STUDIES ON TESTICULAR INOSITOL BIOSYNTHESIS

by

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Eisenberg (J. Biol. Chem., 242, 1375 (1967)) has reported that m-inositol synthesis is primarily a function of the testis in the rat, and that greater than 90% of this activity occurs in the germinal epithelium. The present studies were initiated to ascertain which of the cell types within the seminiferous epithelium are responsible for inositol synthesis, and what some of the controlling factors of this synthesis are.

Inositol synthesis was measured in testis slices using D-glucose as substrate, and in cell-free systems using glucose-6-phosphate as substrate. NAD and $MgCl_2$ served as cofactors in the cell-free systems.

Cryptorchidism was chosen as a tool to alter the cellular population of the testis. Surgically-induced cryptorchidism resulted in 65% reduction in testis weight, and 78% depression in inositol synthesis. The depression in inositol synthesis coincided with loss of the more mature cell types of the germinal epithelium. These data suggest that the spermatids and spermatozoa are the most active cell types with regard to inositol synthesis.

Experiments in which inositol synthesis from glucose-6-phosphate was measured in homogenates suggested

that glucose uptake might be altered by cryptorchidism, since only 46% depression of inositol synthesis was noted in cryptorchid homogenates. Studies of glucose uptake into testis slices revealed a 28% depression in cryptorchid slices. This suggested that depressed glucose uptake might partially account for the decrease in inositol synthesis noted in cryptorchid testis slices.

The depression of inositol synthesis seen in cryptorchid homogenates suggested that enzyme activities may be altered. This possibility was tested by measuring inositol synthesis in the preheated 100,000 x g supernatant fraction which Eisenberg has reported to be the only fraction containing inositol synthetic activity. Heat treatment was necessary to achieve maximal activity. Inositol synthesis was found to be nearly equal per mg protein in the preheated cryptorchid and control supernatants. However, since the cryptorchid supernatant contains 1.5 times as much protein as the control, the cryptorchid testis has a slightly greater enzyme activity per 100 mg wet weight of testis than the control. Thus, cryptorchidism does not result in a loss of enzyme.

Experiments in which inositol synthesis was measured using non-preheated 100,000 x g supernatants revealed that the cryptorchid supernatants are less active per mg of protein than the control. Heat treatment eliminated this differential and increased activity for both the control and cryptorchid supernatants. The increase was

slightly larger in the cryptorchid preparations. This suggests that inositol synthesis is limited in both testis types by the presence of some thermolabile factor.

One such factor is a nonspecific phosphatase which hydrolyzes G-6-P. Phosphatase activity was found to be 43% greater in non-preheated 100,000 x g supernatants obtained from 14 day cryptorchid testes than control. No activity was detectable in preheated 100,000 x g supernatants. The greater activity in the cryptorchid supernatants suggests that this enzyme may normally function to limit inositol synthesis in those cell types which are unaffected by cryptorchidism.

An increase in the utilization of inositol could also account for the depression of inositol synthesis detected as a result of cryptorchidism. Experiments in which the incorporation of inositol into the lipid fraction of rat testis slices was measured showed that cryptorchidism resulted in an 80% depression in phosphatidyl inositol synthesis. Thus, increased incorporation of inositol into lipids does not contribute to the depression in inositol synthesis seen accompanying cryptorchidism.

Triethylene melamine (TEM) has been reported to result in the destruction of spermatogonia followed by maturation depletion of the remainder of the germinal epithelium. Experiments in which inositol synthesis was measured in TEM-treated animals revealed that inositol

synthesis was not depressed prior to 21 days of treatment. This point coincides with loss of the spermatids from the seminiferous epithelium, and provides further evidence to suggest that the spermatids and spermatozoa are the most active cell types with regard to inositol synthesis.

APPROVED Robert N. Morris Ph.D.

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INTRODUCTION

1. Historical Perspective

Twenty years ago there were few who were concerned with the rate of expansion of the world's population although Robert Malthus had predicted over a century and a half ago that the world would soon be overpopulated. The advances in technology which have been developed since that time have postponed, rather than averted, the problems which Malthus had predicted would develop. In 6,500 years, which corresponds approximately to the period of recorded history, Ansley Coale of Princeton predicts that if current growth continues, the descendants of the present world population will form a solid sphere of living bodies expanding with a radial velocity that would equal the velocity of light (1). Others have offered equally dramatic representations of the consequences of continued uncontrolled population growth and have reached the pessimistic conclusion that it may become necessary to match scientific death control with scientific birth control.

G. B. Pincus and coworkers were the first to demonstrate clinically the effectiveness of an oral estrogen-progesterone combination for fertility control in females (2). Subsequent investigations by numerous observers have established beyond any doubt that oral administration of a suitable dose of an estrogen

together with one of a number of progestins from the fifth day of the menstrual cycle for 20 or 21 days can prevent pregnancy with a virtual 100% certainty.

There remains, however, a need for a better contraceptive agent. One possibility is a contraceptive agent for the male which would possess a similar order of efficacy, reversibility, safety, and specificity as that developed for the female. In order to accomplish this, it would be desirable for the drug to possess as its mechanism of action the reversible inhibition of a biochemical pathway which is unique, yet critical, to the testis. However, the specific metabolism of the testis is an area which has received little attention, and as a consequence, a rational basis for the design of an antifertility agent is lacking. The object of the present studies was to examine in depth the testicular synthesis of myo-inositol, a metabolic pathway which appears to be at least quantitatively peculiar to the testis (3).

2. Antispermatogenic Agents

A variety of substances have been developed which are capable of inhibiting spermatogenesis. In general, there have been two approaches taken to the development of such agents. The first is an attack on the pituitary-testis axis, while the second involves the

direct interruption of the germinal epithelium.

In general, the attack on the pituitary-testis axis has involved steroid administration. The most powerful steroid inhibitors of spermatogenesis are the estrogens which act by inhibiting FSH production and release (4). Although spermatogenesis is clearly estrogen-labile, Leydig cell function may also be inhibited since both FSH and ICSH levels are affected by estrogen (5). McGinty and Djerassi have reported that larger doses of progestins are required to inhibit spermatogenesis (6). Conversely, these investigators report that administration of 17-α-ethyl-19-nor testosterone (Nilevar), a progestational steroid, or progesterone to hypophysectomized rats maintains spermatogenesis. Thus steroids appear to have both stimulatory and inhibitory effects on the testis.

Small doses of testosterone in intact male rats suppress spermatogenesis by inhibiting pituitary gonadotrophin release, while large doses of male sex hormone stimulate spermatogenesis directly in spite of the pituitary effect (7). Thus, the effects of sex hormones and their derivatives are difficult to interpret because of the two major components involved. Additional complications result from the metabolic transformation of the steroid administered. Effects on the secondary sex characteristics and libido are quite common, and

suggest that the prospects of fertility control by steroid hormones are not very promising and may well be hazardous due to the risk of producing more general disturbances of the endocrine system (8).

Many compounds of diverse chemical nature have been shown to inhibit spermatogenesis by acting directly on the seminiferous epithelium. Pincus has divided these into: a) substances causing irreversible testis damage; b) nitrofuranes and compounds having similar effects; c) mitosis inhibitors; d) cytotoxic alkylating agents; e) antimetabolites; and f) antibodies (9).

Cadmium salts and erucic acid are examples of those agents which irreversibly damage the seminiferous epithelium. The former causes destruction of the seminiferous tubules with varying damage to the Leydig cells (10). Intraperitoneal injection of the cadmium salts results in many other toxic symptoms, and only when the drug is injected close to or into the testis are the systemic effects avoided (11). Erucic acid fed to rats destroyed the seminiferous epithelium, but left the Leydig cells intact (12). The toxicity of these compounds is too great to be acceptable for human use.

Prior and Ferguson observed that continued oral administration of the nitrofurazones; furacin, furadroxyl and furadantin, produced a fairly specific effect on the germinal epithelium (13). Spermatogenesis is arrested

at the primary spermatocyte stage, and it has been suggested that the action is limited to the meiotic division pachytene stage (14). Changes in glucose utilization, pyruvate oxidation, and citric acid synthesis have been detected with nitrofurazone administration (15), and could be related to the development of the cytotoxicity. There appears to be no effect on Leydig cell function and toxicity is low, but tests for antifertility potential in man have proved unpromising because of the development of numerous side effects (16).

A series of bis-(dichloroacetyl)-diamines which have antimalarial activity have also been found to possess a nitrofurazone-like effect on the testis (17). These compounds seem to affect the germinal epithelium directly, since spermatogenesis is arrested in hypophysectomized animals in which spermatogenesis has been maintained by the administration of testosterone propionate (18). Toxic effects observed in humans were mild, but an antabuse-like reaction accompanying alcohol consumption while under treatment made further use of these compounds impractical. More recently certain dinitropyrroles have been found to possess nitrofurazone-like activity after a single dose (19), but neurotoxicity observed in dogs has discouraged further study (20).

Colchicine and trimethylcolchicinic acid methyl ether are antimitotic agents which have been tested for antifertility properties in males. Although both compounds are successful in inhibiting spermatogenesis (21,22) by inhibiting mitosis in spermatogonia, they have been discarded as potential antifertility agents because of a lack of specificity.

A number of alkylating agents of diverse chemical nature have been shown to be capable of interfering with spermatogenesis. Jackson and Bock first reported the selective effects on male fertility of triethylene melamine (TEM) (23). Since that time the nitrogen and sulfur mustards, various epoxides, ethyleneimines, alkyl sulphonates, and phosphates as well as various diazoalkanes have been found to act in a manner similar to TEM. The pharmacological actions of alkylating agents are rather nonspecific as they react with a variety of chemical groups. As a consequence, compounds of this category present grave difficulties since they inhibit growth in many proliferating cell systems, especially those of the hematopoietic tissues.

Attempts to develop an antimetabolite or antibody which is capable of reversibly inhibiting spermatogenesis have met with little success. Ethionine is capable of damaging the seminiferous epithelium (24), but suffers from a lack of specificity. Attempts to

develop a reversible but effective antibody to sperm have met with little success (25).

The problems involved in developing a selective antifertility agent for the male are compounded by the fact that the testis is composed of a continuum of cell types ranging from the relatively undifferentiated spermatogonia to the highly differentiated spermatozoa. In view of the gross morphological changes occurring between the stem cell and the spermatozoan, as well as the occurrence of reduction of the chromosome number to the haploid condition, it would appear obvious that significant metabolic changes must accompany these phenomena. A better understanding of the metabolic changes occurring between early and late cell types could suggest the presence of crucial metabolic pathways which are unique to this organ, and might represent possible sites of attack for a selective inhibitor of spermatogenesis.

3. Synthesis and Possible Metabolic Role of Inositol

Eisenberg has demonstrated that the synthesis of myo-inositol from glucose occurs at a rate approximately ten times greater in the testis than in any other organ (3). This pathway would appear to be at least somewhat unique to the testis and could provide a possible site of attack

for a potential inhibitor of spermatogenesis. It has yet to be ascertained whether or not inositol has any significant physiological action in the male reproductive tract although one of the richest natural sources of free inositol is the seminal vesicular fluid. Levels of two to three grams per 100 ml have been reported for the boar (26). Seminal plasma obtained from many species including man contains approximately 100 mg per 100 ml (27). These studies have shown that inositol is synthesized in large amounts by the testis and is found in large amounts in the male reproductive tract.

There is some indication that inositol does have a significant physiological role in other systems. In 1901 Wildiers (28) introduced the term "Bios" referring to a substance that was essential for the growth of yeast in a synthetic medium. One of the components of "Bios" was shown by Eastcott to be identical with inositol (29).

The role of inositol in animals is still uncertain. The first mention of myo-inositol as an animal vitamin is to be found in the 1940 publications of Woolley (30). He maintained young mice on an inositol deficient diet and observed inadequate growth accompanied by alopoecia of the back and legs. Death followed two to three weeks after the onset of these symptoms.

Further work by Martin (31) and then by Woolley (32,33) showed that alopoecia and growth retardation could be

overcome to a great extent by the administration of pantothenic acid. In the presence of large amounts of pantothenic acid inositol deficiency symptoms appeared in only a few animals. In those animals which did develop deficiency symptoms the inositol levels in the carcass fell to half the normal level, and the intestinal flora were found to be incapable of synthesizing inositol. The intestinal flora of the resistant animals, however, could synthesize inositol. Thus, bacterial synthesis is sufficient in many cases to supply adequate levels of inositol. Furthermore, there seems to be a synergistic action between pantothenic acid and inositol in mammals.

When the intestinal flora are inhibited by bacteriostatic agents, this source of inositol is lost. Nielsen and Black observed defective growth and hair loss in rats fed a highly purified diet containing all the known vitamins except inositol together with sulphasuccidine (34). Growth became normal and the alopoecia disappeared on administration of 5 mg of inositol per day. Similar experiments by Ershoff and McWilliams (35) using female rats showed that on introduction of sulphaguanidine a regression of fertility resulted. Hamilton and Hogan (36) reported reproductive difficulties in female hamsters maintained on an inositol deficient diet. Therefore, inositol might have a significant although undefined role in reproduction.

Experiments carried out *in vivo* have shown that it is probable that inositol is necessary for normal growth. The exact mechanism by which it exerts its effect has yet to be elucidated. Most of the progress made in mechanistic studies has been made in microorganisms and tissue culture.

Eagle and coworkers have determined that inositol functions as a growth factor for many normal and cancerous cells in tissue culture (37,38). Only one of all the cell types Eagle studied grew indefinitely on an inositol deficient medium. This strain was found to be capable of synthesizing its own supply of inositol (39).

Yeast cells grown on an inositol deficient medium fail to separate from one another, and tend to form aggregates. The walls of these abnormal cells contain two to three times as much glucan as normal cells (40), while other cell constituents are not appreciably altered. An alteration in the cell wall appears to be the primary effect of inositol deficiency in yeast.

Since inositol is required for the maintenance and multiplication of mammalian cells grown *in vitro*, and since mammalian cell walls do not contain the polysaccharides found in yeast cell walls, inositol may have a different function in mammalian cells. Charalampous and coworkers studied the effects of inositol deficiency on KB cells. These investigators reported that during

the first two days of growth on an inositol deficient medium a number of biochemical changes occurred. A decrease in the acid soluble nucleotides and RNA of the cells as well as a decrease in the rate of incorporation of the radioactive carbon of glucose into the guanine of RNA were observed (41). Cell death occurred within two days after the onset of these symptoms. Furthermore, the ability of the deficient KB cells to synthesize nucleotides and RNA from adenine and guanosine was impaired. The incorporation of guanosine was depressed to a greater extent than adenine, an effect which correlated closely with a decrease in guanosine-5'-phosphate pyrophosphorylase activity (42).

Lembach and Charalampous further demonstrated that the activities of several nucleotide pyrophosphorylases and pyrophosphatases were altered (43). At the same time, the de novo synthesis of purine nucleotides and nucleic acids was increased while the ability of the deficient cells to maintain normal amino acid concentrations across the cell membrane was impaired (44). Kinetic studies on the influx of amino acids revealed a decrease in both the initial influx rate and maximal velocity. In addition, deficient cells were unable to increase the steady state accumulation level of α-amino isobutyric acid, a nonutilizable amino acid, to the same extent as normal cells in response to increasing concentrations of

extracellular sodium ion (44). These workers concluded that inositol deficiency impairs the mechanism responsible for the uptake and accumulation of amino acids.

Eagle and coworkers have found that the effects of inositol deficiency could be reversed by the addition of 3×10^{-7} M to 3×10^{-6} M inositol (37,38). These authors noted that this concentration is distinctly higher than that required for the other vitamins and cofactors in the medium, and presumed that inositol, like choline, is utilized principally as a substrate for the synthesis of phospholipids in tissue culture. Lembach and Charalampous (44) concluded that the alterations in amino acid uptake, nucleotide and RNA synthesis, and cell wall constituents could all be related to an altered level of phosphatidyl inositol in the cell membranes.

Many investigators have been unable to demonstrate the necessity for an external supply of inositol. There are a number of possible explanations for this. It should be noted that many diets considered highly purified may still contain significant amounts of inositol. Halliday and Anderson point out, for example, that purified casein, a constituent of most purified diets, contains 1.25 mg of inositol per 100 grams (45). Furthermore, it is certain that bacterial synthesis of inositol can constitute an important supply (34).

Definite proof of inositol synthesis in animals was not achieved until radioactive tracers were available. In 1955, Daughaday, Larner and Hartnett demonstrated the ability of rats to synthesize the cyclitol from ^{14}C-glucose (46). Shortly afterwards, Halliday and Anderson (45) confirmed these results, and clearly demonstrated that the synthesized inositol was not of bacterial origin since treatment with bacteriostatic agents did not significantly decrease the amount of inositol synthesized by the whole animal. Freinkel and Dawson confirmed this in germ-free rats and mice (47).

Various authors (48,49) have reported a difference in incorporation of $1^{14}C$-, $2^{14}C$- and $6^{14}C$-glucose into inositol. This seemed to indicate that the synthesis does not involve a simple cyclization of the glucose molecule. More recent studies in higher plants (50) and the rat (51,52) indicated that the conversion of glucose to inositol does involve a simple cyclization of glucose. All such conclusions remained tentative until a purified enzyme system studied under well defined conditions was obtained. Chen and Charalampous isolated such a system from yeast (53) and found that glucose was cyclized without scission of the six carbon chain (54). These investigators showed that the enzyme system requires a high energy source and showed that optimal conversion could be achieved in the presence of ATP, NAD and Mg^{++}.

ATP seemed to be necessary only to convert glucose to glucose-6-phosphate, while the requirement for NAD indicated the reaction sequence involves one or more oxidations. Mg^{++} was believed to be necessary for the activity of a phosphatase enzyme.

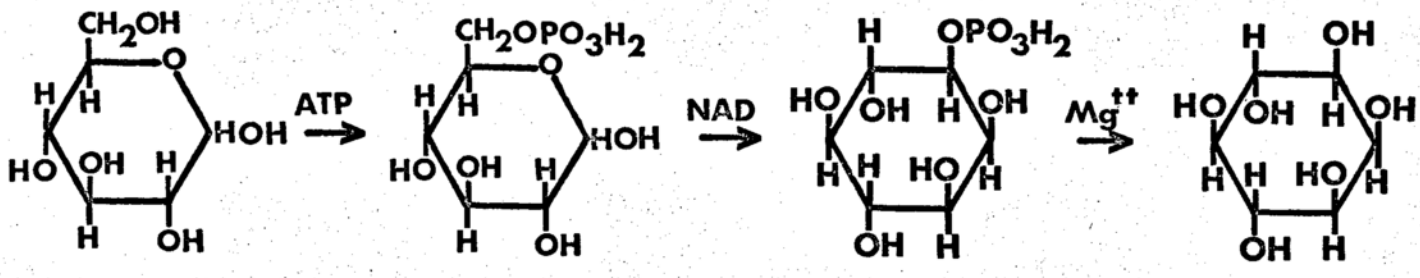
The substrate for this phosphatase enzyme was isolated from yeast (55) and rat testis homogenate (56) and proved to be 1-L-inositol-1-phosphate (I-1-P).

