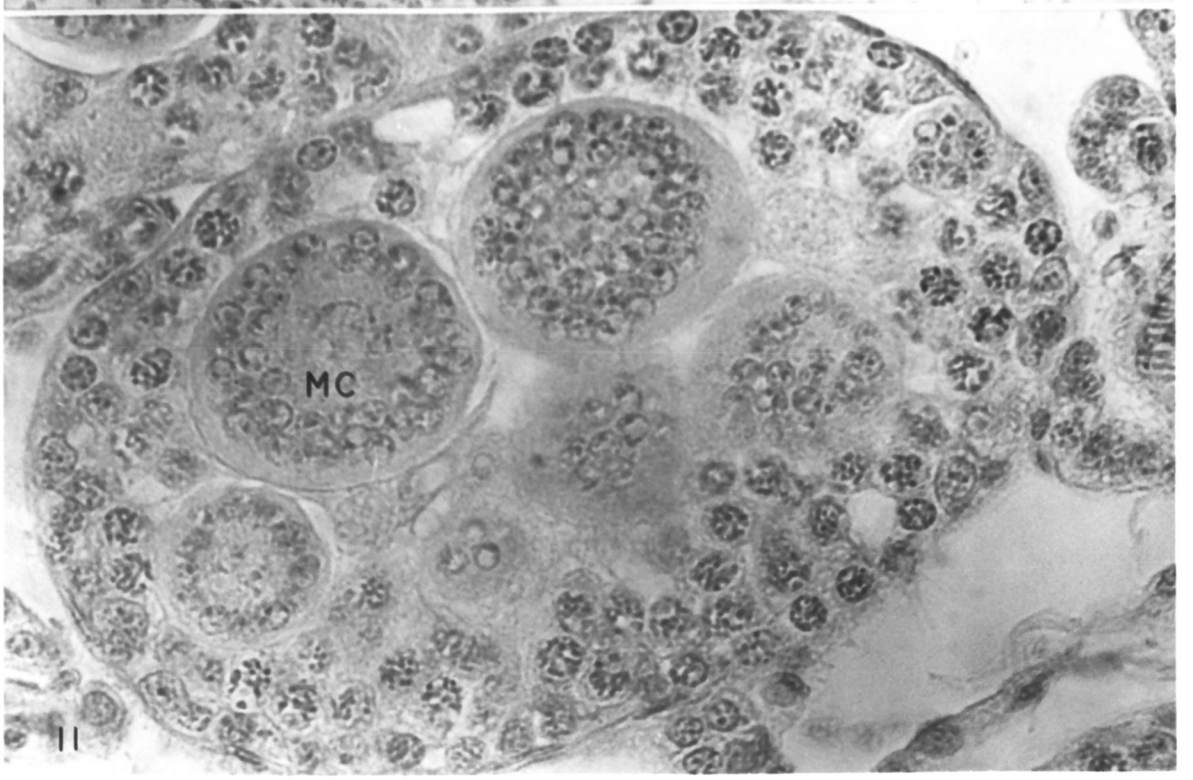
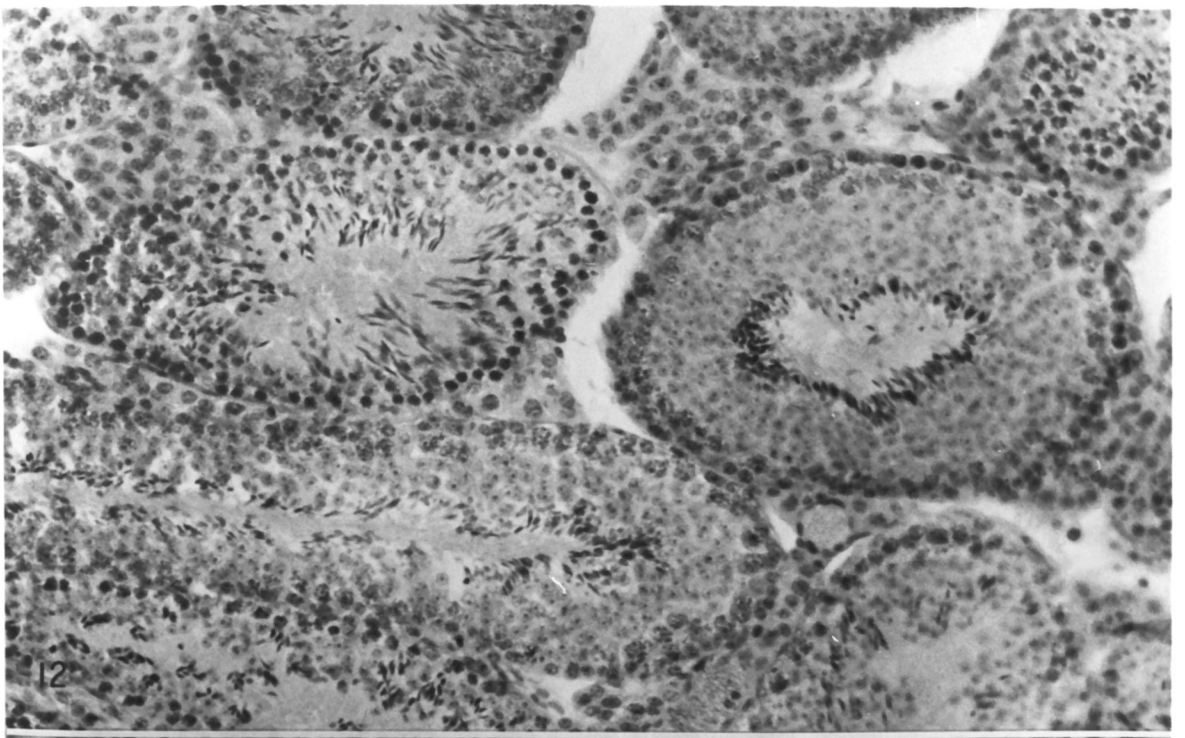

MC
11

Figure 12. Section from testis treated for 42 days and allowed to recover for 15 weeks. Spermatogenesis appears to be occurring normally. Magnification: 180X

Figure 13. Section from the same testis shown in Figure 12. A spermatogenic tubule is indicated by the arrow. Magnification: 180X