Chen and Charalampous reported the separation of two enzymes from the yeast system, one of which cyclized G-6-P to I-1-P. The second enzyme exhibited a specificity for phosphate esters of secondary hydroxyl groups (57). The rate limiting step in the pathway is the cyclization of G-6-P to I-1-P (55).

The suggested pathway of inositol synthesis thus consists of two steps (55). The first step, catalyzed by glucose-6-phosphate cyclase, leads to the formation of 1-L-inositol-1-phosphate, and has an absolute requirement for NAD. In the second step, I-1-P is hydrolyzed to inositol and inorganic phosphate. Mg^{++} is necessary for this step. Figure 1 presents a diagramatic representation of the proposed synthetic pathway starting with D-glucose.

From data obtained using C-6-^{3}H-G-6-P, Chen and Charalampous postulated the following mechanism for the cyclization of G-6-P (58). They proposed that G-6-P is oxidized to 5-keto-G-6-P and NAD is reduced. A C-6-bound H

BIOSYNTHESIS OF M-INOSITOL BY THE RAT TESTIS ENZYME SYSTEM



D-Glucose → Glucose-6-PO_4 → 1-L-Inositol-1-PO_4 → m-Inositol

then dissociates and condensation of C-1 and C-6 occurs. Eisenberg obtained similar data with rat testis preparations and postulated a similar mechanism (59).

4. Sites of Inositol Synthesis

Only limited attempts have been made to ascertain the sites of inositol synthesis within mammalian organisms. Hauser and Finelli (49) provided evidence for the ability of slices of rat brain, liver and kidney to convert labeled glucose to inositol. Eisenberg determined the distribution of the cyclase and phosphatase enzymes in various organs of the rat (3). Extracts of liver, spleen, lung, heart, brain, kidney and testis were prepared and assayed for cyclase and phosphatase activity. It was determined that the phosphatase activity was not confined to testis but is widespread among rat tissues whereas the cyclase activity is approximately ten times higher in testis than in any other organ. Thus, a deficiency in cyclase clearly accounts for the limited production of inositol by other tissues.

Eisenberg (3) also performed several experiments in an effort to locate the inositol synthesizing apparatus among the three major functional divisions within the mature testis; the spermatozoa, the seminiferous tubules in which the spermatozoa are formed, and the interstitial cells which synthesize the majority of the androgenic

hormones. Isolated epididymal spermatozoa were incapable of synthesizing inositol, while immature testes, in which spermatozoa were not demonstrable microscopically, were almost as active as the mature testis. No inositol synthesis could be shown in testes obtained from vitamin E deficient rats in which tubular atrophy had developed. Interstitial cells were nearly inactive. These experiments suggested that at least 90% of testicular inositol synthesis is of tubular origin.

The present study was designed to determine the relative activity of the various cell types within the seminiferous epithelium with regard to inositol synthetic activity and what some of the controlling factors of this synthesis might be.

MATERIALS AND METHODS

1. Animals

Male Holtzman rats, 55 to 60 days of age obtained from the Holtzman Company, Madison, Wisconsin, were used in all experiments. The weight of these animals varied from 230 to 260 grams. They were fed Rockland rat diet *ad libitum*.

2. Surgical Procedures

The experimental induction of bilateral cryptorchidism was carried out under sodium pentobarbital (30 mg/kg) and supplementary ether anesthesia. A technique similar to that developed by Hollinger and Davis was used (60). The abdomen was cleaned with 70% alcohol, and a midline abdominal incision was made. The epididymal fat pad was grasped with a forceps, and each testis was carefully raised from the scrotum. A fine 6-0 surgical silk suture was then passed just under the tunica albuginea, and the testes secured to the ventro-lateral abdominal wall, thereby preventing redescent of the testes into the scrotum. Sutures were placed beyond the point where the testicular artery enters the parenchyma in order to preserve intact the testicular circulation, and minimize the possible development of ischemia. The

abdominal musculature was closed by suturing with 6-0 surgical silk sutures. The skin incision was closed by clamping the skin with two or three 18 mm wound clips obtained from Clay-Adams, Inc. The animals were placed in cages containing clean wood shavings and carefully observed until recovery was complete. Sham-operated control animals were treated similarly, although the testes were not sutured and fixed to the abdominal wall.

3. Incubation Media

Krebs-Ringer bicarbonate buffer was used in all experiments involving the use of testicular tissue slices. The components of this buffer were as follows: 0.11 M NaCl, 0.005 M KCl, 0.0025 M $CaCl_2$, 0.001 M KH_2PO_4, 0.001 M $MgSO_4$ and 0.025 M $NaHCO_3$. A pH of 7.4 was achieved by passing a mixture of 95% O_2 and 5% CO_2 through the buffer for ten minutes. Fresh buffer was prepared immediately before each experiment from concentrated stock solutions of the various constituents.

A 0.05 M tris buffer was used in all experiments in which cell-free systems were used. This buffer was prepared by adjusting the pH of a 0.05 M tris-(hydroxymethyl)-aminomethane (Aldrich Chemical Company) solution to pH 7.4 by the addition of concentrated HCl.

4. Isotopes

Those experiments in which the synthesis of inositol was measured in tissue slices involved the use of [U^{14}C]-D-glucose obtained from the Amersham-Searle Corporation. This isotope had a specific activity of 3.9 millicuries per millimole, and was diluted to a concentration of 3×10^6 disintegrations per minute (dpm) per 0.2 ml with distilled water. An aliquot of 0.2 or 0.4 ml was added to the sidearm of each incubation flask. This was equivalent to 1.36 or 2.72 microcuries per flask. The final concentration of isotope in the 3.2 ml incubation volume was 0.425 or 0.85 microcuries per ml.

Experiments using cell-free systems to measure inositol synthesis used the disodium salt of [U^{14}C]-glucose-6-phosphate, [U^{14}C]-G-6-P, as the tracer. This isotope was also purchased from the Amersham-Searle Corporation and had a specific activity of 2.3 millicuries per millimole. A dilution to 3×10^6 dpm per 0.2 ml in distilled water was made. In these experiments, 0.05 or 0.1 ml were added to each incubation tube. This was equivalent to 0.34 or 0.68 microcuries, and since the incubation volume measured 1.0 ml the concentration was 0.34 or 0.68 microcuries per ml.

Authentic [U^{14}C]-inositol was used to ascertain the identity and purity of the isolated reaction product.

This isotope was also obtained from Amersham-Searle Corporation, and had a specific activity of 160 millicuries per millimole. It was diluted to a concentration of 6,254,600 dpm per ml. Phosphatidyl inositol synthesis was measured by use of 2-^{3}H-m-inositol. The isotope was obtained from the New England Nuclear Corporation and had a specific activity of 3470 microcuries per millimole. It was diluted to an initial concentration of 25 microcuries per ml. An aliquot representing 5 microcuries of initial activity was added to each flask.

5. Biochemicals

For those tissue slice studies requiring D-glucose as substrate, a 0.08 M solution was prepared in pH 7.4 Krebs-Ringer bicarbonate buffer immediately before each experiment. In order to achieve a final concentration of 0.005 M in the incubation mixture, 0.2 ml of this solution was added to the sidearm of the appropriate incubation flasks.

Cell-free systems which were used to measure inositol synthesis utilized the disodium salt of glucose-6-phosphate (G-6-P) as the substrate, and β nicotinamide adenine dinucleotide (NAD) and $MgCl_2$ as cofactors. The G-6-P and NAD were obtained from the Sigma Biochemical Corporation. The G-6-P was prepared immediately before each experiment as a 0.05 M solution

in 0.05 M tris buffer at pH 7.4 while the NAD and $MgCl_2$ were prepared in tris buffer as 0.01 M solutions. The desired concentrations of 0.005 M for G-6-P and 0.001 M for NAD and $MgCl_2$ were achieved by adding 0.1 ml of each to the 1.0 ml incubation mixtures.

6. Preparation of Testis Slices and Cell-Free Systems

The animals were sacrificed 3.5, 7, 14 or 21 days post-operative by a blow on the head and subsequent decapitation. The testes were rapidly removed at 4°C, the capsule excised, and tissue slices prepared with the aid of a Stadie-Riggs microtome. Approximately 400 mg wet weight of testis was used in each incubation.

Cell-free systems were prepared by homogenizing the testes after the capsule had been removed in two volumes of 0.154 M KCl at 4°C using a motor-driven Tenbroeck ground glass homogenizer. The 100,000 times gravity (100,000 x g) supernatants were prepared as follows. The crude homogenates were centrifuged at 15,000 x g for ten minutes at 4°C in a Sorval angle centrifuge to remove cell debris, nuclei, and mitochondria. The supernatant fraction was decanted and centrifuged at 100,000 x g for one hour at 0°C in a Beckman Model L Ultracentrifuge to remove the microsomes. The 40 rotor which accommodates 5/8 by 3 inch Polyallomar tubes (Beckman Spinco Division) was used. The supernatant was decanted and kept at 4°C.

In some experiments, this supernatant was divided into two portions, one of which was heat-treated at 60°C for two minutes. The protein which was precipitated by this treatment was removed by centrifugation at 15,000 x g for ten minutes at 4°C.

7. Protein Determination

The protein concentrations of the various 100,000 x g supernatant fractions were determined using the biuret reaction (61). The biuret reagent was prepared by dissolving 1.50 grams of $CuSO_4 \cdot 5H_2O$ and 6.0 grams of sodium potassium tartrate in approximately 500 ml of distilled water. The solution was made alkaline by the addition of 300 ml of 10% NaOH and made up to 1000 ml by the addition of distilled water.

An aliquot of the protein solution was made up to 1.5 ml with distilled water, and 1.5 ml of the biuret reagent was added. The mixture was incubated for 15 minutes at 37.5°C, and the absorbance read at 540 millimicrons in a Bausch and Lomb Spectronic 20 spectrophotometer. The reagent blank consisted of 1.5 ml of water and 1.5 ml of biuret reagent. A standard curve was prepared with Bovine Serum Albumin Fraction V (Nutritional Biochemical Corporation).

8. Incubation Systems

Tissue slice experiments were performed on a Gilson Warburg incubator, in 20 ml Warburg flasks with a single sidearm. The flasks were agitated at a rate of 80 strokes per minute, and the temperature was controlled with a precision of ±0.01°C.

Cell-free experiments were performed in open test tubes placed in a test tube rack which rested on rubber vacuum tubing which covered the edge of a five gallon aquarium. The aquarium was placed immediately adjacent to a Dubnoff Metabolic Shaker (Precision Scientific Company). The test tube rack was attached to the rotor which had been detached from the shaking pans, and reversed so as to allow shaking of the rack. The aquarium was filled with water and equipped with a Tecam Thermostatic Circulating Immersion Heater (LaPine Scientific Company) which controlled the temperature with a precision of ±0.1°C. This modification of the Dubnoff incubator provided better temperature control than the standard instrument.

9. Inositol Synthesis in Tissue Slices

Approximately 400 mg wet weight of testis was weighed on a Roller Smith Precision Balance, and placed in the main chamber of a Warburg flask containing 2.8 ml of pH 7.4 Krebs-Ringer bicarbonate buffer. The sidearm

was provided with 16 micromoles of D-glucose and 0.2 ml of [$U^{14}C$]-D-glucose which represents 3 x 10^6 dpm. Those experiments using cryptorchid slices had 6 x 10^6 dpm of isotope added to increase the specific activity of the isolated inositol. In this case, only 2.6 ml of buffer was added to the main chamber. The final concentration of D-glucose in all experiments was 0.005 M.

The flasks were placed on the manometers and preincubated under a stream of 95% O_2 and 5% CO_2 for ten minutes. The flasks were incubated at a temperature of 32°C or 37.5°C. The flasks were then closed to the atmosphere and the sidearm contents added to the main chamber. Incubation time was generally 60 minutes, but varied in those experiments in which the time course of inositol synthesis was measured. Reactions were terminated by boiling the flask contents for five minutes.

10. Inositol Synthesis in Cell-Free Systems

Aliquots consisting of 0.3 ml of crude homogenate were incubated in open test tubes at 37.5° in 0.05 M tris buffer pH 7.4 containing 750,000 or 1.5 x 10^6 dpm of the disodium salt of [$U^{14}C$]-glucose-6-phosphate, 5 micromoles of unlabelled disodium G-6-P, 1 micromole of NAD and 1 micromole of $MgCl_2$. These reagents were prepared as described in sections 4 and 5. The final incubation volume was 1.0 ml. Reactions were terminated by placing the tubes in boiling water for two minutes.

Aliquots of the 100,000 x g supernatant fractions containing 3 mg of protein were incubated under identical conditions. Incubation times were generally 30 minutes but varied in those experiments designed to study the time course of inositol synthesis.

11. Isolation of Inositol

A method similar to that developed by Eisenberg (3) was employed. In those experiments which involved the use of testis slices, a homogenization step was necessary. One hundred mg of unlabelled carrier inositol was added and the protein precipitate removed by centrifugation. Cell-free systems were treated similarly except homogenization was unnecessary. The precipitates were washed twice with three ml of distilled water and the supernatant and washings combined in a glass-stoppered bottle containing 500 mg of $BaCO_3$ and one ml of bromine solution to destroy any residual substrate. This mixture was shaken vigorously for 30 minutes. Excess bromine was removed by passing a stream of nitrogen through the suspension followed by the dropwise addition of styrene (J. T. Baker Chemical Company) with shaking. The resulting suspension was then passed over either a 2 x 15 cm column of Rexyn AG 501 (Fisher) mixed bed ion exchange resin or a 2 x 15 cm column consisting of a layer of Dowex 50 W-X-4 (J. T. Baker Chemical Company) H^+

form and a layer of Amberlite IRA 400 (Rohm and Haas Company) which had been converted from the Cl^- to the OH^- form by washing with a 1 N NaOH solution until no AgCl precipitate was formed when the washings were tested with a dilute $AgNO_3$ solution. The effluent, including 100 ml of wash, was evaporated to about 1 ml and ten ml of chilled methanol was added. This mixture was placed in the freezer until crystallization was complete. The crystals were filtered, redissolved in water and recrystallized as above. All samples were routinely recrystallized twice to ensure homogeneity. The inositol was air dried, weighed into a tared 1.0 ml volumetric flask, and dissolved in enough water to make exactly 1.0 ml.

12. Radioactive Assay of ^{14}C-Inositol

A 0.1 ml aliquot of the above solution was added to 15 ml of Bray's solution (62), a dioxane based scintillation fluid, in a 20 ml glass scintillation vial (Packard Instrument Company). The Bray's solution was prepared by dissolving 120 grams of naphthalene, 8 grams PPO (2,5-diphenyloxazole) (Packard Instrument Company) and 400 mg of dimethyl POPOP (1,4-bis-(2,4-methyl-5-phenyloxazolyl)-benzene) (Packard Instrument Company) in 200 ml absolute methanol, 40 ml ethylene glycol, and enough redistilled dioxane to make 2000 ml. The vials

were counted at ambient temperature in a Packard 3002 Tri-Carb liquid scintillation spectrometer. The degree of quenching was determined by the addition of ^{14}C-toluene internal standard (Packard Instrument Company).

13. Cellulose Chromatography of Inositol

The identity and purity of the isolated product was ascertained by cellulose chromatography. A 1 x 21 cm column of standard grade Whatman cellulose powder obtained from E. H. Sargent & Company was prepared. Approximately 8 mg of isolated inositol was dissolved in 0.3 ml of water and carefully placed on the column. The column was eluted with acetone-water, 8:2, and 5 ml fractions were collected. A 0.25 ml aliquot was taken from each fraction and the radioactivity determined in 15 ml of Bray's solution. Peak tubes were combined, the inositol recrystallized, and the specific activity determined and compared with that of the compound placed on the column.

An aliquot of ^{14}C-inositol containing 18,200 dpm was added to 0.3 ml of a solution containing 7.8 mg of unlabelled inositol. This was added to the same column after washing, and was eluted in the same manner. The location and shape of the radioactive peak was compared with that of the isolated product.

14. Nonspecific Phosphatase Determination

The method of Chen, Toribara and Warren was used (63) to determine the nonspecific phosphatase activity in 14 day cryptorchid and control, unheated 100,000 x g supernatant fractions of rat testis. The testes from six cryptorchid and three control animals were used to prepare each supernatant assayed. The supernatants were dialyzed for 24 hours against 1000 volumes of 0.154 M KCl. Aliquots containing 3 mg of protein were incubated with 0.005 M G-6-P and 0.001 M $MgCl_2$ in 0.05 M tris buffer pH 7.4 in a manner identical to that used for measurement of inositol synthesis. However, NAD was not added to the incubation media in order to minimize the inorganic phosphate resulting from the activity of inositol-1-phosphatase. The reaction was terminated by the addition of 4 ml of 5% trichloroacetic acid. The precipitated protein was removed by centrifugation and a 0.5 ml aliquot of the supernatant was removed and added to 3.5 ml of distilled water. To this was added 4 ml of freshly prepared color reagent. This reagent consisted of one volume each of freshly prepared 6 N H_2SO_4, 10% ascorbic acid, and 2.5% ammonium molybdate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$; and two volumes of distilled water. The sample was incubated in Parafilm covered tubes for two hours at 37.5°C. After cooling the absorbance was

read at 820 millimicrons in a Bausch and Lomb Spectronic 20 equipped with a red filter and a P40 tube (E. H. Sargent & Company). The blank consisted of 4 ml of distilled water and 4 ml of color reagent. The absorbances of reagent and tissue blanks for each supernatant were determined, and subtracted from absorbances of the unknowns. A standard curve was prepared using dilutions of a pure monopotassium phosphate solution which had been prepared by dissolving 0.3509 grams of KH_2PO_4 in distilled water to which 10 ml of 10 N H_2SO_4 had been added. This solution was diluted to 1000 ml with distilled water and represented 0.4 mg of inorganic phosphorus per 5 ml.

15. Glucose Uptake Determination

Glucose uptake by cryptorchid and normal testis slices was determined by measuring the disappearance of glucose from the incubation medium using the Glucostat reagent obtained from the Worthington Biochemical Corporation. A modified version of the technique reported by Saloman and Johnson (64) for measuring glucose in blood and urine was used.

Approximately 400 mg wet weight of testis was added to 3.0 ml of pH 7.4 Krebs-Ringer bicarbonate buffer in the main chamber of a Warburg flask. The sidearm was provided with 16 micromoles of D-glucose in 0.2 ml. The flasks were placed on the manometers and preincubated under a

stream of 95% O_2 and 5% CO_2. The flasks were then closed to the atmosphere and the sidearm contents added to the reaction chamber. Incubations were carried out for 15, 30 or 60 minutes. The reactions were terminated by rapidly decanting and filtering the incubation media. A 0.1 ml aliquot of this filtrate was added to 1.9 ml of distilled water and deproteinized by adding 1.0 ml of 1.8% $Ba(OH)_2$ solution followed by the addition of an equal volume of 2% $ZnSO_4$ solution. These two solutions had been prepared so as to exactly neutralize one another. This was achieved by diluting 10.0 ml of the $ZnSO_4$ solution with 50 ml of water, adding two drops of phenolphthalein test solution and titrating with the $Ba(OH)_2$ solution to a faint pink color. The more concentrated solution was then diluted if necessary so that 10.0 ± 0.05 ml of $Ba(OH)_2$ was required for neutralization.