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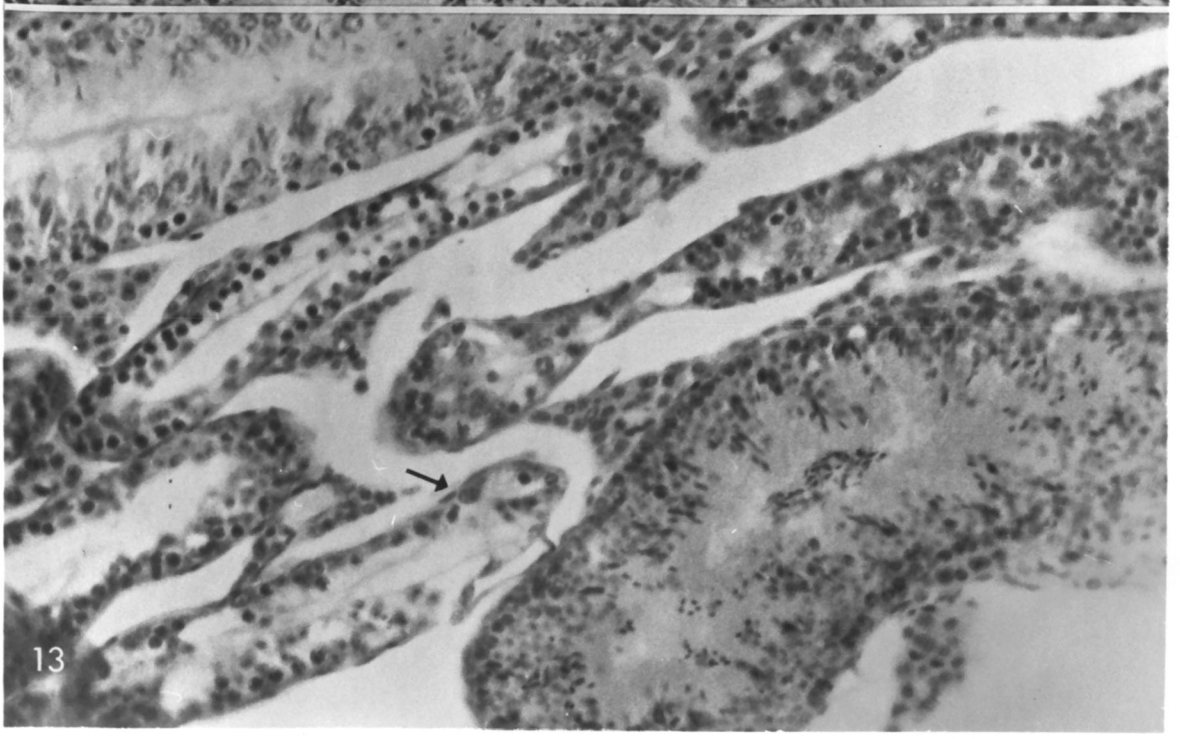
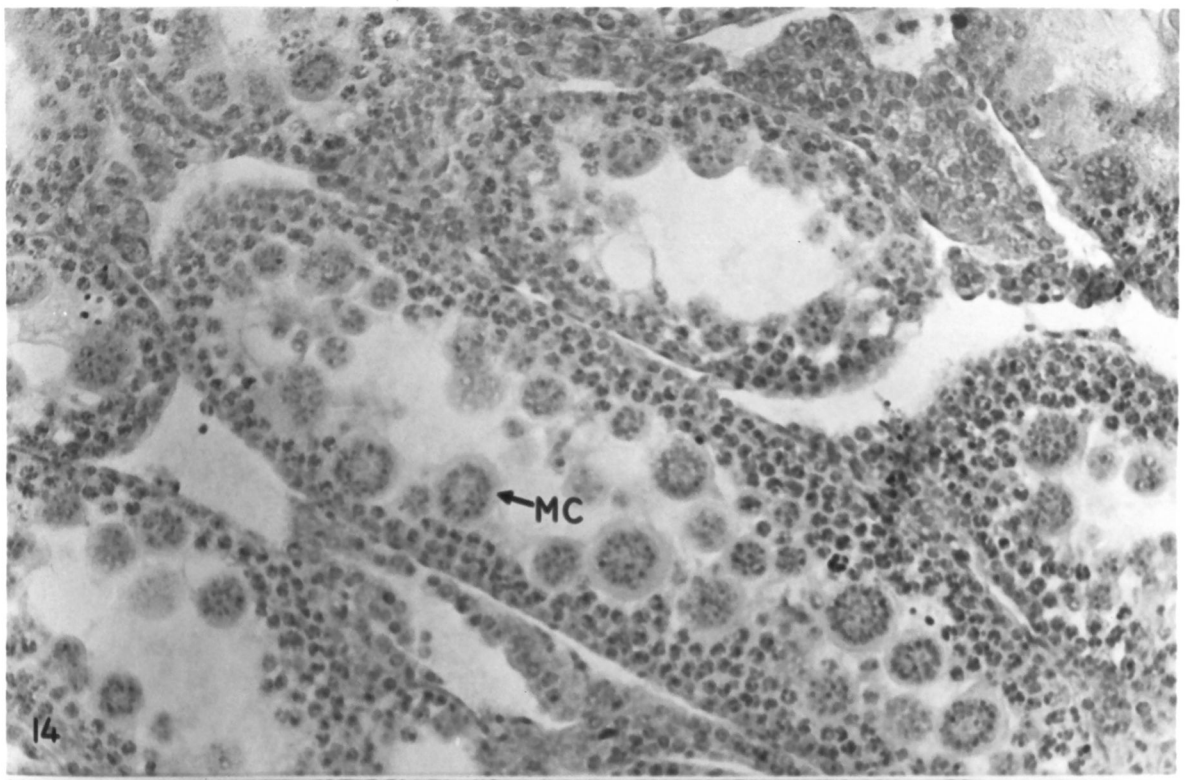


13

Figure 14. Section from testis treated daily for 42 days with an additional 14 days of twice weekly treatment. Note similarly to 42-day treated testis. Arrow indicates area magnified in Figure 15. MC, multinucleated cells. Magnification: 180X

Figure 15. Area from Figure 14 under higher magnification. MC, multinucleated cells. Magnification: 450X



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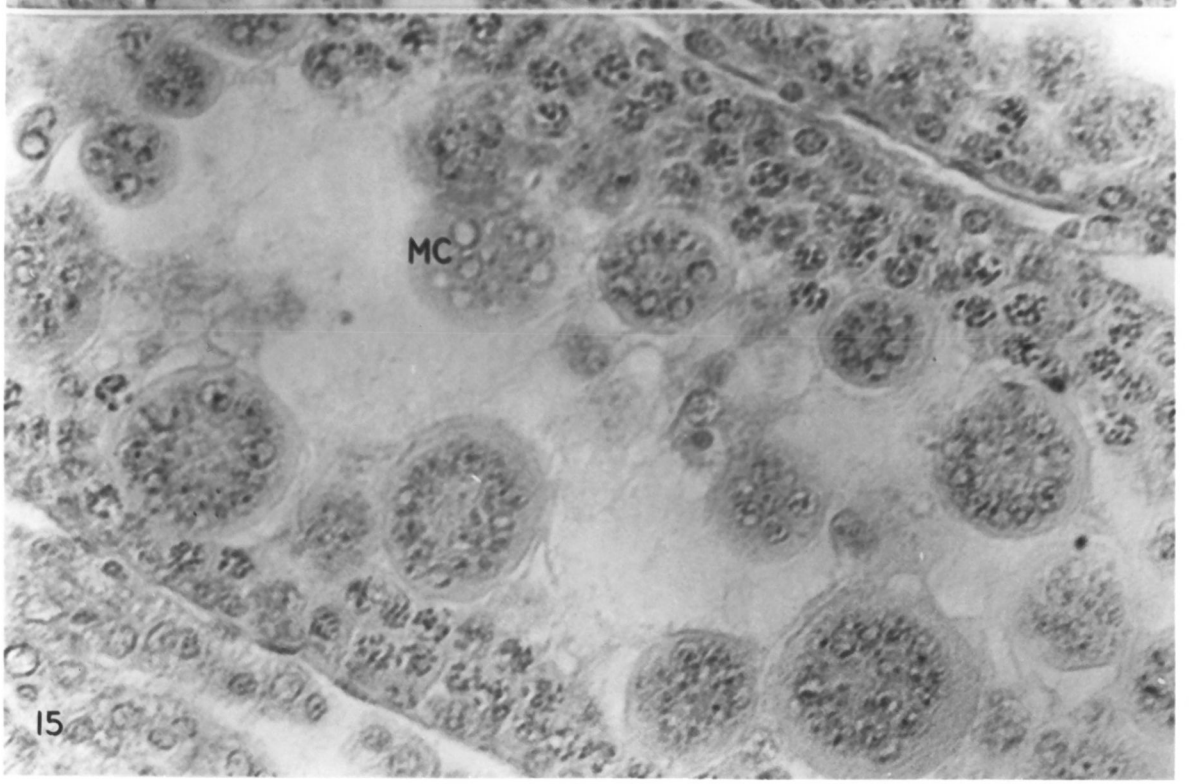


15

Radiorespirametric Studies

The radiorespirametric studies were used as evidence of glucose oxidation, as indicated by evolved $^{14}CO_2$ from labeled glucose, within the tissues. Glycolysis-TCA and PPP are the two major routes of glucose oxidation in both the testis and the liver, as previously mentioned. The relative activity of PPP to glycolysis-TCA can be evaluated by using G-1-^{14}C and G-6-^{14}C in radiorespirametric studies to compute C-1/C-6 (% recovery of $^{14}CO_2$ from G1-^{14}C/ % recovery of $^{14}CO_2$ from G-6-^{14}C). This comparison is possible because the initial carbon cleaved in each pathway is different.

One function of PPP is to form pentoses (e.g. D-ribose-5-Phosphate). In this process, the 1-carbon of glucose is cleaved off and evolves as CO_2 (Figure 16). Depending on several factors, the formed pentose may remain as a pentose or be converted to another sugar and possibly enter the glycolysis-TCA cycle. Glycolysis results in glucose being split into dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. These two compounds continue through the rest of the reactions to form two pyruvates. The first CO_2 evolves when pyruvate is oxidized to acetyl CoA which then enters the TCA cycle. The first two carbons (one from each pyruvate) are the 3- and 4-carbons (Figure 17). The 2- and 5-carbons are next to evolve as CO_2, with the 1- and 6-carbons coming off last. The last four carbons are released during the TCA cycle. Thus, if only glycolysis-TCA pathway is functioning the C-1/C-6 would equal 1. Anytime this ratio is greater than 1, both pathways are in operation.

There exist a degree of uncertainity associated with the fate of

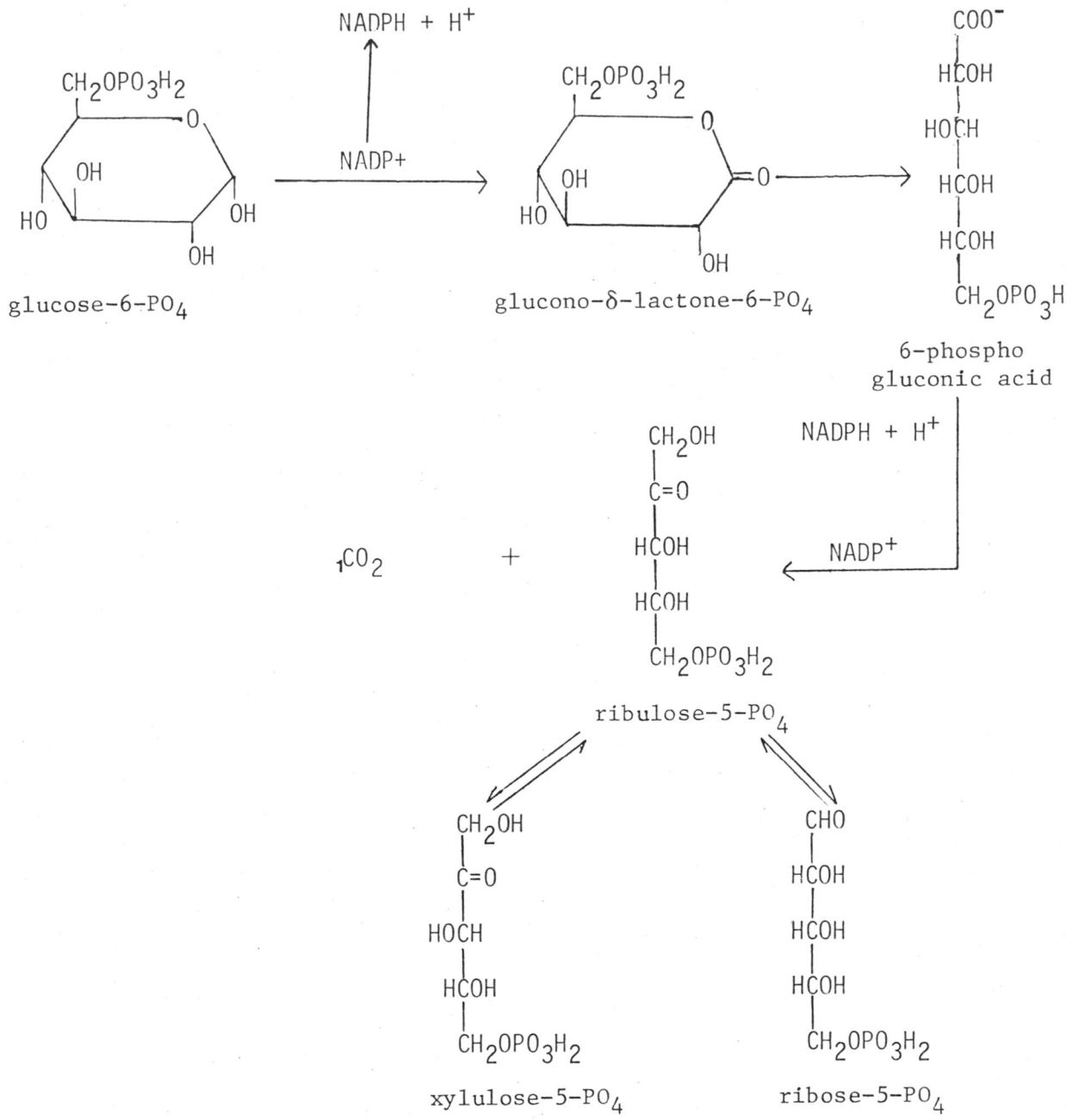


Figure 14. Diagram of the Pentose Phosphate Pathway showing when CO_2 is cleaved off.