The precipitate formed by the addition of the $Ba(OH)_2$ and $ZnSO_4$ was removed by centrifugation. A 2.0 ml aliquot of the supernatant was placed in a colorimeter tube and 2.0 ml of Glucostat reagent was added. The Glucostat reagent was prepared immediately before each experiment by dissolving the chromagen and glucose oxidase in water and diluting to 50 ml. The unknowns, reagent blank, and a standard which contained 0.1 mg of glucose were incubated for 30 minutes at 37.5°C. The

reaction was terminated and the color stabilized by the addition of one drop of 4 N HCl. The absorbance was read at 425 millimicrons in a Bausch and Lomb Spectronic 20. A standard curve was prepared using samples which contained 0.025 to 0.1 mg of D-glucose.

16. Phosphatidyl Inositol Synthesis

Phosphatidyl inositol synthesis by 14 day cryptorchid and control testis slices was measured using a technique similar to that developed by Hokin and Hokin (65) for pancreas slices. Approximately 400 mg wet weight of testis were added to 2.8 ml of Krebs-Ringer bicarbonate buffer pH 7.4 in the main chamber of a Warburg flask. The sidearm was provided with 16 micromoles of D-glucose in 0.2 ml and 5 microcuries of 2-^{3}H-myo-inositol prepared as described in section 4. The actual number of dpm added to each flask was determined for each experiment. After ten minutes of preincubation, the sidearm contents were added to the reaction chamber. Incubations were carried out for 15, 30 and 60 minutes and were terminated by the addition of 3.2 ml of 10% trichloroacetic acid. The tissue was then homogenized and centrifuged at 15,000 x g for ten minutes at 4°C. The supernatant was decanted and discarded. The pellet was washed three times with 3 ml of 5% trichloroacetic acid. The washed pellet was resuspended in 16 ml of 1:1, chloroform:ethanol. The suspension was

transferred to a 40 ml centrifuge tube, placed in the cold room at 4°C and extracted with shaking overnight. The addition of 20 ml of 0.1 N HCl resulted in the formation of two phases. After 15 minutes of shaking, these phases were separated by centrifugation. A 4.0 ml aliquot of the chloroform layer was removed and placed in a glass scintillation vial and evaporated to dryness under a stream of nitrogen. The vials were then placed in a vacuum dessicator provided with KOH for two hours. This step reduces any quenching that residual trichloroacetic acid might cause. The radioactivity of the lipid fraction was determined by adding 15 ml of a toluene based scintillator fluid containing 3% PPO (2,5-diphenyloxazole) and 0.1% POPOP (2,2'-paraphenylene-bis-5-phenyloxazole). These compounds were obtained from the Packard Instrument Company. Quenching was determined by the addition of ^{3}H-toluene internal standard.

17. Preparation of Histological Plates

The preparation of histological slides was done in the laboratories of Dr. John Anderson of the Department of Anatomy. The testes were fixed by the method developed by Lillie (66), which involves initial fixation in Bouin's fixative followed by drying in various alcohol water mixtures. This is followed by a softening procedure which involves treatment with cedar oil and benzene. The tissue was then embedded in paraffin and

Para Plast Plus and sectioned into ten micron sections. The tissue was then stained with Delafield's hematoxylin (67) and counterstained with eosin (68). Photographs of the resulting slides were taken and prints prepared. A 350-fold magnification is achieved on the photographic plates presented.

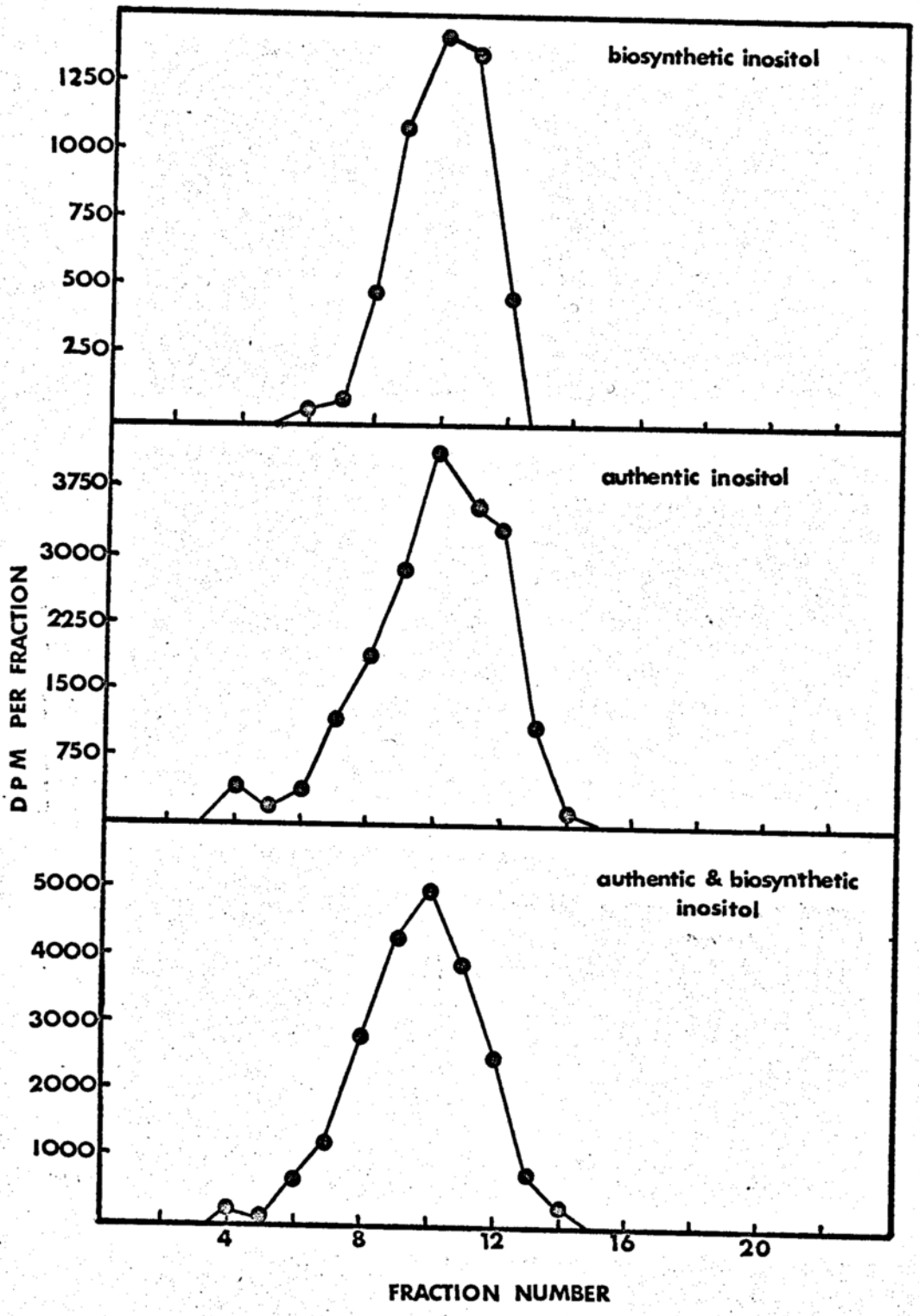
RESULTS AND DISCUSSION

1. Verification of Radiochemical Purity of Biosynthetic m-Inositol

An investigation of the conversion of [U^{14}C]-D-glucose and [U^{14}C]-glucose-6-phosphate (G-6-P) to inositol in various preparations of rat testis, *in vitro*, was of major concern in the present studies. Therefore, it was necessary to ascertain the radiochemical purity of the biosynthesized inositol isolated from the incubation media. The isolated [U^{14}C]-inositol was recrystallized to constant specific activity and chromatographed on a cellulose column (45). Figure 2 depicts the results of a typical experiment in which biosynthetic inositol, authentic [U^{14}C]-inositol, and a mixture of the two were chromatographed consecutively on the same column. The column was thoroughly washed with 8:2, acetone:water between samples.

The fraction which contained maximal radioactivity was identical for each of the three samples, and 95-102% of the radioactivity added to the column could be accounted for in this peak. The shape of the curve was similar for all three samples. Inositol recrystallized from the pooled fractions constituting the peak had an identical specific activity to that of the sample placed

Cellulose Chromatography of ^{14}C m-inositol



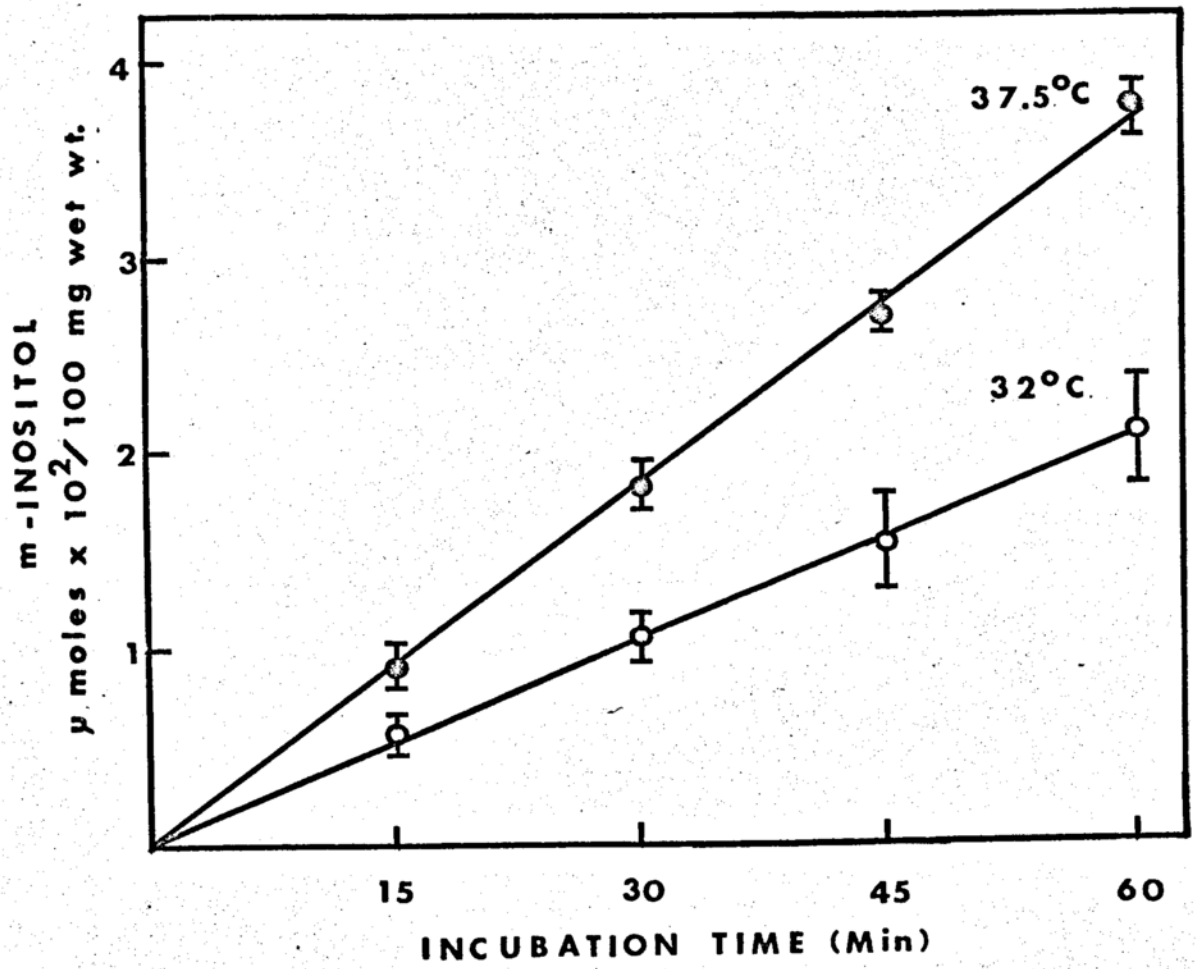
on the column. Therefore, recrystallization to constant specific activity was considered to provide adequate radiochemical purity of biosynthetic inositol.

2. Effect of Incubation Temperature on Inositol Synthesis in Rat Testis Slices

Normal spermatogenic activity is apparently facilitated by the lower ambient temperature provided by the scrotal position of the testes in most mammals (69). Metabolic studies by Davis, et al. have shown that the ability of rat and rabbit testis slices to incorporate lysine into protein, in vitro, is maximal at the scrotal temperature of 32°C, whereas the normal body temperature of 37.5°C is optimal for other tissues such as liver, kidney, and spleen (70). Similar results have been obtained with other species (71). Consequently, it seemed essential to compare inositol synthesis at the scrotal and body temperatures in order to determine which would be an optimal incubation temperature. This was achieved by measuring the capability of rat testis slices to synthesize inositol at incubation temperatures of 32°C and 37.5°C.

Figure 3 illustrates the effect of incubation temperature on the rate of conversion of [U^{14}C]-D-glucose to inositol by rat testis slices. In the presence of 0.005 M D-glucose, inositol synthesis was linear throughout the 60 minute incubation period for both

CONVERSION OF D-GLUCOSE (0.005 M) TO m-INOSITOL IN RAT TESTIS SLICES



temperatures. Each point represents the mean of four experiments at 37.5°C and eight experiments at 32°C.

The rate of conversion was 1.67 times greater at 37.5°C than at 32°C. At an incubation temperature of 37.5°C, inositol was synthesized at the rate of 0.0375 ± 0.009 micromoles (μM) per 100 mg wet weight of testis per hour, whereas 0.0224 ± 0.0027 μM were synthesized per hour at 32°C. Unlike protein synthesis, the testicular synthesis of inositol is more active at 37.5°C. As a result, 37.5°C was chosen as the incubation temperature for all subsequent experiments.

These data suggest that either the inositol synthesizing enzymes are not as heat labile as the protein synthesizing enzymes appear to be, or that the addition of exogenous glucose in substrate quantities to the incubation medium serves to protect this system from the higher temperature. The latter explanation seems possible in view of the apparent protective effect of glucose on protein synthesis which has been reported by Davis and Morris (72).

3. Effect of Surgically-Induced Cryptorchidism on Inositol Synthesis in Rat Testis Slices

Transplantation of the testes from the scrotum to the abdominal cavity results in atrophy of the germinal epithelium (73). The spermatogonia, Sertoli cells, and

interstitial cells seem to be relatively unaffected by cryptorchidism (74,75), while more mature germinal cells, the spermatocytes, spermatids and spermatozoa, appear to be very susceptible to deterioration in this condition (75,76). Cryptorchidism was chosen as a tool for alteration of the cellular population in the present studies. Sixty-day-old male rats were made bilaterally cryptorchid by surgical translocation of the testes from their scrotal position to the abdominal cavity. Animals which had been sham-operated served as controls in these experiments.

The reduction in testis weight which occurred following surgically-induced cryptorchidism is shown in Figure 4. Day zero on this graph presents the average testis weight of sham-operated animals. Cryptorchidism resulted in a 70% reduction in testis weight within 21 days after translocation. The greatest reduction in testis weight occurred within the first seven days. By 14 days after surgery, the weight of the testis appears to have become essentially stable.

Table I presents the loss in testis weight as a result of cryptorchidism in terms of per cent reduction from sham-operated control testis weight. The data presented in this table suggest that most of the atrophy and morphological changes which occur in the germinal epithelium as a result of cryptorchidism would be expected within the first 14 days after translocation.

Reduction in Testis Weight Following Surgically – Induced Cryptorchidism

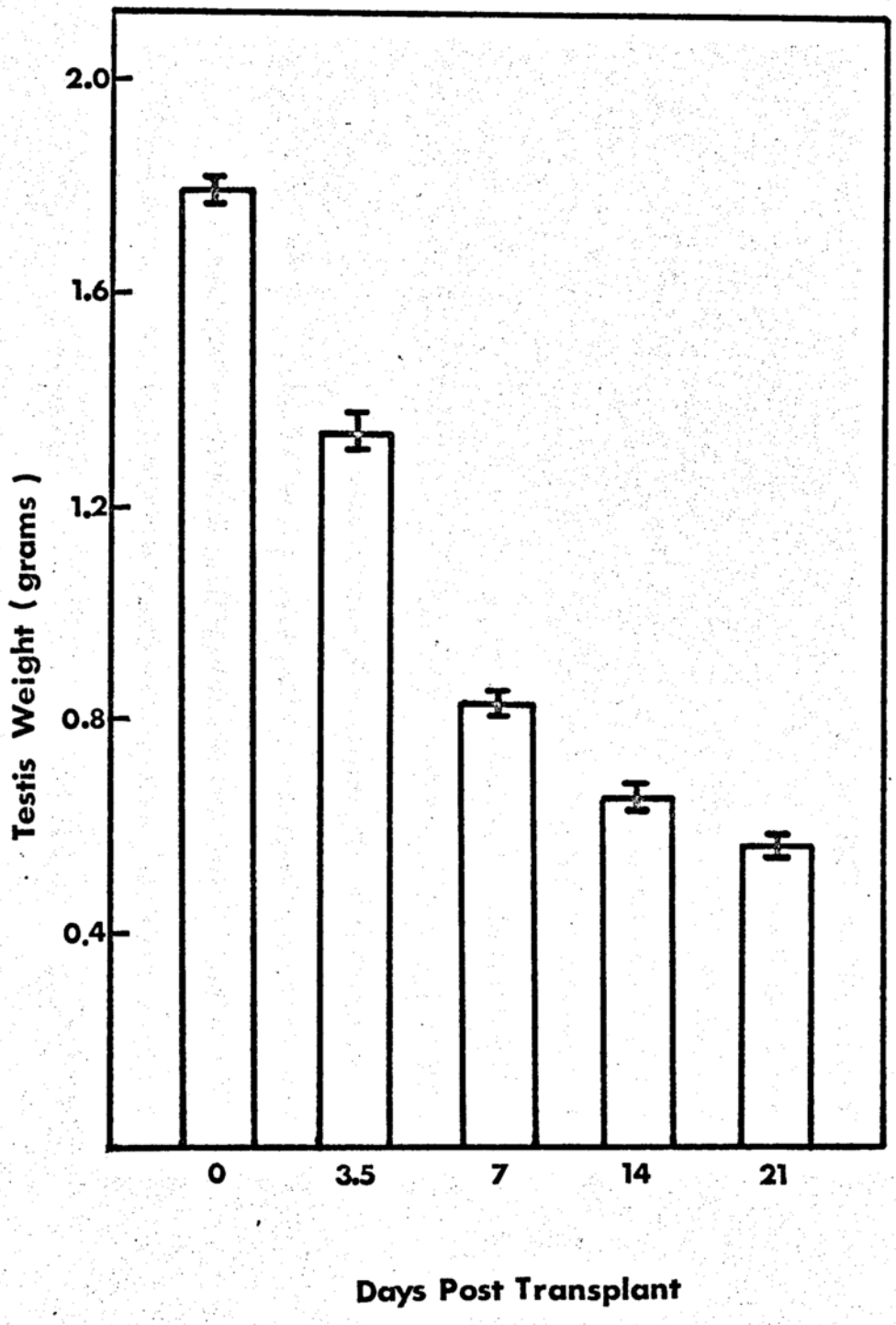


TABLE I

REDUCTION IN TESTIS WEIGHT FOLLOWING SURGICALLY-INDUCED CRYPTORCHIDISM

Days Post Transplant	Testis Weight (mg) ±s.e. of mean	n	% Reduction Testis Weight
Control	1797±32	12	--
3.5	1348±42	12	25.0
7	832±21	14	53.7
14	646±37	20	64.1
21	565±22	22	68.6

Examination of Plates 1 through 4, in which the histology of control, 3.5, 7, and 14 day cryptorchid testes are presented, suggests that histological stability has been achieved within 14 days after translocation. By 14 days after translocation, the cryptorchid testis has lost all of the cell types normally found in the germinal epithelium with the exception of the spermatogonia and Sertoli cells. The interstitial cells are not altered as a result of cryptorchidism.