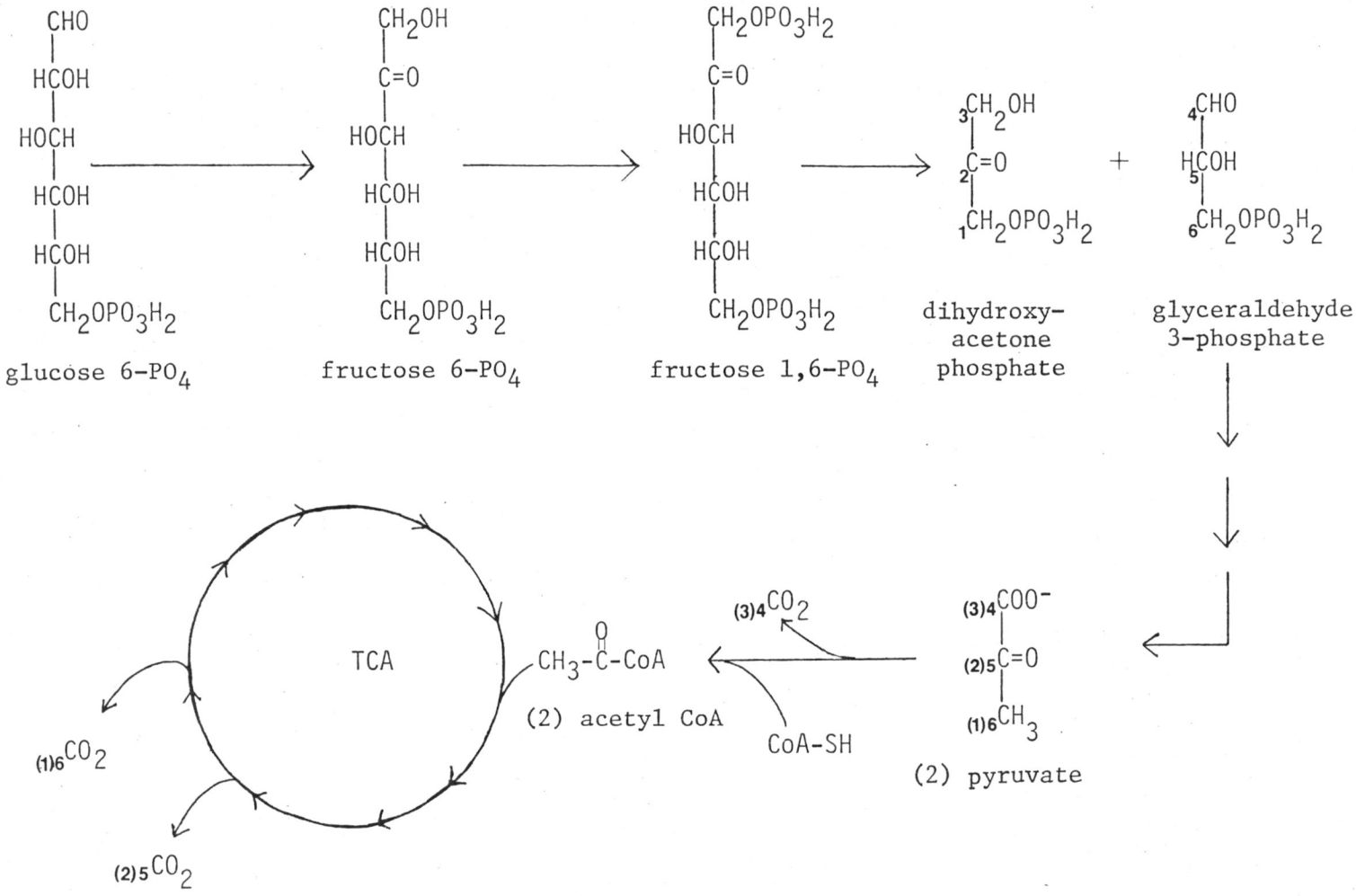


Figure 15. Schematic representation of glycolysis-TCA cycle demonstrating the order that CO_2 evolves.

the pentose formed in PPP. If the pentose formed from G-6-^{14}C enters the TCA cycle, $^{14}CO_2$ would evolve. When this occurs, the eventual % recovery of $^{14}CO_2$ from G-1-^{14}C and from G-6-^{14}C would be equal and C-1/C-6 would not give useful information. Therefore, only the initial % recovery (generally from the first hour) is used to calculate the C-1/C-6 value. Additionally, the rate of evolution $^{14}CO_2$ has been shown to drop after the first hour in the testis when gluconate-1-^{14}C was used to gauge the PPP activity. Thus, PPP appears to be inhibited in vitro due to some limiting factor (Free and Vandemark, 1969). If the fate of pentose, however, does not involve further degradation, the final % recovery of $^{14}CO_2$ from G-1-^{14}C will be greater than % recovery from G-6-^{14}C.

The radiorespirametric data on the liver tissue for the individual treatment periods were plotted to show the cumulative percent recovery of $^{14}CO_2$ for each hour sampled (Graphs 1-5). The total percent recovery of $^{14}CO_2$ for each labeled glucose was statistically examined using one factor ANOV (Table 2; Appendix C). No significant differences existed between the treated and the control in total percent recovery of $^{14}CO_2$ from either of the labeled forms of glucose with one exception. In the 14 day group, the G-6-^{14}C treated were lower than the control group at 0.01 significant level. The overall importance of this one difference is probably little, since it did not continue and it did not occur in the G-1-^{14}C group. If the tissues had actually been damaged from the 5-TDG treatment, glucose metabolism would have remained reduced for all subsequent treatment periods.

Graphs 1-5. Rate of evolution (cumulative) and yield of $^{14}CO_2$ from specifically labeled glucose by liver tissue from 5-TDG and control mice during each treatment period. Each point represents the mean of 5 animals. Graph 1, 7 days; Graph 2, 14 days; Graph 3, 21 days; Graph 4, 28 days; Graph 5, 42 days. ———— treated, ------- control.

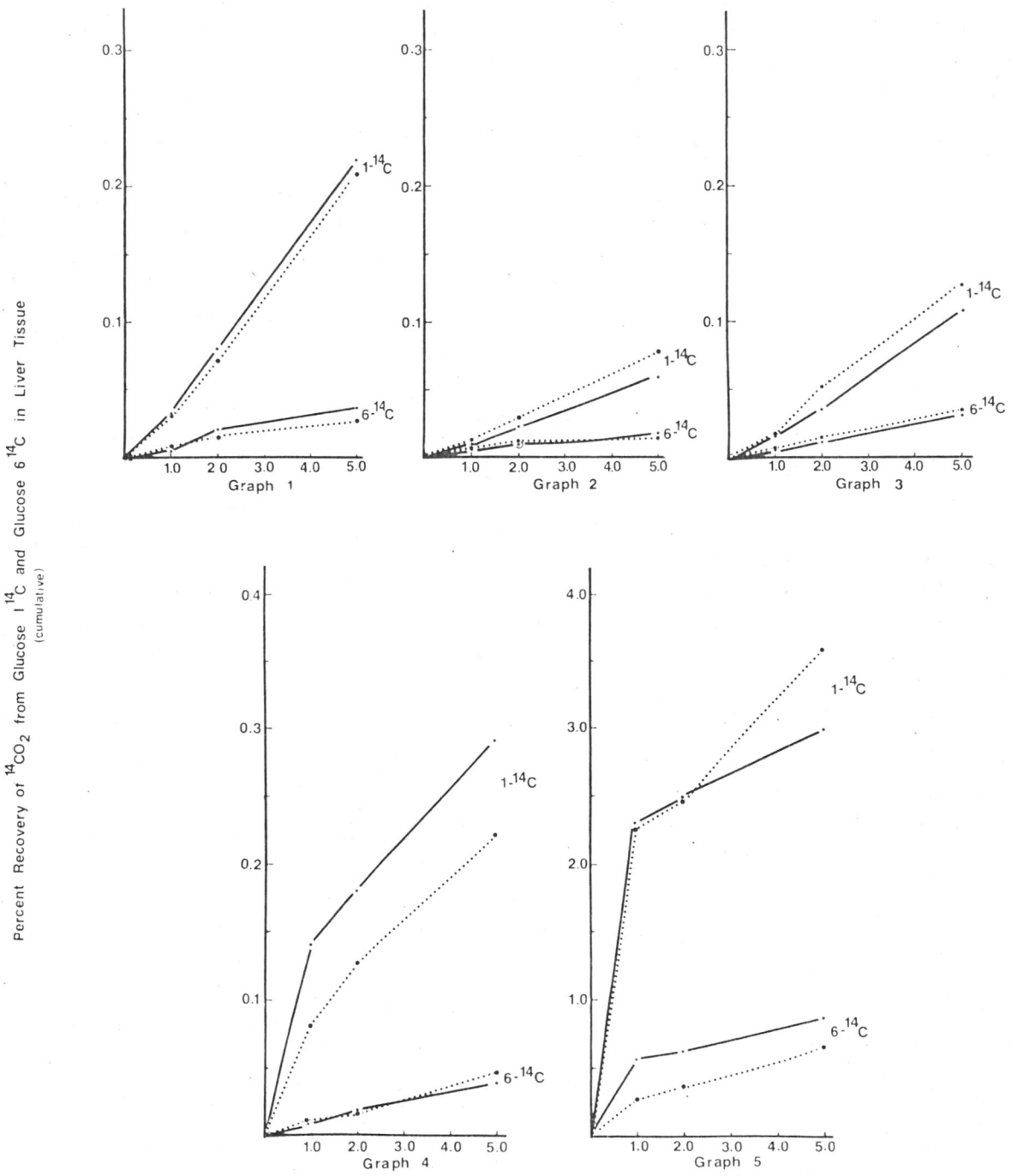

Percent Recovery of $^{14}CO_2$ from Glucose $1^{14}C$ and Glucose $6^{14}C$ in Liver Tissue
(cumulative)
0.3
0.2
0.1
1-^{14}C
6-^{14}C
1.0 2.0 3.0 4.0 5.0
Graph 1
Graph 2
Graph 3
0.4
4.0
Graph 4
Graph 5
Time in Hours