The 3.5 and 7 day cryptorchid testes undergo rapid morphological and histological changes. Within the first 3.5 day period a considerable reduction in the number of spermatids and spermatozoa occurs. At the same time, a number of large, polynucleated cells appear, while the presence of a considerable amount of cellular debris indicates that cellular necrosis has developed. The 7 day cryptorchid testis is similar except that a considerable reduction in the number of primary and secondary spermatocytes has occurred. In the following seven day period the few remaining spermatocytes disappear from the cryptorchid testis.

The histological appearance of the 14 day cryptorchid testis is quite diffuse in that many, large, unoccupied spaces may be seen within the germinal epithelium. The cell types which remain, the interstitial cells, Sertoli cells, and spermatogonia, are those which

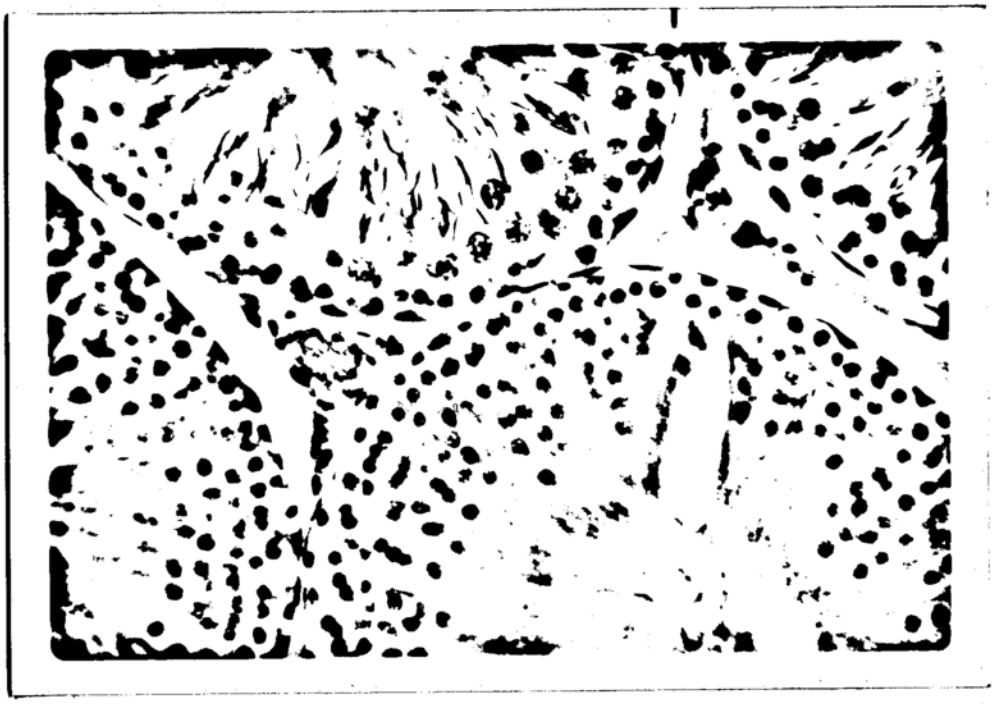


Plate 1

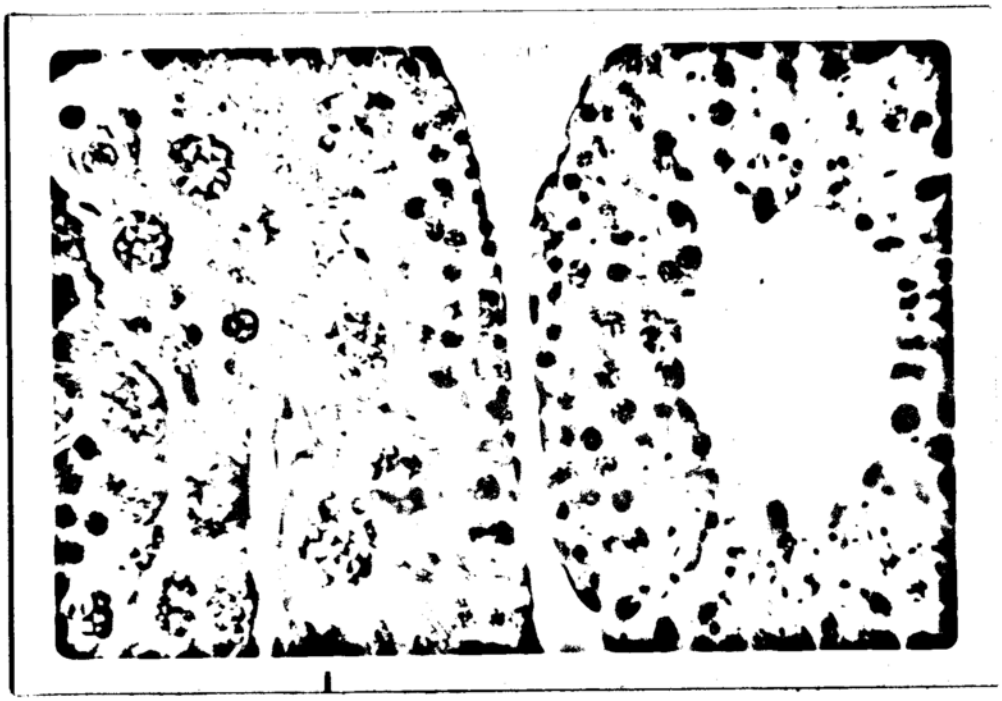


Plate 2

Plate 3

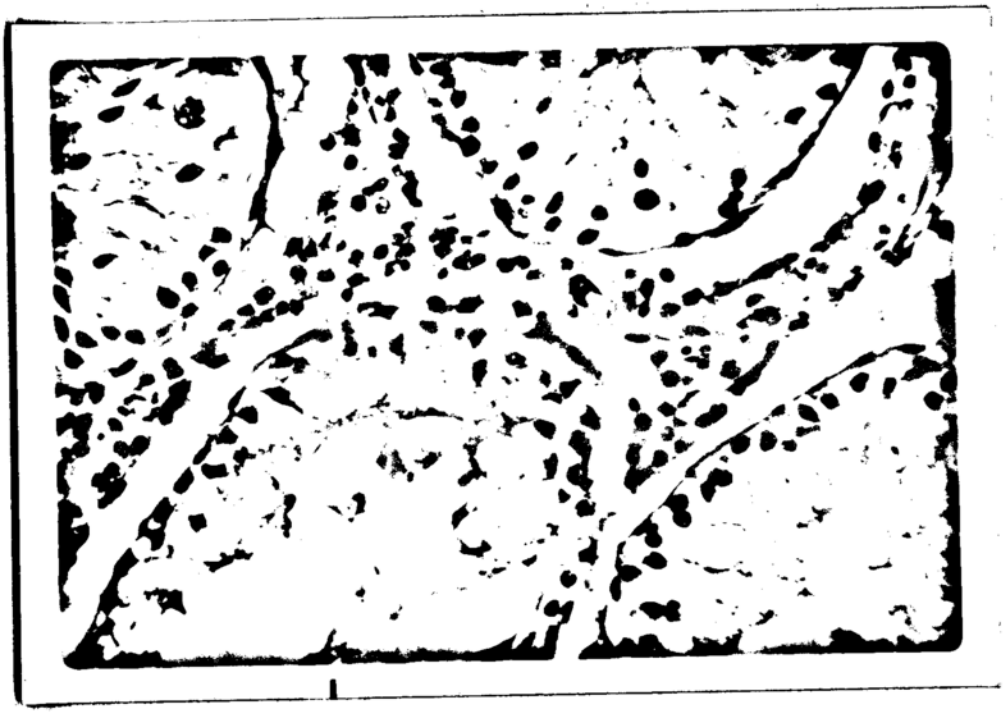


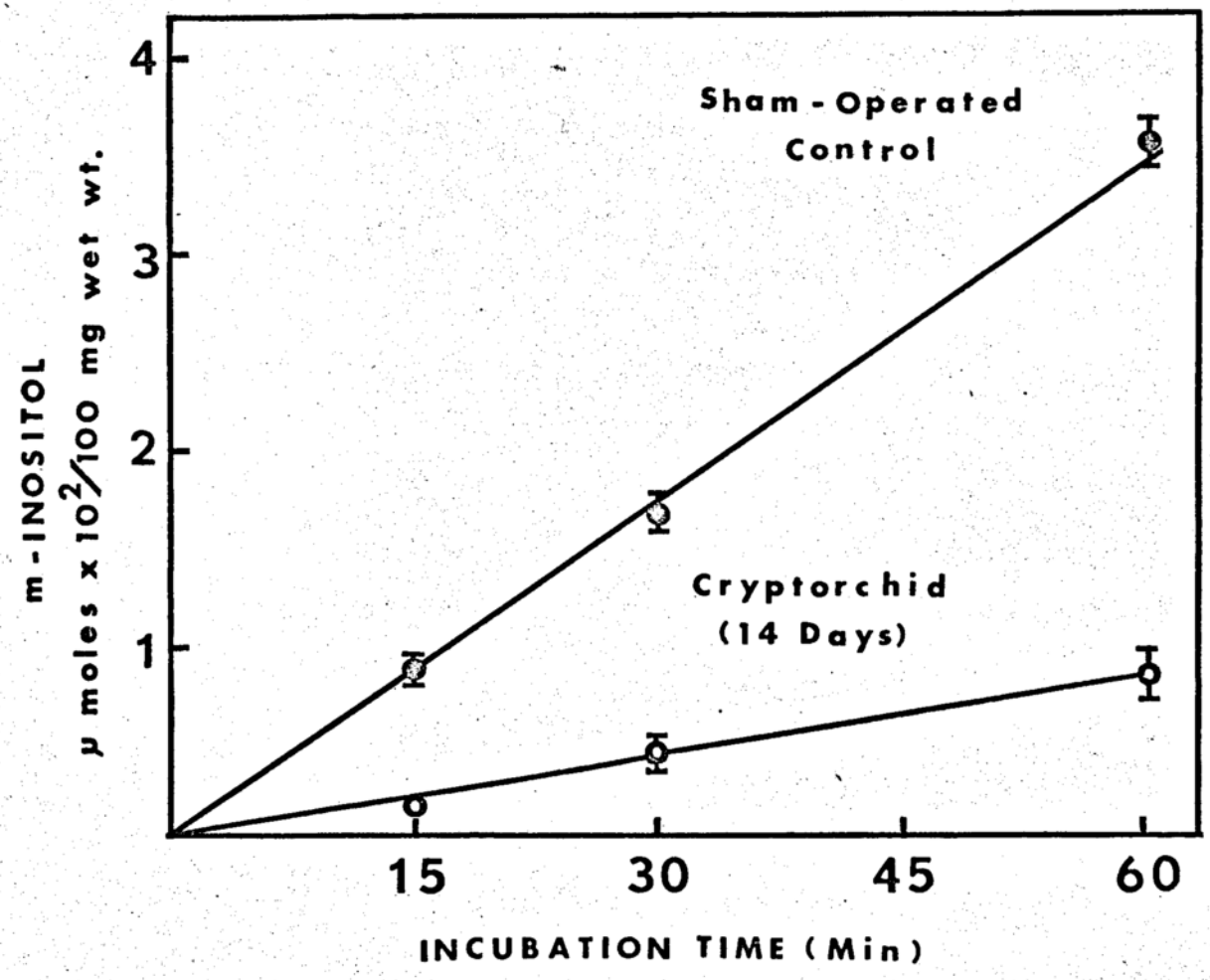
Plate 4

have been reported to be unaffected by experimentally-induced cryptorchidism (74,75). Thus, any further loss in testis weight may be the result of removal of the cellular debris.

Figure 5 depicts the rate at which sham-operated control and 14 day cryptorchid rat testis slices synthesize inositol from [U^{14}C]-D-glucose, in vitro. Each point represents the mean of four to twelve experiments. The synthesis was linear throughout the one hour incubation period for both testis types. The rate of synthesis for sham-operated controls was identical with that of the untreated controls reported in Figure 3.

Cryptorchidism resulted in a 78% reduction in synthesis rate. Testis slices from sham-operated control animals synthesized 0.0372±0.0008 μM of inositol per 100 mg wet weight of testis per hour while 14 day cryptorchid testis slices synthesized 0.0083±0.0016 μM per 100 mg wet weight of testis per hour. This suggests that one or more of the cell types which were lost as a result of exposure to the temperature of the abdominal cavity contained a major portion of the inositol synthetic activity. An alternative possibility is pathways which ordinarily regulate inositol synthesis may be altered. These possibilities are not mutually exclusive.

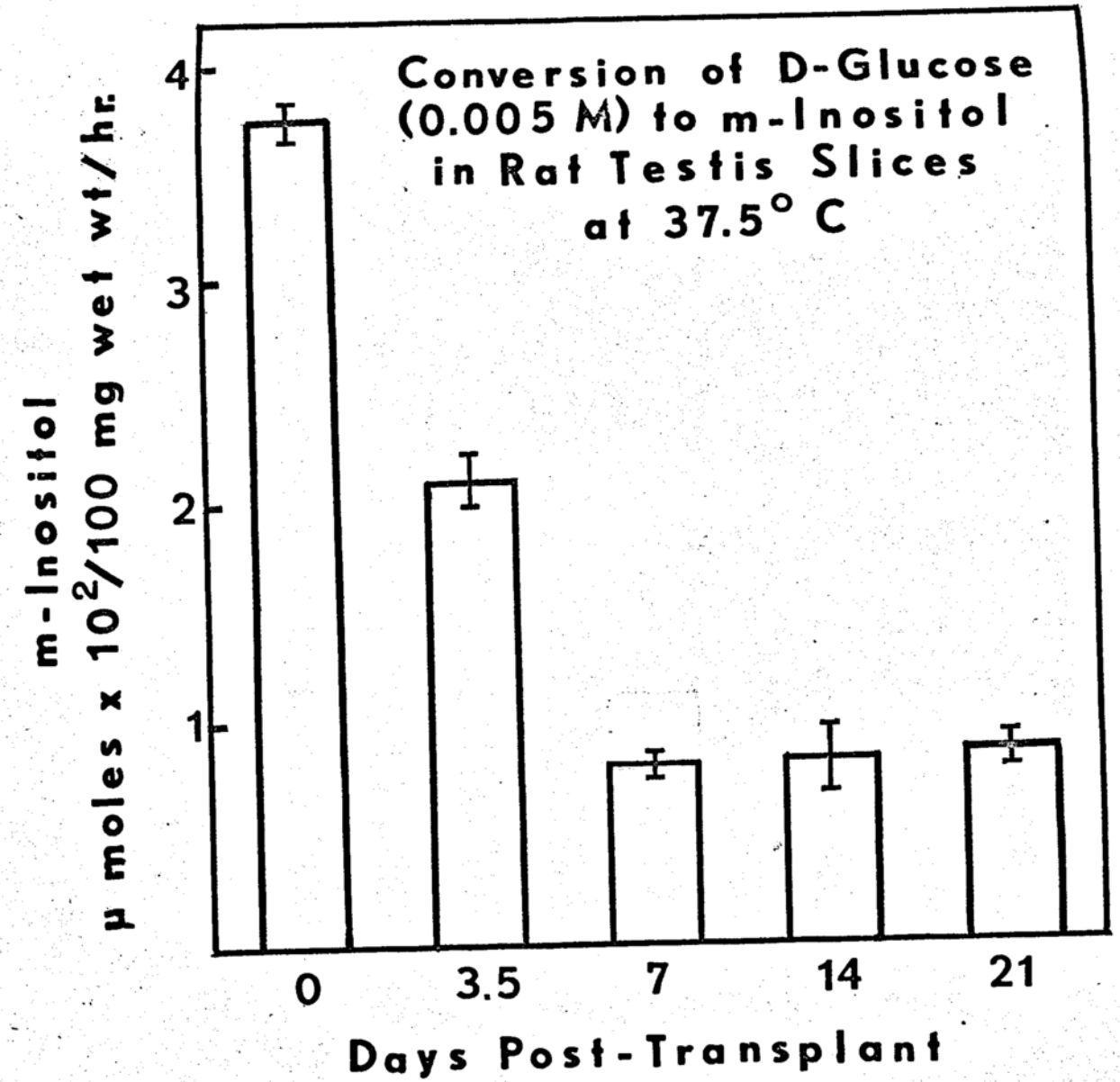
CONVERSION OF D-GLUCOSE (0.005 M) TO
m-INOSITOL IN RAT TESTIS SLICES
AT 37.5° C



The rate of onset of depression in inositol synthesis which occurred as a consequence of cryptorchidism is illustrated in Figure 6. The sham-operated control data are represented by the zero day bar. Maximal depression of inositol synthesis developed within the first seven days after translocation of the testes, and remained at a constant level throughout the remaining period of observation. The development of depression of inositol synthesis paralleled the reduction in testis weight observed with cryptorchidism (Figure 4). These data further suggest that the major portion of inositol synthetic activity resides in one or more of the more mature cell types which partially constitute the germinal epithelium.

4. Synthesis of Inositol in Homogenates of Control and Cryptorchid Testes

A variety of biochemical alterations are theoretically plausible which might explain or partially account for the observed depression of inositol synthesis in cryptorchid testis slices. Underlying any observation of altered glucose utilization, is the possibility of an alteration in glucose uptake by the tissue in question. A possible difference in glucose uptake between slices of control and cryptorchid testes was suggested by the results of experiments in which inositol synthesis from [U^{14}C]-glucose-6-phosphate (G-6-P) was measured in rat testis


Conversion of D-Glucose
(0.005 M) to m-Inositol
in Rat Testis Slices
at 37.5° C
m-Inositol
μ moles x 10²/100 mg wet wt/hr.
4
3
2
1
0
3.5
7
14
21
Days Post-Transplant

homogenates.

Figure 7 depicts the alteration in rate of inositol synthesis from [U^{14}C]-G-6-P by testis homogenates from control and cryptorchid animals. Six animals were used to prepare each cryptorchid homogenate, while three were used for each control. The rate of inositol synthesis from G-6-P in control homogenates was 0.0701 μM per 100 mg wet weight testis per 30 minutes while homogenates of 3.5 to 21 day cryptorchid testes exhibited a rate of 0.0382 to 0.0450 μM per 100 mg wet weight per 30 minutes. This represents a 40 to 46% reduction in inositol synthesis as a result of cryptorchidism. Although this is a significant reduction in inositol synthetic activity, it does not approach the 70 to 78% depression noted in testis slices. The data for slices and homogenates are summarized in Table II.

5. Glucose Uptake in Control and Cryptorchid Testis Slices

Although synthesis of inositol in the absence of cell membranes is depressed in the cryptorchid testis, the degree of depression is less than that observed in testis slices. This suggests the possibility that differences in glucose uptake between the control and cryptorchid testis might also exist. Thus, studies of glucose uptake in testis slices were undertaken.

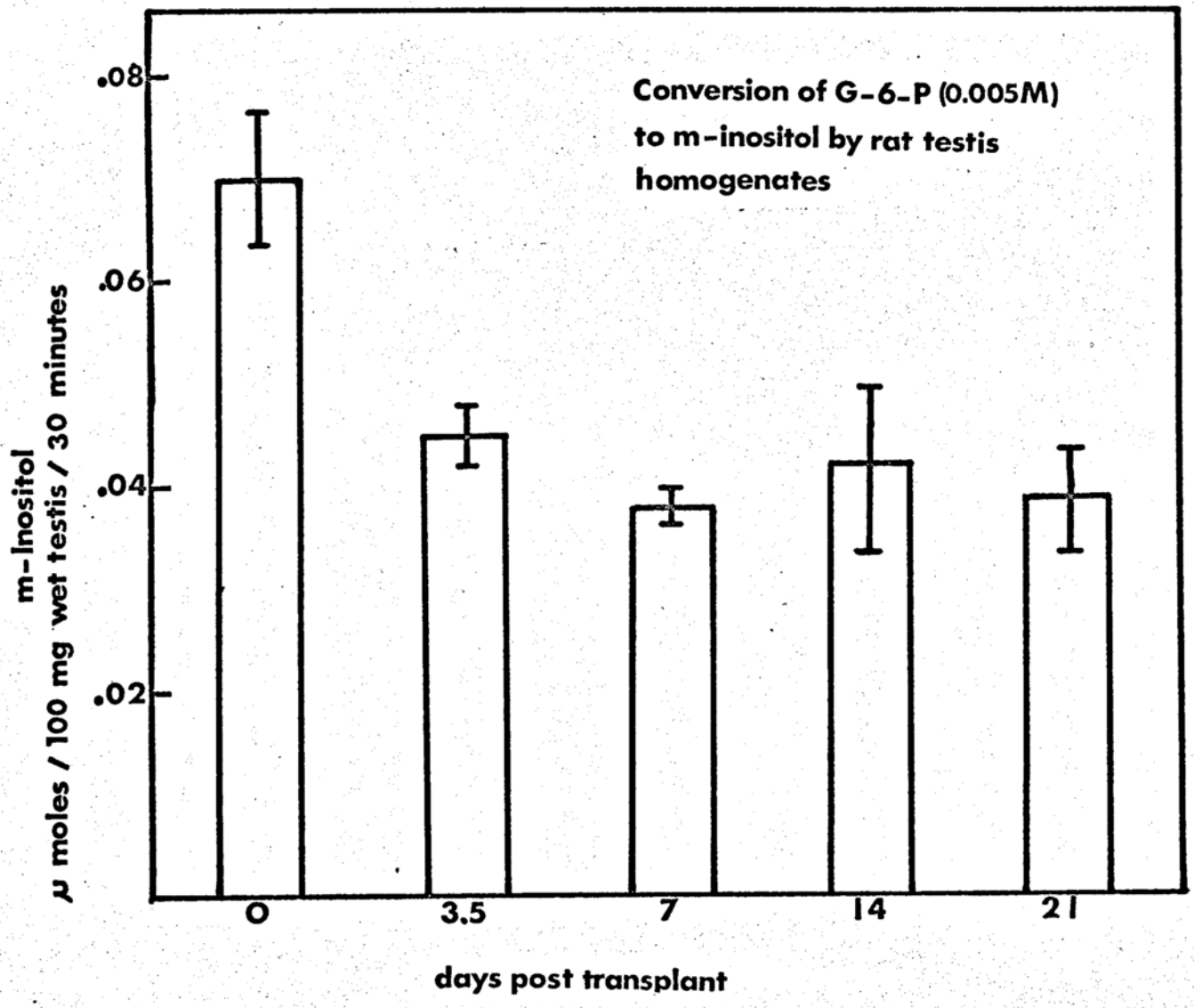

Conversion of G-6-P (0.005M)
to m-inositol by rat testis
homogenates
m-Inositol
μ moles / 100 mg wet testis / 30 minutes
.08
.06
.04
.02
0
3.5
7
14
21
days post transplant

TABLE II

SYNTHESIS OF m-INOSITOL BY RAT TESTIS SLICES AND HOMOGENATES

Testis Slices

Days Post Transplant	μM m-Inositol 100 mg wet wt/hr ±s.e. of mean	n	Per Cent Depression
Control	.0372±.0008	(12)	--
3.5	.0210±.0013	(10)	43.5
7	.0111±.0002	(5)	70.2
14	.0083±.0016	(5)	77.7
21	.0084±.0003	(5)	77.4

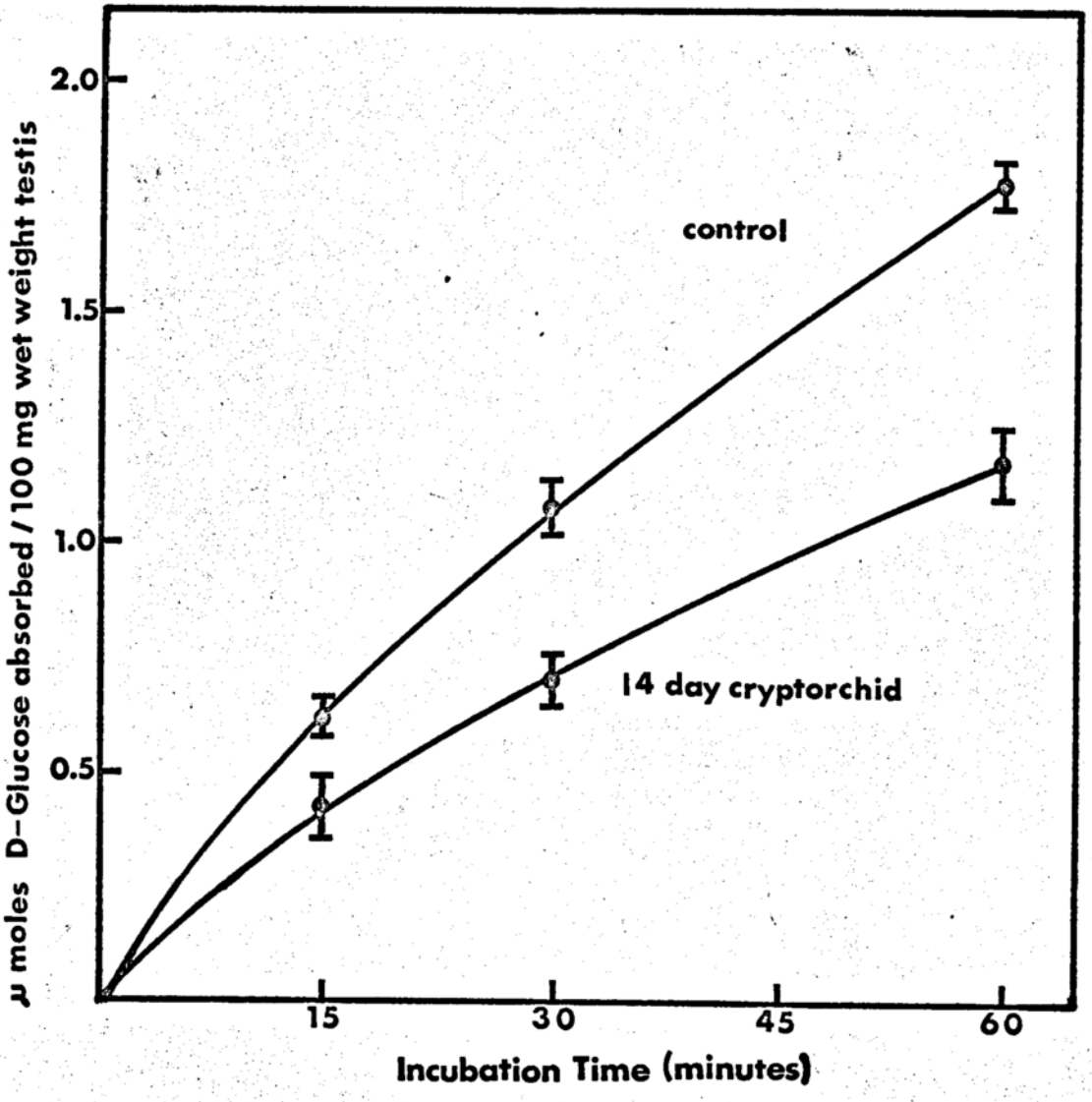
Homogenates

Days Post Transplant	μM m-Inositol 100 mg wet wt/hr ±s.e. of mean	n	Per Cent Depression
Control	.0701±.0065	(8)	--
3.5	.0450±.0030	(3)	35.8
7	.0382±.0018	(2)	45.5
14	.0422±.0080	(4)	39.8
21	.0390±.0050	(2)	44.4

Figure 8 illustrates the rate of glucose uptake by control and 14 day cryptorchid testis slices, *in vitro*. Uptake was determined by measuring residual glucose in the incubation medium at various times. The difference between the quantity of residual, exogenous glucose at each time point and the quantity of exogenous glucose at time zero was presumed to reflect glucose uptake by testis slices. Glucose was taken up at the rate of 1.785 ± 0.049 µM per 100 mg wet weight of testis per hour by control slices, while uptake by 14 day cryptorchid slices proceeded at the rate of 1.290 ± 0.087 µM per 100 mg wet weight of testis per hour. This represents a 27.8% decrease in glucose uptake. These data lend further support to the possibility that the rate of glucose uptake is a controlling factor in inositol synthesis by testis slices.

Inspection of Figure 8 also reveals that glucose uptake is nonlinear with respect to time during the first few minutes of incubation. A possible explanation for the early nonlinearity might be that passive equilibration between the intra- and extracellular glucose was occurring. Gey has demonstrated that rat testes contain endogenous levels of less than 0.1 mg of glucose per gram of tissue (77). This concentration is substantially lower than that of the exogenous glucose concentration of 0.9 mg per ml used in these studies.

Uptake of D-Glucose by Rat Testis Slices, in vitro

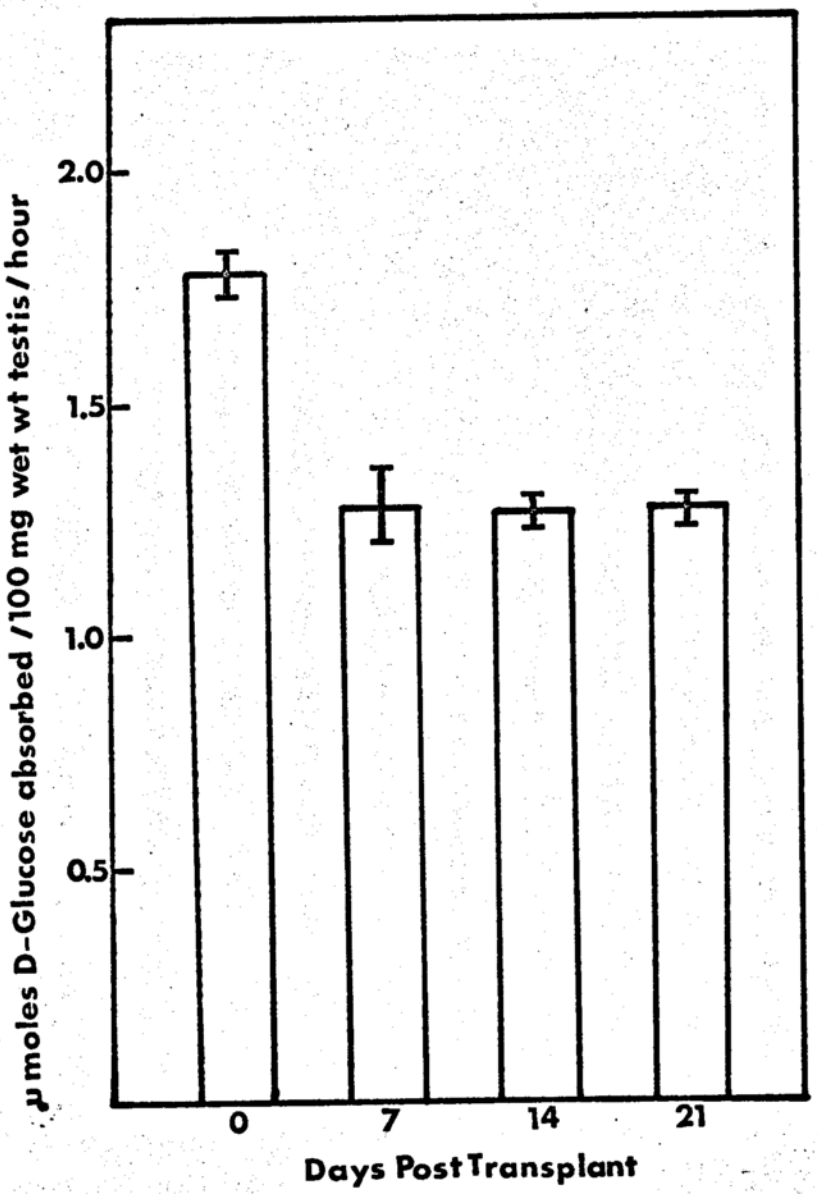


Thus, a large concentration gradient existed across the cell membrane and could have provided a driving force for passive diffusion of glucose into the cell.

The time course of development of impaired glucose uptake into cryptorchid testis slices is shown in Figure 9. Maximal depression of glucose uptake develops before the seventh day after translocation of the testes. There is no further depression beyond this time. This suggests that those cell types which are lost early in cryptorchidism, primarily the spermatids and spermatozoa, might be the most active cell types with regard to glucose uptake, and that decreased inositol synthesis might be partially due to depressed glucose uptake.

Several lines of evidence suggest the importance of glucose to testicular metabolism and function. Several investigators have reported a large reduction in oxygen uptake by the mature testis following omission of glucose from the incubation medium (78-81). Similar results have been obtained following the induction of experimental hypoglycemia (82). Hypoglycemia has also been reported to result in histological damage to the germinal epithelium of the rat testis. The atrophic changes occur predominately in the spermatids (83). In contrast, the addition of glucose to the incubation medium has been found to result in a 600% increase in the incorporation of [U^{14}C]-lysine into the protein of normal rat testis slices (72).

Uptake of D–Glucose by Rat Testis Slices, *in vitro*



The cryptorchid testis does not appear to be as dependent upon glucose as the normal testis. Hollinger and Davis have reported a 45% decrease in CO_2 production from [U^{14}C]-D-glucose as a result of cryptorchidism (60). In contrast, these investigators demonstrated a 4.6-fold increase in protein labeling from glucose in the cryptorchid testis (60) even though the addition of glucose to the incubation medium did not stimulate the incorporation of lysine into protein of the cryptorchid testis (84). Davis and coworkers concluded that the enzymatic systems involved in the incorporation of lysine into protein of the spermatids display the greatest sensitivity to the addition of exogenous glucose (84).

6. Inositol Synthesis in 100,000 x g Supernatant Fractions of Control and Cryptorchid Rat Testis Homogenates

A decrease in glucose uptake by cryptorchid rat testis slices may partially account for the depression in inositol synthesis noted in intact tissue slices. However, the depression in synthesis seen in cryptorchid homogenates suggests that intracellular factors such as enzyme levels may be altered. Eisenberg (3) has reported that the enzymes glucose-6-phosphate cyclase and inositol-1-phosphatase are present only in the 100,000 x g supernatant fraction of rat testis homogenates. Furthermore, Eisenberg (3) observed that maximal activity

could be obtained in this fraction only after preheating at 60°C for two minutes. In the present studies, inositol synthesis was measured in preheated 100,000 x g supernatant fractions obtained from control and cryptorchid rat testis homogenates in an attempt to ascertain whether enzyme activity had been lost in the cryptorchid testis.

Figure 10 shows the rate of inositol synthesis from [U^{14}C]-G-6-P per mg of supernatant protein in the preheated 100,000 x g supernatant fractions obtained from control and 14 day cryptorchid rat testis homogenates. The supernatants were preheated at 60°C for two minutes prior to incubation with substrate and cofactors. The testes from three control and six cryptorchid animals were used to prepare each supernatant. Each point in Figure 10 represents the mean of two to twelve experiments. Synthesis was linear throughout the incubation period for both control and cryptorchid supernatants.