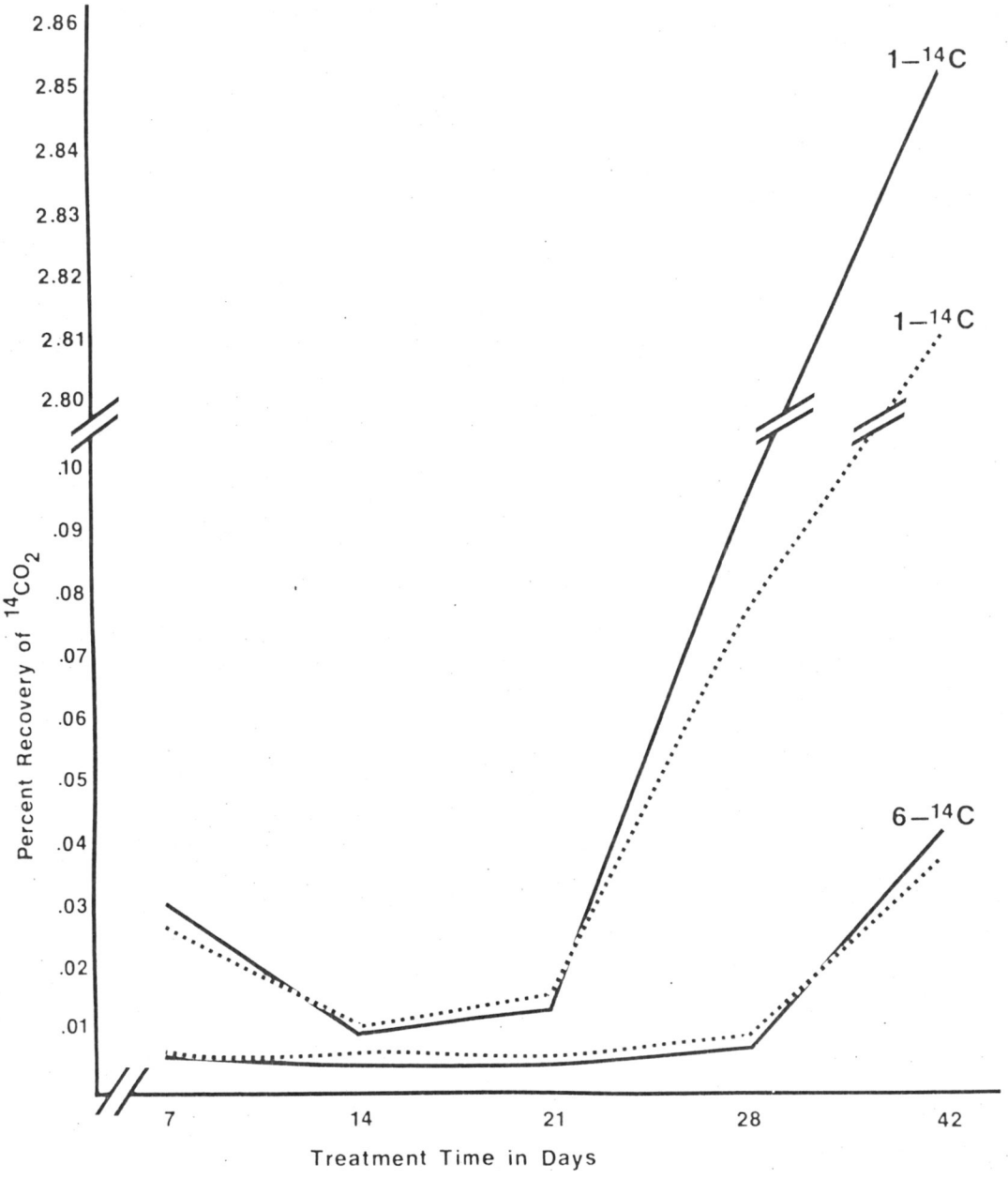
Table 2. Mean and standard error (n=5) for total percent recovery of $^{14}CO_2$ from glucose-1-^{14}C and glucose-6-^{14}C in liver tissue.

Treatment Period	Glucose-1-^{14}C		Glucose-6-^{14}C	
	Treated	Control	Treated	Control
7	0.213±0.042	0.214±0.017	0.035±0.007	0.026±0.004
14	0.060±0.012	0.067±0.014	0.016±0.001*	0.033±0.005
21	0.108±0.025	0.125±0.015	0.030±0.002	0.031±0.003
28	0.244±0.064	0.213±0.050	0.039±0.006	0.046±0.006
42	3.503+0.652	3.532±0.881	0.861±0.304	0.641±0.203

*Significantly different at 0.01 level

In addition to cumulative percent recovery graphs, the percent recovery of the first hour for all treatment periods was plotted (Graph 6). The graph indicates little difference between the treated and the control data. However, an overall increase in percent recovery is present during the final two treatment periods. From Graph 4 and 5, it is evident that the major portion of this increase is due to greater recovery during the first hour. Furthermore, the steep slopes of the lines on these graphs imply increased rates of $^{14}CO_2$ recovery, that is, increased glucose utilization. The reason for this increase is uncertain. However, since it occurred in all groups (G-1 treated, G-1 control, G-6 treated, G-6 control), it is apparently a result of some factors affecting all groups and not from the 5-TDG treatment.

Graph 6. Percent yield of $^{14}CO_2$ after one hour of incubation from specifically labeled glucose by liver tissue from 5-TDG treated and control mice for each treatment period. Each point represents the mean of 5 animals. ——— treated, ------- control.


2.86
2.85
2.84
2.83
2.82
2.81
2.80
.10
.09
.08
.07
.06
.05
.04
.03
.02
.01
Percent Recovery of $^{14}CO_2$
1—^{14}C
1—^{14}C
6—^{14}C
7
14
21
28
42
Treatment Time in Days

The C-1/C-6 values computed for the liver tissue (Table 3) were statistically analyzed by one-factor ANOV. The F-values indicated no significant difference between the treated and the control (Appendix C). The ratio values are comparable to values recorded in

Table 3. Summary of the C-1/C-6 ratios for the liver tissue.

Treatment Period	C-1/C-6 Treated	Control
7	5.14	4.41
14	2.22	1.91
21	2.47	3.21
28	10.08	9.08
42	6.56	7.35

*C-1/C-6 = $^{14}CO_2$ from glucose-1-^{14}C/ $^{14}CO_2$ from glucose-6-^{14}C

the literature for rat liver. The mean ratio for all control tissue was 4.91 (Range: 2.22-10.08). Bloom et al. (1955) after 2 hours of incubation obtained a mean of 4.7 (Range: 1.54-8.33) when the tissues were incubated for 3 hours.

It is important that the 5-TDG treatment had no significant effect on glucose metabolism in the liver. If a drug is to be useful as a male contraceptive, it must selectively affect the the testis. Although this study does not examine all aspects of liver metabolism and function, it does show that 5-TDG does not impair glucose metabolism.

The data on the percent recovery of $^{14}CO_2$ from the studies of the testis (Table 4; Graphs 7-13) were statistically analyzed using one-factor ANOV. The F-values (Appendix C) indicate no significant

Table 4. Mean and standard error (n=5) for total percent recovery of $^{14}CO_2$ from glucose-1-^{14}C and glucose-6-^{14}C in testis tissue.