Table III presents a statistical comparison of the rate of inositol synthesis in control and 3.5, 7, 14, and 21 day cryptorchid, preheated 100,000 x g supernatants. The P values presented in Table III were calculated using the procedures developed by Steel and Torrie for calculating Student's t (85). These data reveal no significant difference between preheated cryptorchid and

SYNTHESIS OF m-INOSITOL-C^{14} FROM GLUCOSE-6-PO_4-C^{14} (0.005 M) BY THE 100,000 X G SUPERNATANT FRACTION OF RAT TESTIS HOMOGENATES

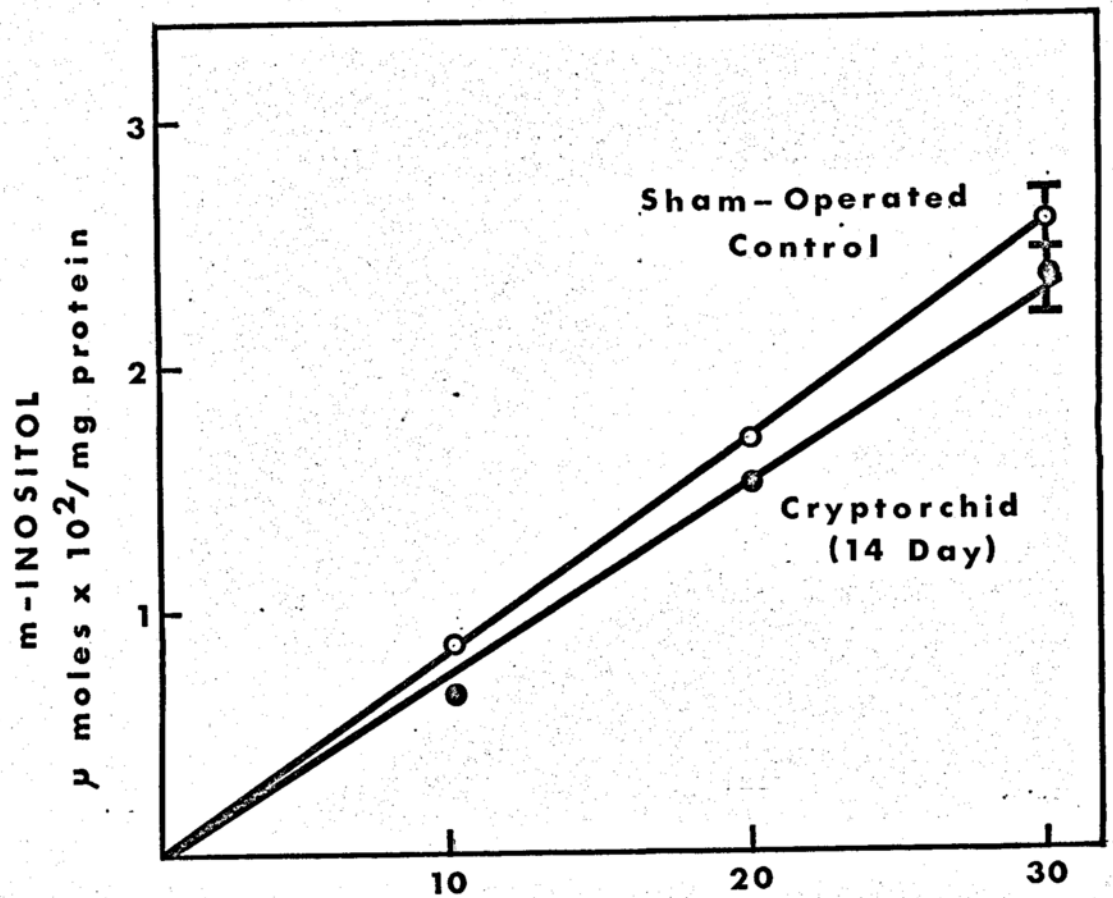


TABLE III

SYNTHESIS OF m-INOSITOL BY THE PREHEATED 100,000 x g SUPERNATANT FRACTION OF RAT TESTIS HOMOGENATES

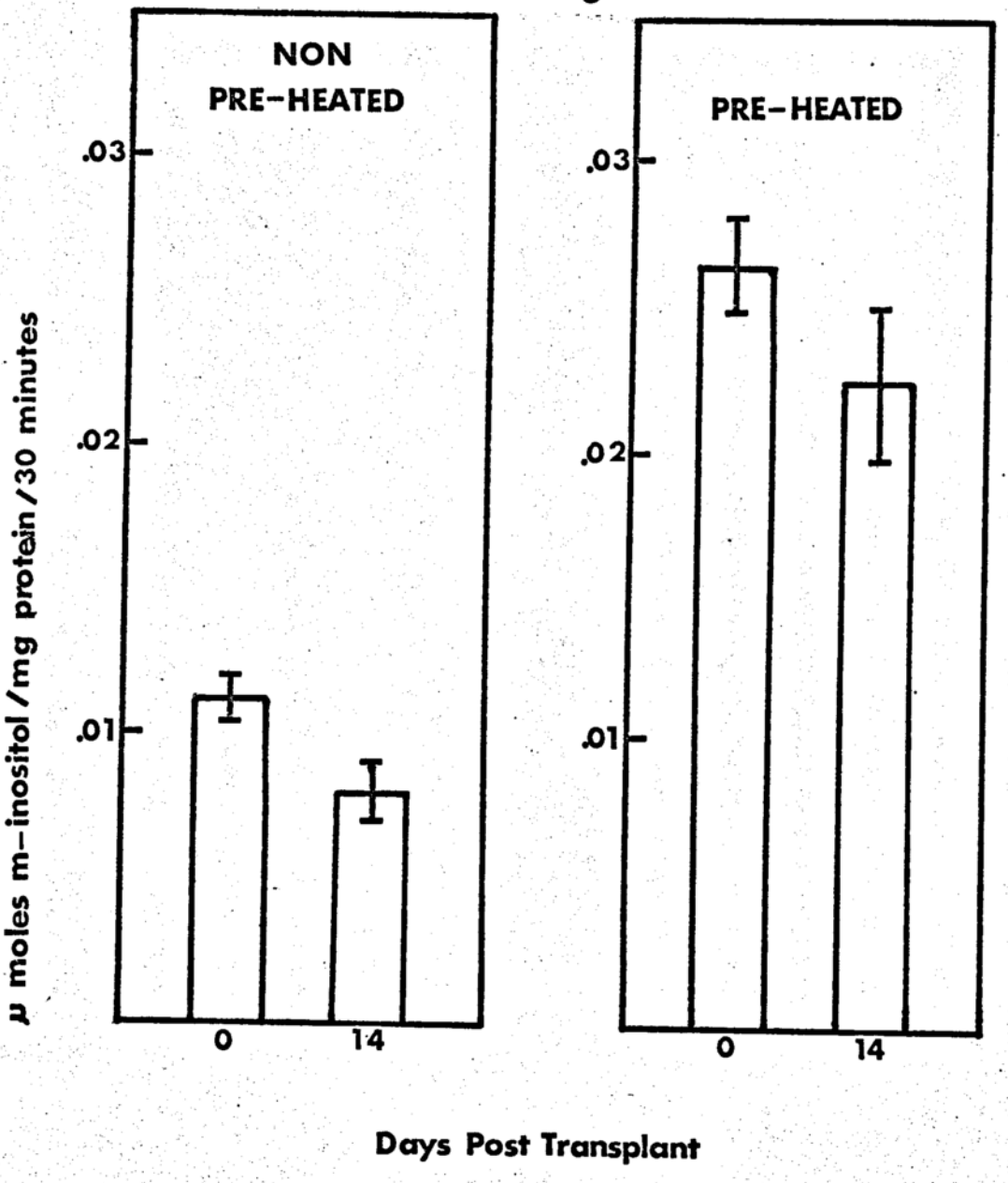
Days Post Transplant	μM m-Inositol/mg protein/30 min ± s.e. of mean	n	P
Control	.0264±.0015	(11)	--
3.5	.0289±.0015	(3)	0.4
7	.0248±.0007	(3)	0.5
14	.0223±.0026	(5)	0.2
21	.0242±.0022	(3)	0.5

control supernatants in inositol synthetic activity, per mg of supernatant protein.

Preheating the 100,000 x g supernatant fractions may have served to partially unmask the inositol synthesizing activity possessed by those cell types which are unaffected by cryptorchidism. The effects of preheating the 100,000 x g supernatant fractions on inositol synthesis was determined by measuring inositol synthesis in non-preheated 100,000 x g supernatants obtained from control and cryptorchid rat testis homogenates.

The results of experiments in which six control and three 14 day cryptorchid non-preheated supernatants were assayed for inositol synthetic activity are presented in Figure 11 along with the results obtained from the equivalent heat treated supernatants. In the non-preheated preparations, the cryptorchid supernatants were significantly ($P < 0.05$) less active per mg of protein than the control supernatants. Heat treatment not only eliminated this differential, but also resulted in a 2.36-fold increase in activity for the control supernatants, and a 2.63-fold increase for the cryptorchid supernatants. Heat treatment therefore, resulted in the inactivation of a thermolabile factor which serves to limit inositol synthesis in both testis types. The slightly greater increase in inositol synthesis observed in the cryptorchid supernatants

Synthesis of m-inositol from Glucose-six-Phosphate (0.005M) by the 100,000 x G Supernatant Fraction of Rat Testis Homogenates

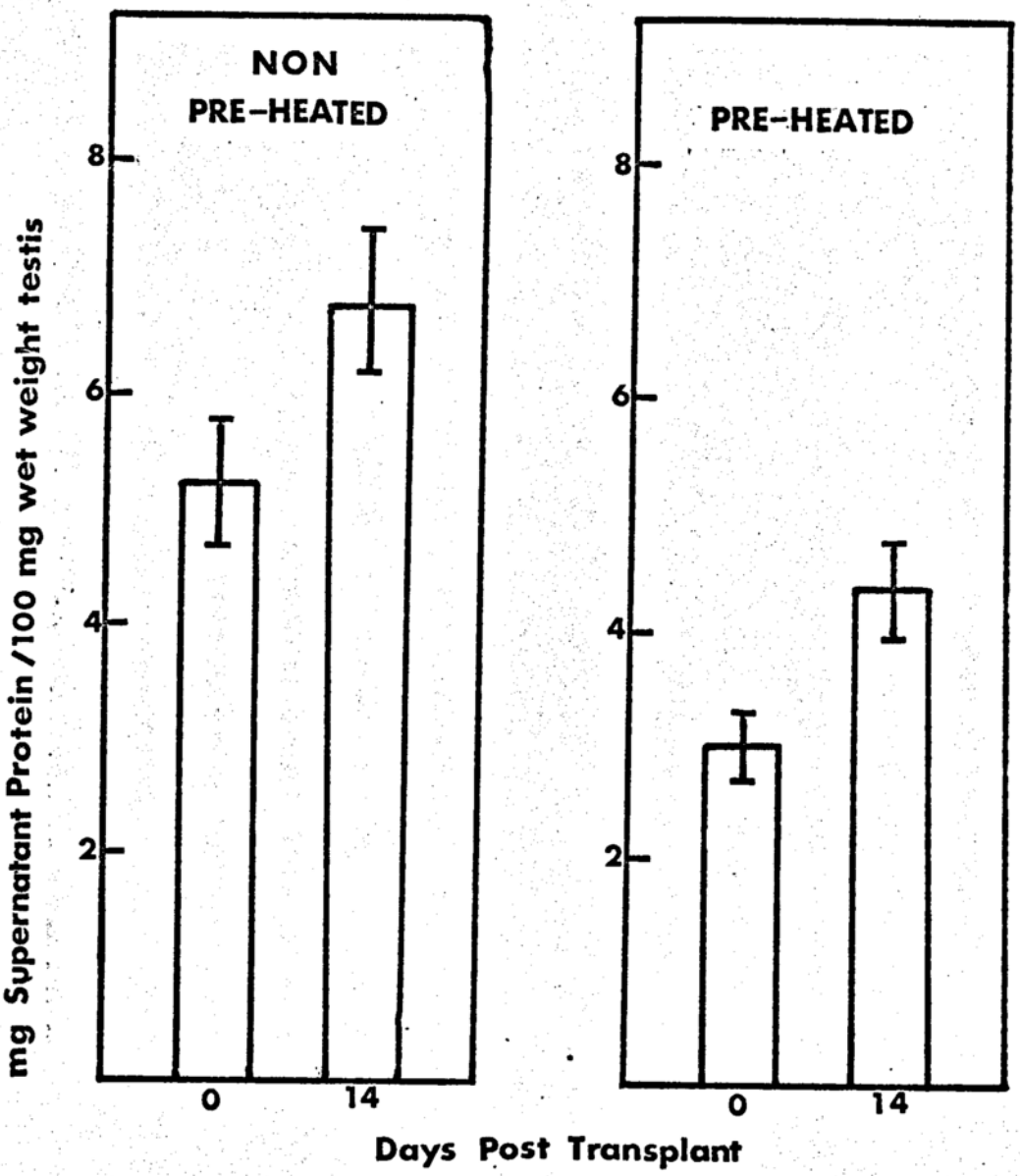


suggests the possibility that the thermolabile factor might be more concentrated in the cryptorchid testis.

Heat treatment resulted in the precipitation of a considerable amount of protein from the supernatant fractions. As a consequence, the protein content of aliquots representing 100 mg wet weight of testis was determined for both the non-preheated and preheated supernatants obtained from control and cryptorchid rat testis homogenates. Figure 12 illustrates the results of these measurements for the control and 14 day cryptorchid supernatants. In both cases, the 14 day cryptorchid supernatants had a greater protein content than the control. A ratio of 1.3 was observed in the non-preheated preparations while a ratio of 1.5 was seen in the preheated supernatants. Approximately 2.25 mg of protein per 100 mg wet weight of testis was lost from both of the supernatant types as a result of heat treatment. This suggested that the thermolabile factor which served to limit inositol synthesis in non-heated preparations might be a protein.

Since the cryptorchid supernatants contained a greater protein content than the control supernatants per 100 mg wet weight of testis, the inositol synthetic activity per 100 mg wet weight of testis in the supernatant fractions is different than the activity per mg of protein. The results of calculations in which synthetic activity per 100 mg wet weight of testis were

100,000 x G Supernatant Protein Concentrations



determined are presented in Figure 13. These calculations reveal that there is no significant difference in the synthetic activity of the non-preheated 100,000 x g supernatants, while in heated preparations the 14 day cryptorchid supernatants were actually more active than the control.

In Table IV, the synthesis of inositol per 100 mg wet weight of testis by the non-preheated and preheated 100,000 x g supernatants is shown along with a statistical analysis of the difference between the control and cryptorchid.

The data presented in Table IV reveal that non-preheated 100,000 x g supernatants obtained from cryptorchid and control rat testis homogenates are equiactive in terms of inositol synthesis per 100 mg wet weight of testis. This suggests, either that the enzymes responsible for inositol synthesis are uniformly distributed throughout all of the cell types within the seminiferous epithelium, or that the thermolabile controlling factor as well as the inositol synthesizing enzymes are concentrated in those cell types which are unaffected by cryptorchidism. In view of the greater increase in synthesis seen in the cryptorchid supernatants on the basis of wet weight of tissue following heat treatment, the latter conclusion seems more probable.

Synthesis of m-inositol from Glucose-6-PO_4 (0.005M) by the 100,000 x G Supernatant Fraction of Rat Testis Homogenates

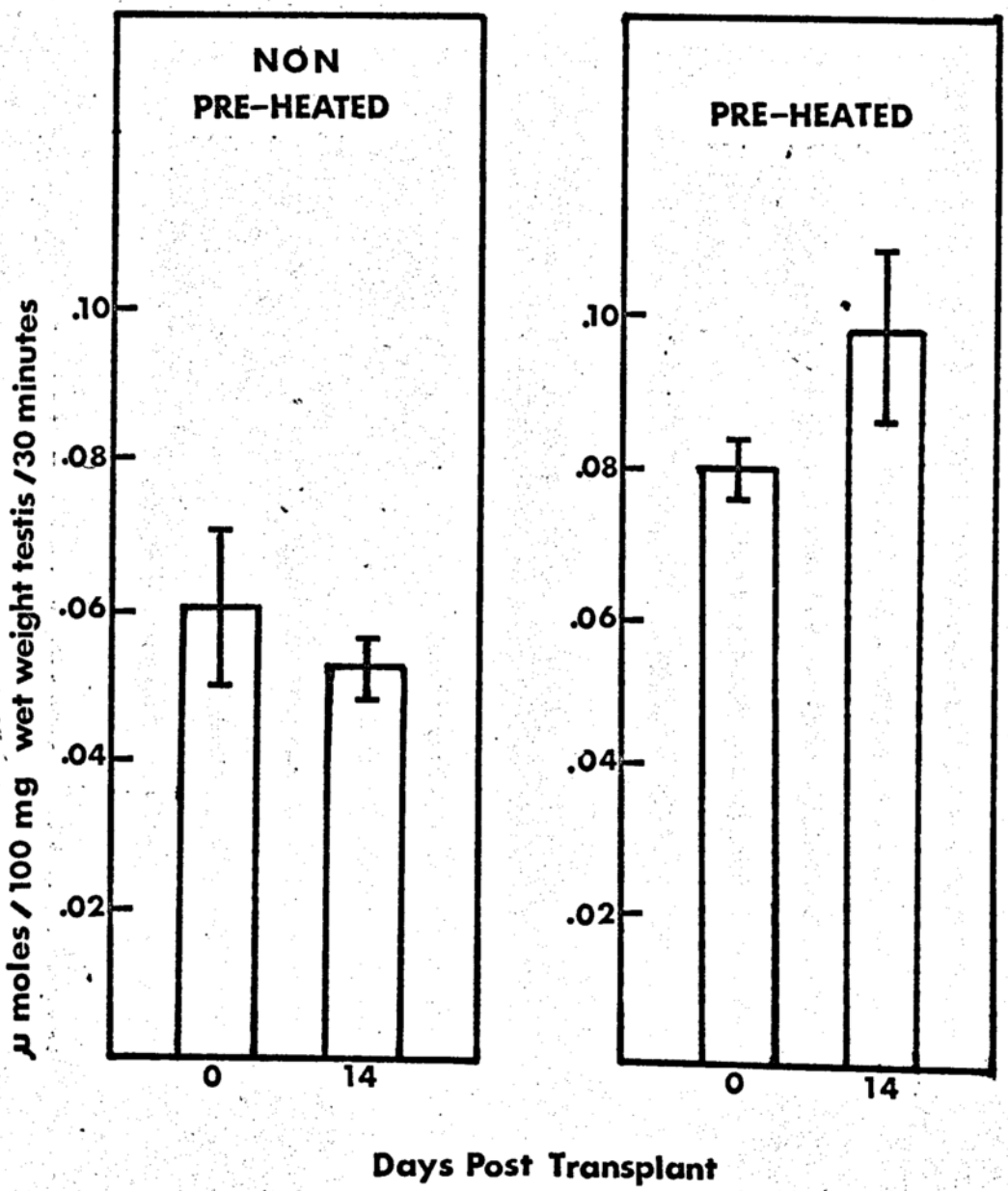


TABLE IV

SYNTHESIS OF m-INOSITOL BY NON-PREHEATED AND PREHEATED 100,000 x g SUPERNATANTS OF RAT TESTIS HOMOGENATES

Days Post Transplant	µM m-Inositol / 100 mg wet wt/30 min ± s.e. of mean	n	µM m-Inositol / 100 mg wet wt/30 min ± s.e. of mean	n
Control	.0608±.0104	(6)	.0800±.0044	(11)
3.5	.0708±.0069	(3)	.1097±.0029**	(3)
7	.0462±.0120	(2)	.1131±.0032**	(3)
14	.0525±.0060	(3)	.0972±.0109*	(5)
21	.0594	(1)	.1089±.0099**	(3)

Numbers in parentheses refer to the number of experiments. *P = < .05
**P = < .01

7. Determination of Nonspecific Phosphatase Activity in Control and 14 Day Cryptorchid 100,000 x g Supernatants

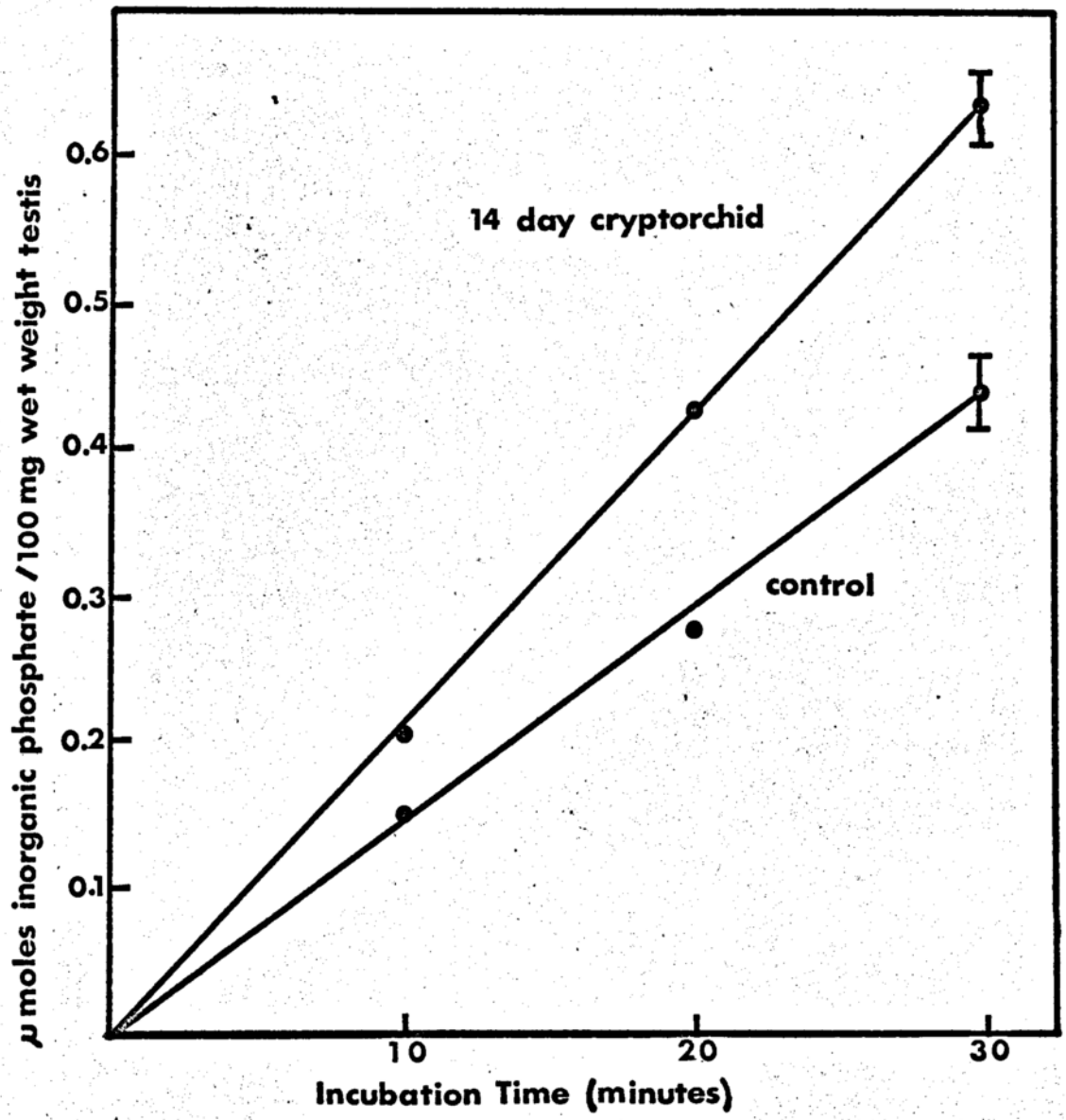
Eisenberg has postulated that the thermolabile factor which must be inactivated in order to achieve maximal inositol synthesis is a nonspecific phosphatase which hydrolyzes G-6-P (3). Furthermore, Tice and Barrnett have demonstrated that nonspecific phosphatase activity is concentrated at, or near, the basement membrane of the seminiferous epithelium (86).

In order to determine whether nonspecific phosphatase activity was elevated following cryptorchidism, the hydrolysis of G-6-P by control and 14 day cryptorchid supernatants was measured. Figure 14 illustrates the results of these experiments for five non-preheated control and cryptorchid supernatants in terms of micromoles of inorganic phosphate formed per 100 mg wet weight of testis. The testes from six animals were used to prepare each cryptorchid supernatant while three animals were used for each control supernatant. The rate of hydrolysis of G-6-P was linear over the entire incubation period and was 43% greater in the cryptorchid supernatants than in controls. Preheated supernatants had no measurable phosphatase activity.

Figure 15 depicts the phosphatase activity per mg of supernatant protein, protein concentrations per 100 mg

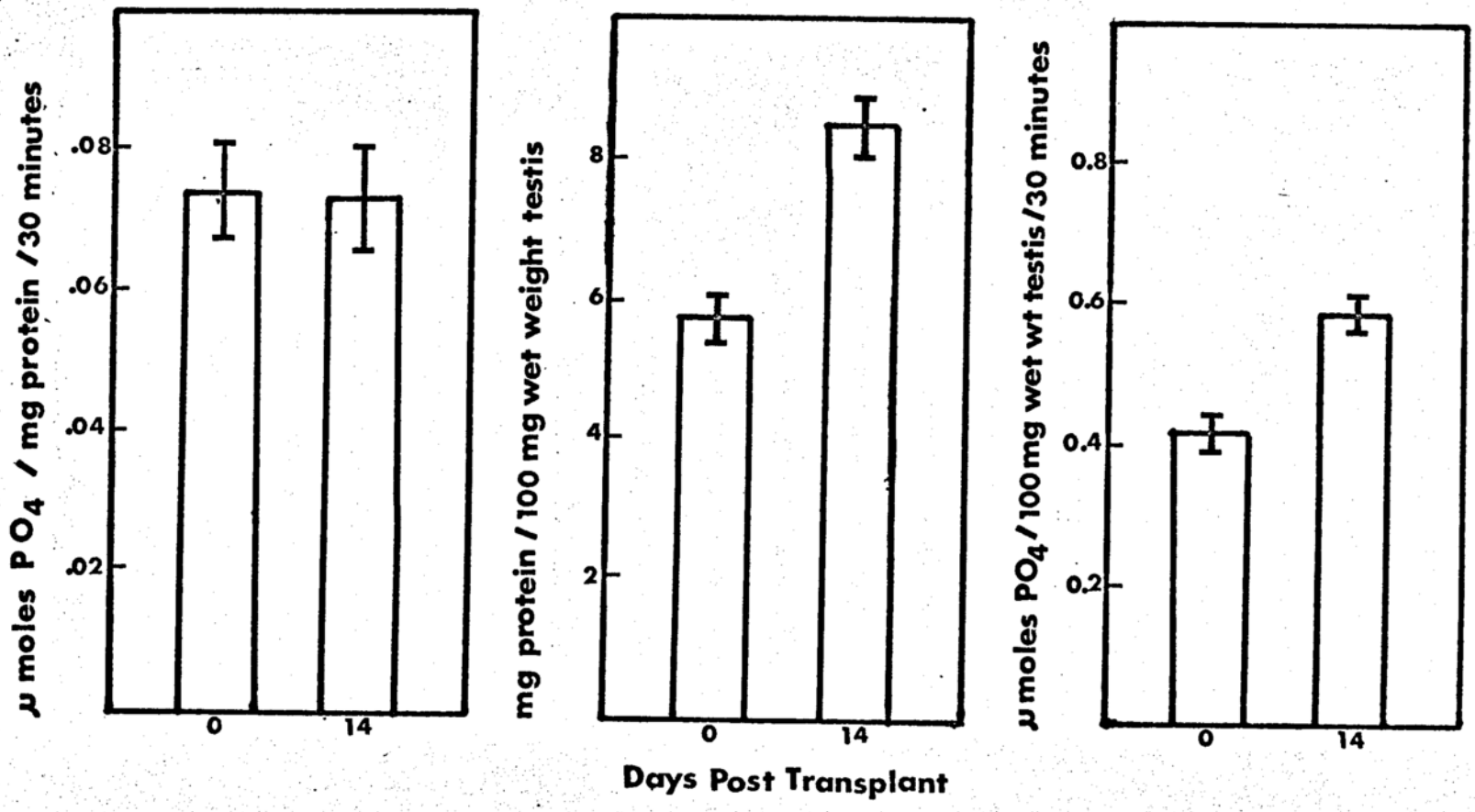
Hydrolysis of Glucose-6-PO_4 by the 100,000 x G

Supernatant Fraction of Rat Testis Homogenates



Hydrolysis of Glucose-6-PO_4 by the 100,000 x G

Supernatant Fraction of Rat Testis Homogenates



wet weight of testis, and phosphatase activity per 100 mg wet weight of testis for the control and 14 day cryptorchid, non-preheated 100,000 x g supernatants. Both preparations possessed identical phosphatase activity per mg of protein. However, because of the differential in protein concentration, the phosphatase activity per 100 mg wet weight of testis was greater in the cryptorchid supernatants. This provides further evidence that the nonspecific phosphatase activity is concentrated at or near the basement membrane since the seminiferous tubules of the 14 day cryptorchid testis consists of basement membrane and those cell types such as spermatogonia and Sertoli cells which are associated with it. In addition, these data suggest that nonspecific phosphatase might be a thermolabile factor which serves to limit inositol synthesis in those cell types which are unaffected by cryptorchidism.

Thus decreased glucose uptake and increased nonspecific phosphatase activity might be related to the decreased free inositol synthesis measured in cryptorchid testis slices. It is also possible, however, that alterations in the metabolic fate of inositol occur as a result of cryptorchidism.

8. Incorporation of Inositol Into the Lipid Fraction of Control and Cryptorchid Rat Testis Slices

Howard and Anderson (87) have demonstrated that inositol is catabolized to glucuronic acid, and that this pathway is limited to the kidney. It would, therefore, seem improbable that inositol metabolism would occur in the cryptorchid testis.

Inositol is known to be incorporated into lipids (88), primarily as phosphatidyl inositol which has been isolated from a number of sources (89-91). The inositol-1-phosphate structure is common to the phosphatidyl inositols isolated from all sources.

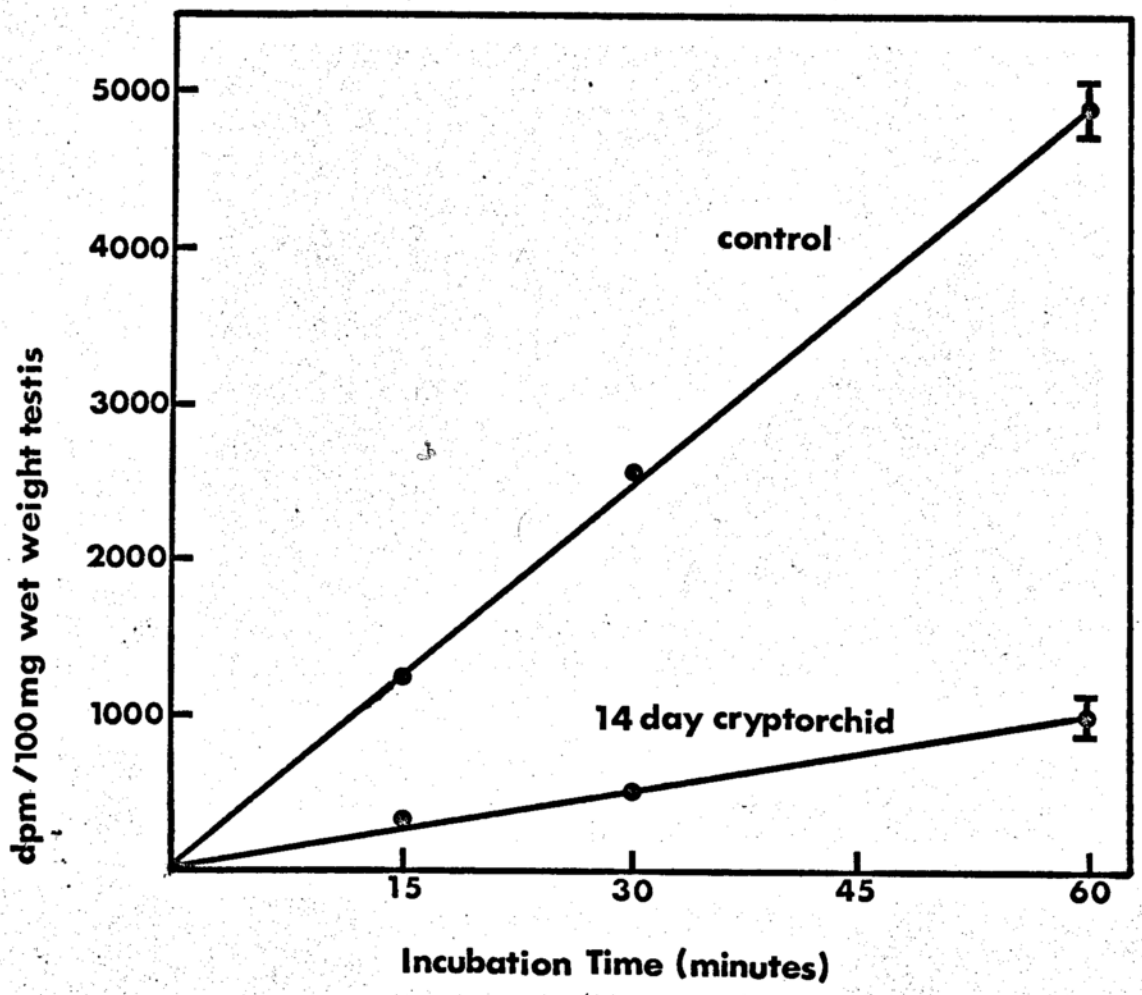
The biosynthesis of phosphatidyl inositol has been studied in several mammalian tissues including liver, kidney, and brain. Agranoff and coworkers have established that the synthesis involves the activation of phosphatidic acid by cytidine triphosphate (92). The resulting CDP-diglyceride then combines with free inositol to produce phosphatidyl inositol and cytidine monophosphate (93). Thus, any increase in phosphatidyl inositol synthesis would result in a decreased free inositol level.

In order to test the hypothesis that the detection of free inositol in tissue slice preparations might be limited by the activity of this pathway, the incorporation

of 2-^{3}H-m-inositol into the lipid fraction of rat testis slices was measured. Figure 16 depicts the results of these experiments for control and 14 day cryptorchid rat testis slices. Each point represents the mean of two experiments for the 15 and 30 minute incubations, whereas the 60 minute incubation point represents the mean of five experiments for both testis types. The incorporation of inositol into the lipid fraction was linear over the 60 minute incubation period. Since only tracer quantities of radioactive inositol were added to the incubation media, the data are reported as disintegrations per minute per 100 mg wet weight of testis. The cryptorchid testis slices had a much lower incorporation rate than control. Eighty per cent depression in incorporation of inositol into the lipid fraction was seen.

Although the per cent depression in incorporation of inositol into lipids is approximately the same as the per cent depression in inositol synthesis seen as a result of cryptorchidism, it is questionable whether these phenomena are directly related. However, in view of the decreased rate of glucose uptake observed in cryptorchid testis slices, a partial explanation for the decrease in inositol incorporation into lipids could be a decrease in the uptake of inositol from the incubation

Incorporation of 2-^{3}H-m-inositol into the Lipid Fraction of Rat Testis Slices, in vitro



medium. Future studies should take this possibility into consideration.

The cryptorchid testis, unlike the normal testis, is composed of cells which are not rapidly dividing. As a result, a lower rate of membrane synthesis would be expected in the cryptorchid testis. Since phosphatidyl inositol is found primarily in membranes (94), it is tempting to postulate that the decreased incorporation of inositol into the lipid fraction reflects a decrease in membrane synthesis.

9. Effects of TEM Treatment on Inositol Synthesis by Rat Testis Slices

The data thus far presented indicate that although the inositol synthesizing enzyme system is found in those cell types of the testis which are unaffected by cryptorchidism, it is probable that one or more of the more mature cell types synthesize the major portion of inositol under physiological conditions. In order to determine if this is the case, an attempt was made to destroy the less mature cell types with the alkylating agent, triethylene melamine (TEM). Bock and Jackson have reported that TEM treatment induces and maintains sterility in male rats when administered in small doses (95). Steinberger, et al. subsequently reported that higher doses (0.2 mg/kg) resulted in destruction

of the spermatogonia, followed by maturation depletion of the seminiferous epithelium (96).

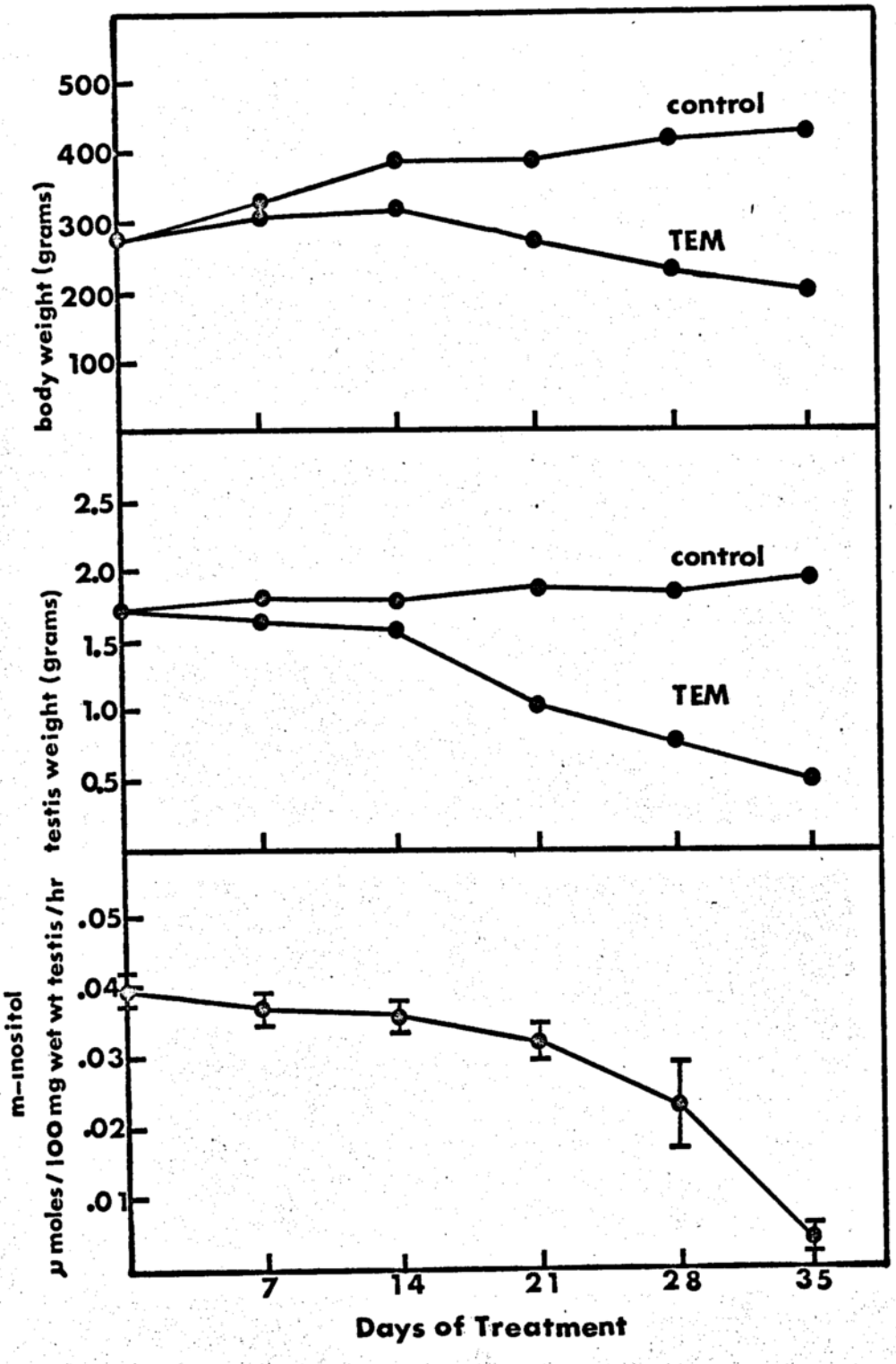
Thus, 30 adult, male rats were injected with 0.2 mg/kg of TEM intraperitoneally once a day, six days per week. Saline treated animals served as controls. Treatment continued for as long as 35 days and inositol synthesis was measured in tissue slices, at seven day intervals.