Treatment Period	Glucose-1-^{14}C		Glucose-6-^{14}C	
	Treated	Control	Treated	Control
7	3.114±0.132	2.926±0.397	0.932±0.187	0.721±0.153
14	1.021±0.218	1.321±0.422	0.419±0.048	0.496±0.096
21	1.999±0.375	2.026±0.399	1.513±0.368	1.068± 0245
28	1.764±0.133	1.662±0.159	0.873±0.064	0.805±0.181
42	3.673±0.457*	1.447±0.209	1.588±0.	1.071±0.152

*Significantly different at 0.05 level

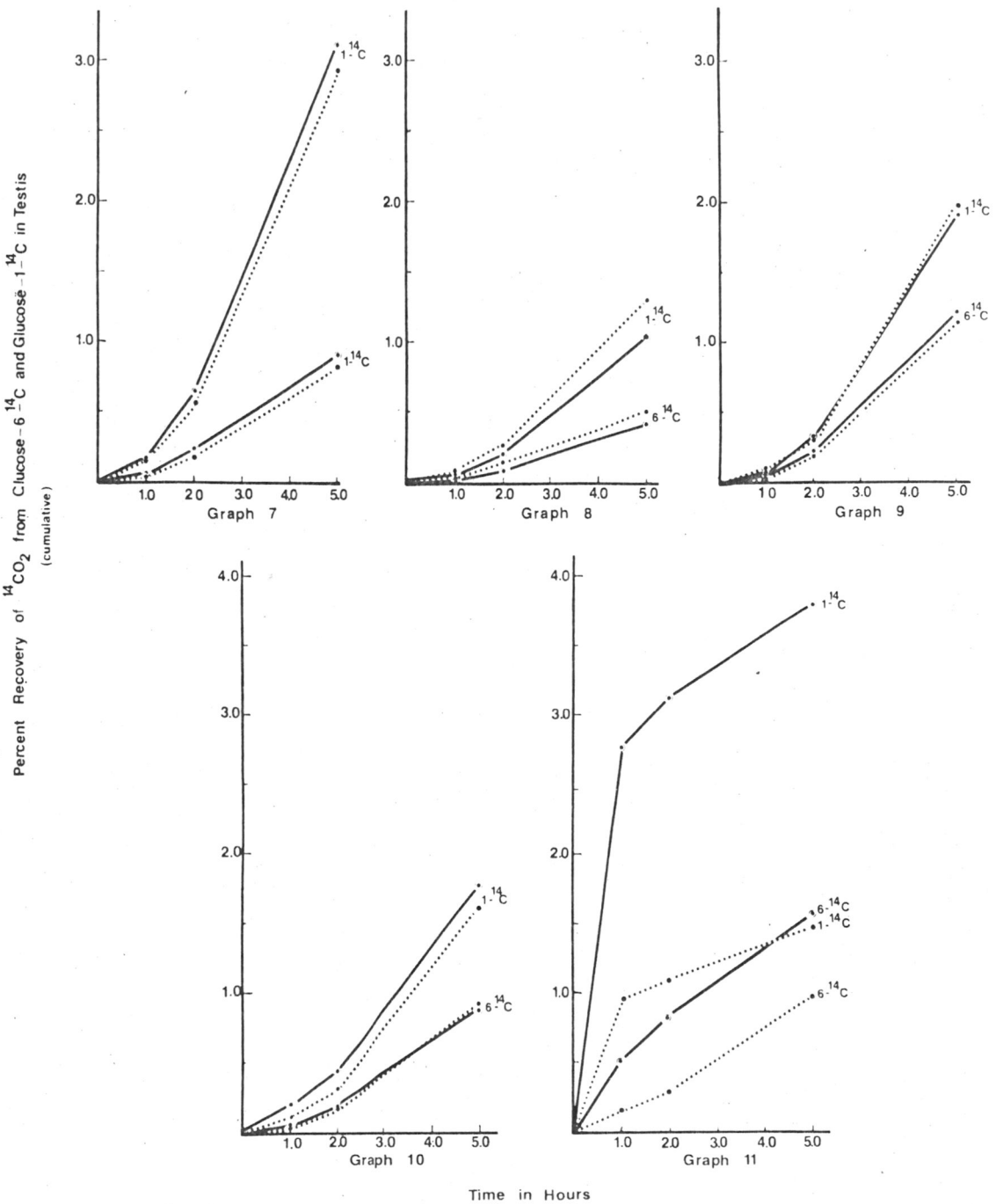
difference between control and treated tissues in total percent recovery of $^{14}CO_2$ from G-6-^{14}C during any of the treatment period. Recovery from G-1-^{14}C showed no significant difference until the 42 day treatment period. At this time, the treated group had a statistically higher percent recovery than the control group. The greater glucose oxidation in testicular tissue of 42 day treated animals is contrary to results for other testicular disorders with similar spermatogenic degeneration such as cryptorchid testis. This contradiction will be discussed in more detail later. Also, during the 42 day treatment period, it should be noted that while for the control, the total percent recovery values from G-1-^{14}C and from

G-6-^{14}C were close (1.447 to 1.071), this was not true for the treated group. The total percent recovery from G-1-^{14}C exceeds the value for G-6-^{14}C (3.673 to 1.588). This difference indicates more PPP activity in the treated testes.

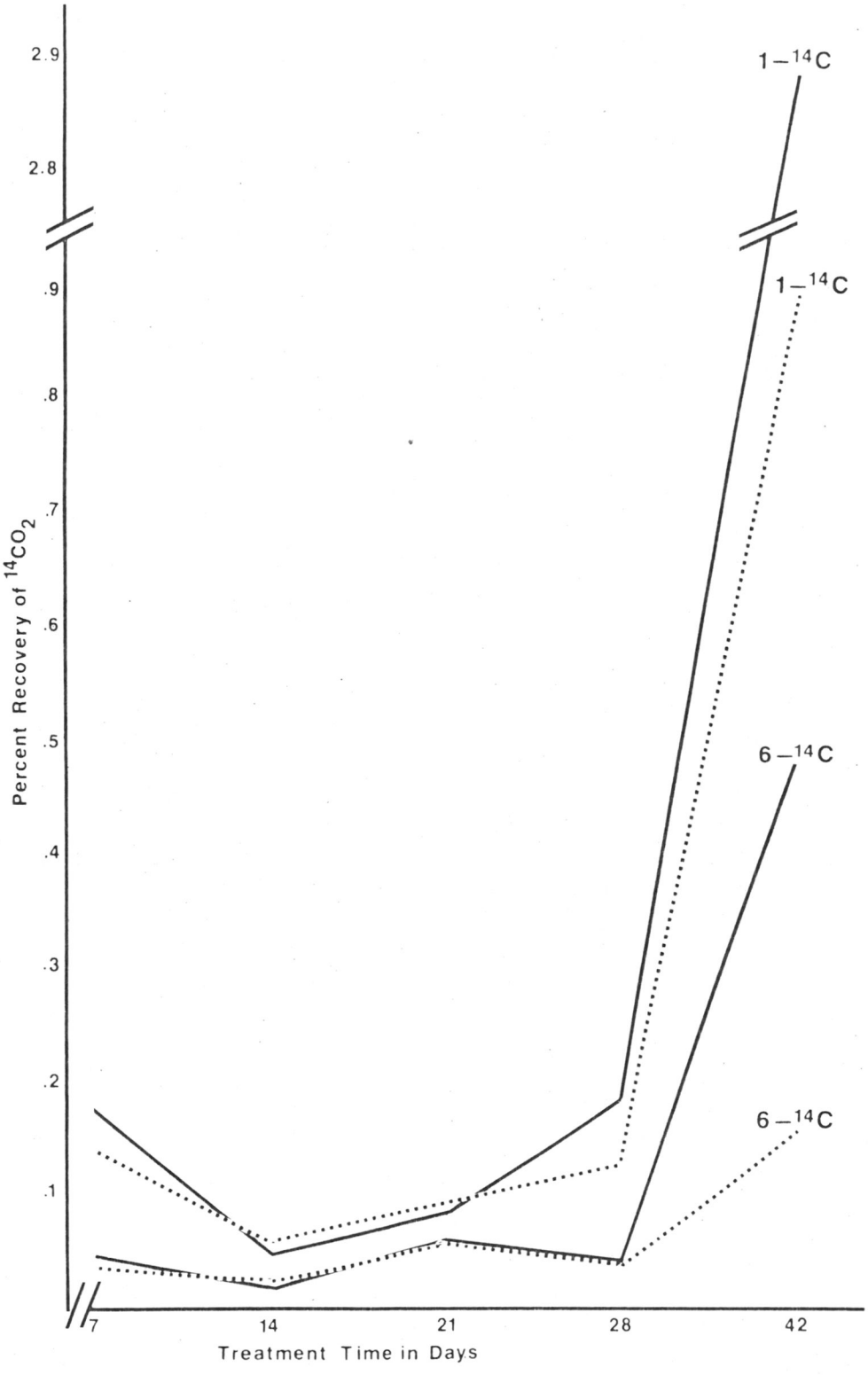
The testis radiorespirametric data were plotted by cumulative percent recovery for each treatment period (Graphs 7-11) and by the first hour percent recovery for an all treatment periods (Graph 12). In Graph 12, there is an overall increase during the final treatment period similar to the one that is observable in liver tissue (Graph 6). Examination of Graph 11 shows, as in the liver, that the majority of the increase occurred during the first hour of incubation. The presence of the increase in both tissue types further indicates that the increase is due to a common factor affecting all tissues. There is an interesting deviation in Graph 12 involving G-6-^{14}C. While the slopes of the treated and controls are similar for G-1-^{14}C, this is not true for G-6-^{14}C. The slope for the treated is approximately 3 times greater than the control. This seems to imply greater initial glycolysis-TCA activity in the treated testes. The total recovery, however, is not significantly different. The cause or significance of this apparent increase is uncertain.

The C-1/C-6 values computed for the testicular tissue are recorded in Table 5. The mean of all the control testes was 3.42 with a range of 1.56 to 5.26. Although the ratio for mouse testis has not been previously assessed, the values of this study are apparently within the realm of feasibility as estimated by relative enzyme activity of the PPP. The relative activity of the enzymes of

Graph 7-11. Rate of evolution (cumulative) and yield of $^{14}CO_2$ from specifically labeled glucose by testis tissue from 5-TDG and control mice during each treatment period. Each point represents the mean of 5 animals. Graph 7, 7 days; Graph 8, 14 days; Graph 9, 21 days; Graph 10, 28 days; Graph 11, 42 days. ———— treated, -------- control.


Percent Recovery of $^{14}CO_2$ from Glucose-6-^{14}C and Glucose-1-^{14}C in Testis
(cumulative)
Graph 7
Graph 8
Graph 9
Graph 10
Graph 11
1-^{14}C
6-^{14}C
Time in Hours

Graph 12. Percent yield of $^{14}CO_2$ after one hour of incubation from specifically labeled glucose by testis tissue from 5-TDG treated and control mice for each treatment period. Each point represents the mean of 5 animals. ———— treated, ---------- control.


2.9
2.8
.9
.8
.7
.6
.5
.4
.3
.2
.1
Percent Recovery of $^{14}CO_2$
1—^{14}C
1—^{14}C
6—^{14}C
6—^{14}C
7
14
21
28
42
Treatment Time in Days

the PPP to those of the glycolysis-TCA pathway have been determined for mouse testis (Blackshaw, 1963), as well as for the testes of ram, rat, chicken, and rabbit (Free, 1970). Among these species the order of increasing activity of the PPP in the testis is the following: chicken<rat<rabbit<mouse<ram. If relative activity is a legitimate estimate of C-1/C-6 ratio, the mean of this study (3.42) would seem reasonable compared with the value of 3.30 for the rabbit, (The recorded values for the rabbit testis are 2.61 (Free et al., 1968) and 3.30 (Bloom, 1955).)

Table 5. Summary of the C-1/C-6 ratios for the testis tissue.

Treatment Period	C-1/C-6 Treated	Control
7	3.88	3.79
14	2.42	3.28
21	1.51	1.56
28	4.33	3.19
42	8.37*	5.26

*Significantly different at 0.025

The C-1/C-6 values of treated testis were statistically compared to the control by one-factor ANOV (Appendix C). There was a slight increase in the treated tissue over the control during the 28-day treatment period, and by the 42-day period, the treated was significantly higher. The increased ratio indicated increased PPP activity in the treated cells. Overall, there was little difference between

the control and the treated ratios. The mean of the treated testes was 4.10 (Range: 1.51-8.37) as compared to 3.42 of the control.

On an overall basis, the glucose oxidation was higher in the treated testes during the 42-day treatment period. This is implied when the combined percent recovery from G-1-^{14}C and G-6-^{14}C for the treated (5.261) was compared to the combined value for the control (2.518). Part of the increase can be attributed to the increased PPP activity. The $^{14}CO_2$ percent recovery data from G-1-^{14}C (Table 4) was significantly higher in the treated testes during the 42-day period. A similar increase in recovery from G-1-^{14}C was observed in the cryptorchid and testosterone treated testes by Davis et al. (1968). An examination of the G-6-^{14}C percent recovery data for the treated (1.588) and the control (1.071) indicate that glycolysis-TCA activity in the treated testes was normal although, the initial rate in the treated did appear greater. The treated value, although slightly higher, was not significantly higher. Thus, the data imply that the overall increase is due mainly to the higher PPP activity, with a normal amount of glycolysis-TCA activity.

This increase of glucose metabolism in the 5-TDG testis raises one major question. How can an increase occur when the cells responsible for the greatest amount of glucose oxidation are degenerating? As previously stated, this increase contradicts studies with other testicular disorders where spermatogenic degeneration occurs. Examination of the data from these studies gives some insight into the answer. In studies with cryptorchid testis (Free et al., 1969) and with nitrofuran treated testis (Paul et al., 1953), a decrease

in glucose metabolism corresponded to the decrease in spermatogenic cells. In the cryptorchid testis an increase in PPP activity did, however, occur. (The PPP activity was not measured in the nitrofuran testis) Thus, while the PPP increased in both cryptorchid and 5-TDG testis, the glycolysis-TCA showed a decrease in cryptorchid and appeared normal in 5-TDG testis. The reason for the variation can only be surmised at this time; however, two obvious differences exist between the conditions.

One noticeable difference in the cryptorchid and the nitrofuran testes from the 5-TDG testis is the more rapid rate of degeneration. In both of these conditions, the testes are devoid of all spermatozoa and spermatids, and most spermatocytes by 14 days into the treatment (Free et al., 1969; Paul et al., 1953). Spermatocytes and spermatids were still present in the testes of this study even after 42 days of treatment. It is possible that the metabolic activity by these cells plus the PPP increase could account for the overall increase.

Another difference is the absence of the multinucleated masses which are present in the 5-TDG testes. If these giant cells were still active, they could account for some of the metabolic activity. Spermatids with two or more nuclei have been observed continuing their development to form giant spermatozoa (Maximow and Bloom, 1957). This observation implies that, at least in some cases, these multinucleated cells remain active metabolically.

The observed glycolysis-TCA activity is apparently due to the remaining spermatogenic cells and possibly to any active multinucleated cells. The overall increase is a combination of this

glycolysis-TCA activity and the increased PPP activity.

Two possible explanations exist for increased PPP activity in the 5-TDG and cryptorchid testes. These explanations involve two different functions of the PPP the production of pentoses and the generation of NADPH. As previously mentioned, the Leydig cells and spermatogonia are the sites of most of the PPP activity in the testis. The importance of the PPP to the Leydig cells is thought to involve steroidogenesis. Steroidogenesis has been shown to be dependent on NADPH for hydroxylation reactions in many tissues including the testis (Grant and Brownie, 1955; Halkerston et al., 1961; Hall, 1970). Moreover, the importance of PPP activity in steroidogenic endocrine tissues has been demonstrated (Field et al., 1960; Hall, 1970). The implication of this information is that PPP in the Leydig cells is needed to generate NADPH for androgen production. Therefore, if the degeneration of the spermatogenic cycle were to cause a compensatory increase in androgen production, a corresponding increase in the PPP activity could occur.

This explanation is supported by 5-TDG sterility studies by Fick (1979). These histochemical studies showed that androgen production was slightly higher in the 5-TDG testis. A similar increase was observed by Kormano et al. (1964) in cryptorchid testes of rats. Thus, it appears that, at least part of the increased PPP activity was due to increased steroid production in the cells of Leydig. Since the germinal cells are also involved, to some degree in steroid production, an additional increase in PPP could take place in these cells.