The results are shown in Figure 17. TEM treatment resulted in a marked impairment of growth. The control animals gained approximately 150 grams over the 35 day period, while the 35 day TEM treated animals lost approximately 80 grams. Simultaneously, the control animal testis weights increased nearly 200 mg whereas the TEM treated animal testis weights decreased 1250 mg. Most of the loss in testis weight in the TEM treated animals occurred after the fourteenth day of treatment.

Inositol synthesis remained near control levels throughout the first 21 days of TEM treatment. After this time, the testes from TEM treated animals rapidly lost inositol synthetic activity. This suggests that those cell types which were lost between 21 and 35 days of treatment were the most active with respect to inositol synthesis.

Examination of Plates 5 through 8, which present the histology of the 14, 21, 28 and 35 day TEM treated testes reveals that the primary cell types lost between

Effects of TEM Treatment on the Rat



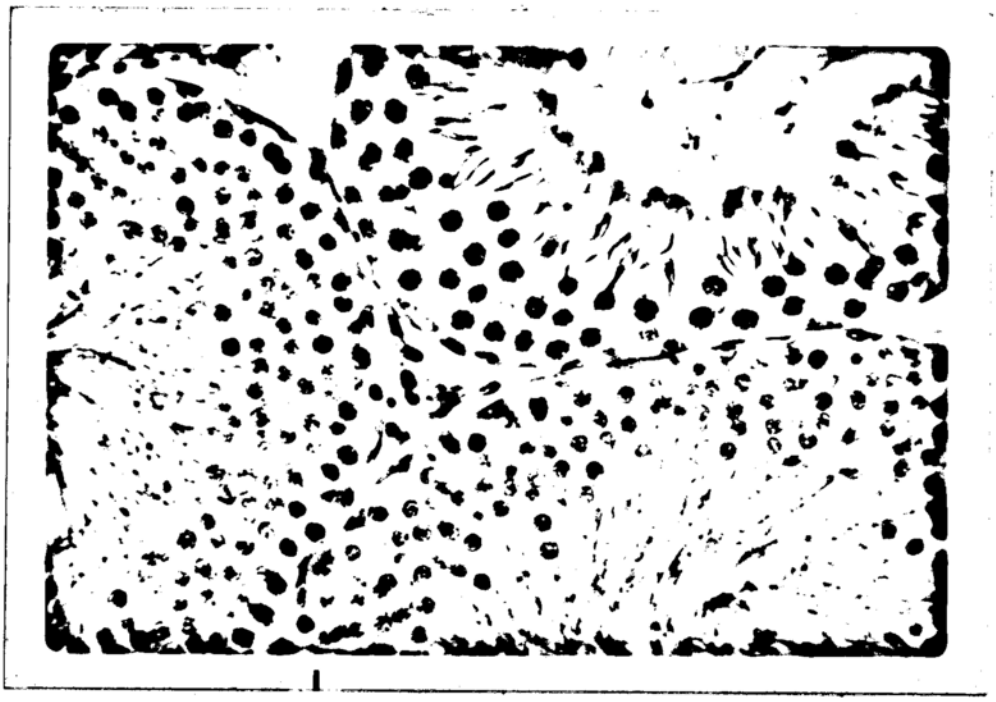


Plate 5

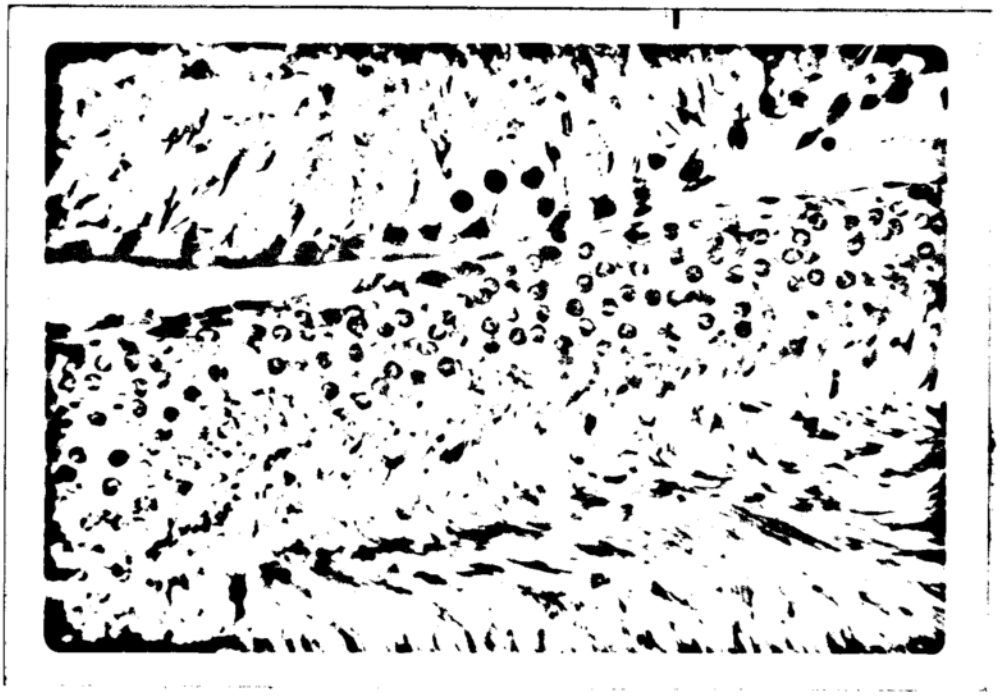


Plate 6

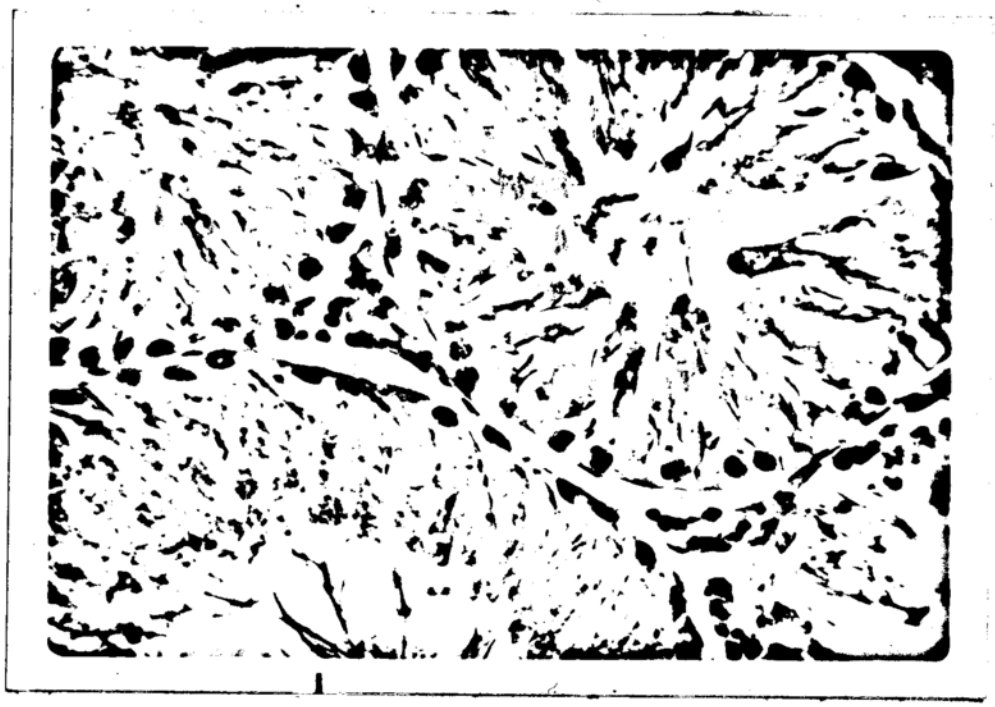


Plate 7

Plate 8

21 and 35 days are the spermatids and spermatozoa. The sharpest fall in synthesis coincides primarily with the loss of the spermatids. These data suggest that the spermatid is the most active cell type with regard to inositol synthesis.

CONCLUSIONS

The studies of Eisenberg have provided evidence for a previously unsuspected pathway of glucose utilization in the mammalian testis (97). This pathway involves the conversion of D-glucose to glucose-6-phosphate (G-6-P) which is then converted by the enzyme G-6-P cyclase to 1-L-inositol-1-phosphate. This compound is then dephosphorylated by the enzyme inositol-1-phosphatase to yield free inositol (Figure 1). Further studies by Eisenberg (3) in which the two main functional units of the testis, the germinal epithelium and interstitial cells, were separated by microdissection suggest that inositol synthesis is primarily a function of the germinal epithelium.

The present studies were designed to determine whether this pathway is restricted to any particular cell type within the germinal epithelium. Cryptorchidism and triethylene melamine (TEM) treatment were used as tools to alter the cellular population of the germinal epithelium. Examination of the histology of testes obtained from rats subjected to these treatments reveals that cryptorchidism first destroys the spermatozoa and spermatids and then the spermatocytes, while TEM treatment results in destruction of the spermatogonia, followed by maturation depletion of the germinal

epithelium within 35 days of treatment. Comparison of the effects on inositol synthesis produced by these two treatments was expected to provide data which would indicate whether any particular cell type within the germinal epithelium is responsible for inositol synthesis.

The measurement of inositol synthesis from [U^{14}C]-D-glucose in control and cryptorchid testis slices revealed that a 78% depression in inositol synthesis had developed as a result of cryptorchidism. Since this depression developed within the first seven days after translocation of the testes to the abdominal cavity, it is proposed that those cell types which were completely lost during this period, the spermatids and spermatozoa, account for the greater portion of inositol synthesis.

The metabolism of the cryptorchid testis has been shown in several cases to be different from normal testicular metabolism. The incorporation of lysine into protein (84), the incorporation of thymidine into DNA and uridine into RNA (98), and protein labelling from radioactive glucose (60) are all enhanced in the cryptorchid testis, while CO_2 production from glucose is decreased in cryptorchid testis slices (60). Although protein biosynthesis in the normal testis is increased six-fold by the presence of glucose (72), the cryptorchid testis is relatively independent of glucose (84). This suggested the possibility that glucose uptake might be

depressed in those cell types which survive cryptorchidism. Since inositol synthesis in the testis requires glucose as a precursor, the rate of glucose uptake might be rate limiting. In the present studies, the observation that cryptorchidism depressed inositol synthesis to a greater extent (78%) in slices than in homogenates (46%) also suggested that a glucose uptake difference might exist between the normal and cryptorchid testis.

Experiments in which glucose uptake was measured reveal that the cryptorchid testis is approximately 27.8% less active. Thus, a portion of the depression in inositol synthesis seen with cryptorchidism might be due to depressed glucose uptake. However, since the amount of glucose taken up by a tissue is probably a reflection not only of the uptake process, but also of utilization, the results of these experiments should be viewed with caution. Further studies using a carbohydrate which is not metabolized by the testis seem to be warranted before it can be said with assurance that impaired uptake is a contributing factor to the depression in inositol synthesis seen with cryptorchidism.

Experiments using the preheated 100,000 x g supernatant fraction of control and cryptorchid rat testis homogenates were performed in order to ascertain whether the enzymes responsible for inositol synthesis are less concentrated in the cryptorchid testis. These

experiments demonstrated that the cryptorchid testis is equiactive with the control in terms of inositol synthesized per mg of protein. Moreover, since the cryptorchid testis has 1.5 times as much protein in the aliquot representing 100 mg wet weight of testis, the preheated 100,000 x g cryptorchid supernatants are actually slightly more active than the control. This suggests that the inositol synthesizing enzymes are found in those cell types which are unaffected by cryptorchidism, but some factor serves to limit inositol synthesis in these cell types.

Eisenberg has demonstrated that heat treatment is necessary to obtain maximal inositol synthetic activity in the 100,000 x g supernatant fraction (3). In the present studies, the effect of heat treatment on inositol synthesis was determined in non-preheated supernatants. Cryptorchid, non-preheated supernatants were less active than control in inositol synthesis per mg of protein. Heat treatment not only eliminated this difference but also resulted in a greater than two-fold increase in activity for both the control and cryptorchid supernatants. The increase in activity following heat treatment was slightly greater in the cryptorchid testis. Thus, the cryptorchid testis possesses the enzymes necessary for inositol synthesis in at least the same concentration as normal testis. However, the complete

potential for inositol synthesis in the cryptorchid testis may not be realized because of the presence of a greater activity of the thermolabile limiting factor.

The presence of this thermolabile factor in the control supernatants suggests that it normally functions to limit inositol synthesis in those cell types which possess it. The smaller increase in inositol synthesis seen in the control supernatants following heat treatment further suggests that the thermolabile factor is not as active in the more mature cell types of the germinal epithelium which are lost following surgically-induced cryptorchidism. Thus, it seems probable that the more mature cell types which make up the germinal epithelium are more active in regard to inositol synthesis, not because they possess a greater quantity of enzyme activity, but because the thermolabile limiting factor is not as active in these cell types as in those which are not affected by cryptorchidism.

Eisenberg (3) has suggested that this thermolabile factor is a nonspecific phosphatase. Phosphatase activity was therefore measured in various 100,000 x g supernatants obtained from control and cryptorchid rat testis homogenates. No phosphatase activity was detectable in any of the preheated 100,000 x g supernatants. Non-preheated supernatants exhibited considerable activity, however. The cryptorchid supernatants possessed 43% greater

phosphatase activity than the control per 100 mg wet weight of testis. These data suggest that nonspecific phosphatase is a thermolabile factor which might limit inositol synthesis. Furthermore, it seems probable that the phosphatase activity is concentrated in those cell types which are unaffected by cryptorchidism.

The experimental procedures involved in determining inositol synthesis measured the amount of free inositol isolated from the incubation system. Alterations in the utilization of inositol are not detectable by this technique. Therefore, the further metabolism of inositol was studied. Howard and Anderson have demonstrated that only the kidney is capable of catabolizing inositol (87). Thus, this pathway was not considered a possible cause of a decrease in free inositol. However, the incorporation of inositol into testicular lipids might be a possible controlling factor. Experiments in which the incorporation of radioactive inositol into testicular lipids was measured revealed that an 80% decrease in incorporation occurs as a result of cryptorchidism. Consequently, an increase in phosphatidyl inositol synthesis does not serve to decrease the amount of free inositol in testis slices. Furthermore, this phenomenon is probably a reflection of decreased membrane synthesis in the cryptorchid testis slice rather than a result of the observed decrease in inositol synthesis.

The data thus far presented suggest that inositol synthesis is greatest in the more mature cell types which constitute the germinal epithelium. However, since the cryptorchid testis is a rapidly changing organ, both histologically and biochemically, it is possible that some factor is responsible for the decrease in inositol synthesis seen with cryptorchidism other than loss of the most active cell types.

Steinberger and coworkers have demonstrated that treatment with TEM results in specific destruction of the spermatogonia (96). After this has been achieved, the process of maturation slowly depletes the germinal epithelium of the remaining cell types. TEM treatment therefore provides a tool by which the synthesis of inositol in all of the cell types within the germinal epithelium could be studied. If one cell type is more active than the others, a large decrease in inositol synthesis should be seen when that cell type is lost from the testis.

Experiments in which the effects of TEM-induced maturation depletion on inositol synthesis in testis slices was measured reveal that synthesis was very near control levels until the spermatids began to disappear from the testis. After this time a sharp decrease of inositol synthesis developed. This suggests that the spermatids and perhaps to a lesser extent the spermatozoa

are the most active cell types with regard to inositol synthesis. Furthermore, these experiments provide more evidence to suggest that the less mature cell types are relatively inactive with respect to inositol synthesis. Presumably, in the normal physiological condition, some factor or factors such as nonspecific phosphatase serve to limit inositol synthesis in these less mature cell types. Only those cell types which do not possess significant nonspecific phosphatase activity can synthesize inositol.

The data from the present studies contribute very little to an understanding of the function of m-inositol in the male reproductive tract. The presence of large amounts of inositol in rete testis fluid (99) and seminal plasma (26) suggests that inositol which is produced within the testis might exert an effect outside the testis. Further investigations as to the source of seminal inositol and its function seem to be warranted.

One possible mechanism by which the source and function of seminal inositol might be determined is the development of an inhibitor of testicular inositol synthesis. Chen and Charalampous have reported the inhibition of the partially purified enzyme system obtained from yeast by 2-deoxyglucose-6-phosphate, ribose-5-phosphate, erythrose-4-phosphate and 6-phosphogluconate (54). It may be significant that three of these compounds are intermediates of the hexose

monophosphate (HMP) shunt which has been reported by Field and coworkers to be very active in the testis (100). Thus, the HMP shunt may constitute another physiological mechanism by which testicular inositol synthesis could be regulated.

The use of any of the phosphorylated compounds listed above as *in vivo* inhibitors of inositol synthesis does not seem promising in view of the fact that highly charged molecules such as these are not well absorbed. Thus, a structural analog of inositol with an electron withdrawing functional group at C-1 would appear to be a possible inhibitor of inositol synthesis.

Most of the currently investigated inhibitors of spermatogenesis exert their effects on the less mature cell types which comprise the germinal epithelium. If inositol synthesis is more important in the spermatids and spermatozoa as the data presented in this thesis suggest, and if inositol synthesis is necessary for spermatogenesis to continue, a compound which inhibits spermatogenesis at the spermatid stage may be more desirable. This seems especially true since one of the potential dangers of inhibiting spermatogenesis early in the maturation process is the production of genetic damage.

Inositol seems to be necessary for the normal uptake of amino acids and nucleic acids (44). However, the

investigations conducted by Davis and coworkers (84,98) have shown an increase in protein and nucleic acid synthesis in cryptorchid testis slices. Since the present studies have shown inositol synthesis to be depressed in the cryptorchid testis it seems possible that inositol may serve a different function in the testis. However, since an impairment in uptake of amino acids and nucleic acid precursors was seen only in inositol deficient cells (44), it seems probable that the cryptorchid testis is not an inositol deficient organ. Thus, the greater protein and nucleic acid synthesis seen in the cryptorchid testis are probably a reflection of an increased enzyme concentration or activity in the cryptorchid testis.

Further studies concerning the role of inositol in reproduction should be addressed partially to the questions discussed above.

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