The other explanation involves the spermatogonia and pentose

production. Although most of glucose metabolism is attributed to spermatids and spermatocytes, glucose is also thought to be the major metabolite of spermatogonia (Free, 1970) with the glucose being oxidized mainly in these cells by PPP pathway. With one function of PPP being the production of D-ribose 5-phosphate for nucleic acid synthesis, it is not surprising that spermatogonia, which are responsible for spermatogenic cell renewal, would have high PPP activity. The capacity of high PPP activity in these early spermatogenic cells is also evident by the fact that immature calf testes have been shown to have an extremely high C-1/C-6 ratio of 56 (Fields et al., 1960).

The observed increase of PPP in the 5-TDG and cryptorchid testes plus the large amount of activity in the immature testis tends to imply that PPP activity is connected to cell division. Moreover, in this study the G-1-^{14}C percent recovery was greater than G-6-^{14}C for the treated testes. Thus, the pentoses formed in PPP were apparently being used and not recycled through the TCA cycle. This information along with histological observations previously mentioned indicates the spermatogonia of the 5-TDG testes were involved in cell renewal and cell division. Therefore, the increases in PPP pathway may be partially due to a compensatory type of action. That is, an unmasking of the spermatogonia by the degeneration of the more mature cells, resulting in increased cell division and, thus, increased PPP activity.

An unmasking effect was described by Davis et al. (1965) in relationship to increased protein synthesis in the rat cryptorchid testis. This increase was observed after the spermatids had degenerated. One factor cited by Davis et al. for this increase was an

unmasking of the more undifferentiated cells (spermatogonia and spermatocytes), which had a higher degree of protein synthesis.

These explanations are related to each other by functions and hormones. Androgens (testosterone) are partially responsible for the initiation of spermatogenesis. Consequently, if increased androgen production would occur, a corresponding increase in cellular division of spermatogonia might result. The higher PPP activity, therefore, is most likely due to a combination of more NADPH generation and greater ribose production, with the overall increase resulting from the greater PPP activity.

A study by Davis et al. (1968) supports the idea that PPP activity in both the interstitium and the tubules are involved in the increase. They measured the CO_2 recovery from G-1-^{14}C and G-6-^{14}C in cryptorchid testes (devoid of most germinal cells) and testosterone propionate treated testes (depleted interstitium). In both conditions they observed significantly higher $^{14}CO_2$ recoveries from G-1-^{14}C in the treated testes. This data implies an interrelationship between the two parts of the testes which is reflected by the PPP.

The increased glucose oxidation, whatever the source, rules out one mechanism of action. It eliminated the possibility that 5-TDG works through reduced intercellular testicular glucose, as originally thought. This conclusion is supported by previous 5-TDG studies. Burton and Wells (1977) found that 5-TDG treatment caused increased myo-inositol levels in the testis which indirectly reflects greater intracellular glucose levels. Furthermore, when the diabetogenic effect of 5-TDG was counteracted with insulin in another study, the antispermatogenic action

was not affected (Lobl and Porteus, 1978). Thus, it appears that the interference with glucose transport by 5-TDG is not sufficient to cause sterility.

The data of this study could also be interpreted to imply that 5-TDG is not competitively inhibiting any of the enzymes of glucose oxidation, since the activity of the pathways were either normal or increased. However, in light of two factors such a conclusion would be extremely hasty. First, the incubations were done with whole testis section. Hence, 5-TDG could be effecting one cell type with the effect being hidden by the activity of the remaining cells. This type of effect was observed by Nakamura and Hall (1976), when working with amino acid incorporation. They found no inhibition from 5-TDG in incubation of the whole testis. However, when the different cell types were separated and the individual fraction were incubated, inhibition occurred in some of the fractions. The other factor is the time element. The action of 5-TDG is probably very short termed, since 5-TDG is only present in the system for a short period (3-6 hours) before it is removed. Thus, the action of 5-TDG could be on one or two spermatogenic cell types and only for the brief period when 5-TDG is in direct contact with the testis. To investigate this concept additional research using centrifugal elutriation to separate cell fraction would be required.

SUMMARY

Male mice were treated with 5-TDG of either 7, 14, 21, 28, or 42 days. At the end of each period, radiorespirametric studies were done on testis and liver sections, to monitor glucose metabolism. The C-1/C-6 ratio was computed to determine the relative activity of PPP to glycolysis-TCA.

The $^{14}CO_2$ data showed no significant effect on the glucose metabolism in the liver tissue from the 5-TDG treatment. The PPP was also uneffected.

The 5-TDG treatment caused increased glucose metabolism in the testis. Most of the increase was attributed to greater PPP activity. The PPP activity was accredited to a compensatory (unmasking) action of the remaining cells especially the Leydig cells (steroidogenesis) and spermatogonia (cell division and renewal). The glycolysis-TCA cycle appeared to be functioning normal; although, the cells responsible for this activity was not determined. The treated testis contain many abnormal cells (multinucleated masses), which may have been metabolically active.

The glucose metabolism data complicate the understanding of 5-TDG's mechanism of action. The results tend to eliminate one of the simpler explanations - the interference with D-glucose transport. Before the actual mechanism(s) of 5-TDG will be ascertained, much additional research will be required.

Although, the results of this study provide little insight into the mechanism of 5-TDG's antispermatogenic action, the study does

serve as the foundation for further research with 5-TDG and glucose metabolism. Additionally, the radiorespirametric studies on the liver provide some insight into 5-TDG effect on nontesticular tissue. These results are encouragingly supportive to the idea that 5-TDG may be selectively affecting the testis. The study also suggested an alternate treatment program, which could possibly reduce the permanent damage observed in some of the seminiferous tubules in several cases. Finally, the study tends to support the concept of compensatory action. The increased PPP activity alluded to possible compensatory action by both the Leydig cells and the spermatogonia.

APPENDIX A

PREPARATIONS FOR SOLUTIONS USED IN THIS STUDY

Krebs-Ringer Phosphate Buffer (pH 7.4)

1. 0.90% NaCl
2. 1.15% KCl
3. 1.22% $CaCl_2$
4. 3.82% $MgSO_4 \cdot 7H_2O$
5. 0.1M Phosphate Buffer (pH 7.4)

Solutions 1-5 are made up separately. They will keep for months at 5°C. Before use, mix solutions in the following ration: 100:4:3:1:20.

Carnoy's fixative (100ml quantity)

Glacial acetic acid	10ml
Absolute ethanol	60ml
Chloroform	30ml

APPENDIX B

HISTOLOGICAL PROCEDURES

Dehydration

95% Ethanol	1.5 hours
100% Ethanol	1.0 hours
100% Ethanol	1.0 hours

Clearing

100% Ethanol: Xylene	1.5 hours
Xylene	16 hours

Staining (Hematoxylin & Eosin)

Xylene	5 min
Xylene	2 min
100% Ethanol	2 min
95% Ethanol	2 min
70% Ethanol	2 min
Water	2 min
Hematoxylin	2-5 min
Running tap water	3 min
Acid Alcohol (1% HCl in 70% Ethanol)	2-3 dips
Running tap water	5 min
Eosin (1% solution)	1-3 min
95% Ethanol	2 min
100% Ethanol	2 min
100% Ethanol	2 min
Xylene	2 min
Xylene	5 min

APPENDIX C

Summary of F-values for the ANOV's comparing the total % recovery of $^{14}CO_2$ from G-1-^{14}C and from G-6-^{14}C and comparing the C-1/C-6 ratios between the treated and the control.*

Treatment Period	Testis			Liver		
	G-1-^{14}C	G-6-^{14}C	C-1/C-6	G-1-^{14}C	G-6-^{14}C	C-1/C-6
7	0.148	0.767	0.011	0.002	0.876	0.359
14	0.400	0.402	1.706	0.114	12.181**	0.286
21	0.001	1.016	0.003	0.345	1.167	4.789
28	0.251	0.121	2.324	1.160	0.789	0.444
42	32.097***	3.530	8.010**	0.001	0.367	0.104

*$F_{0.05\ (1)1,8} = 5.32$

**Significantly different at 0.025 level
***Significantly different at 0.0005 level

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