EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM UPON RAT TESTIS METABOLISM

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table | Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Evolution led to progressively higher body temperature in terrestrial vertebrates. These higher body temperatures increased chemical reaction rates which permitted the greater activity needed for survival in the harsh terrestrial environment (33,62,91). Since male gametogenic tissue is damaged by high environmental temperature (33), an evolutionary answer was required to alleviate the need for high temperature for somatic tissues but lower temperatures for spermatogenic cells. The development of the scrotum among many mammalian species and the migration of the testes from the body cavity into the scrotum may represent such an answer. Proof of the importance of this adaptive mechanism is the failure of spermatogenesis in those mammalian testes which fail to migrate into the scrotum during puberty in those species possessing scrotal testes (160).

Temperatures higher than those encountered in the scrotum cause histological degeneration (49) accompanied by a reduction in specific stages of spermatocytes and spermatids in steps 1 and 2 of spermiogenesis (23,35). Davis and co-workers (35,36,38) have shown a reduction in protein synthesis *in vitro* at temperatures higher than scrotal temperature. Other investigators showed that protein biosynthesis *in vitro* by testicular tissue was dependent upon and was stimulated by exogenous glucose (37,108). Means and Hall (108) indicated that glucose availability

appeared to be directly correlated with protein synthesis and testicular ATP concentration. Davis (35) has shown that the spermatocytes and spermatids are extremely dependent upon exogenous glucose for protein synthesis. These investigations suggest a possible relationship in testis among temperature effects, protein synthesis, specific cellular degeneration and the utilization or availability of glucose. Means and Hall (108) have suggested that the deleterious effects of hyperthermia upon spermatogenesis may be attributed to an impairment in the capacity of the testes to utilize glucose.

Ewing and Schanbacher (49) noted some signs of testicular degeneration at 24 hours but others (23) did not find distinct cellular derangement associated with heat until 48 hours after experimental or artificial cryptorchidism. I rationalized that biochemical alterations responsible for this testicular degeneration were operative well in advance of the first appearance of cytological derangement. The present research was designed to elucidate how soon after temperature treatment changes in biosynthesis of lipid and protein occur <u>in vitro</u> in artificially cryptorchid rat testis. In addition, a study of the relationship of glucose metabolism to biosynthetic reactions was undertaken.

CHAPTER II

LITERATURE REVIEW

Unusual Testicular Characteristics That Contribute to our Understanding of Heat Effects on Testis

A Japanese maxim states that "Charcoal burners have no children" (33). Since heat emission from hibachis is low, it is purported that the males' testes receive excessive heat resulting in reduced fertility, when they attempted to warm themselves by standing close to the heat. Decreased fertility similar to that in charcoal burners occurs among those whose professions involve working under similar conditions of excess localized heat e.g. steam press operators, pants pressers and foundry workers (33). This effect of temperature on testis function is paradoxical in view of the requirement of warm blooded organisms for specific internal temperatures which exceed the optimal temperature for spermatogenesis. In general, higher body temperatures are accompanied by increased chemical reaction rates which permit greater activity (33,62,91). Evolution led to progressively higher body temperatures in terrestrial organisms since the harsh terrestrial environment requires a higher level of activity for survival. Cowles (33) states that the limiting factor to the adoption of higher and higher body temperatures by evolving organisms may be the susceptibility of gametogenesis to high temperature

particularly in the male. This susceptibility of gametogenesis to increased temperature exists in a wide variety of both animals and plants (3,10,18,19,24,34,41,59,80,85,104,110,113,123,125,133,135,138,140,155, 171,178,179). In fact it has been suggested that warm temperatures prevalent during the Cretaceous period may have induced sterility leading to the end of the Age of Reptiles (34).

Natural or artificial cryptorchidism causes sterility in the guinea pig (110), man (140), and rat (19,24). Moreover, applying heat to mammaliam testes (18,113,178) or maintaining the mammals at high temperature (41,104,125) induces male sterility without apparent injury to the animal. This phenomenon has been shown to occur in other vertebrate groups, including birds (3,10,135) and reptiles (34,59,171). The fact that this effect occurs in insects (123,133,179) and even in plants (80, 85,138,155) shows that it is not peculiar to vertebrates but appears to be widely distributed among all organisms exhibiting sexual reproduction.

Mutagenic Effects of High Temperature

High, but nonetheless common, temperatures appear to affect chromosome function as shown by sex reversal in plants (121,157), insects (5), amphibians (175), and reptiles (105). A number of investigators (117, 129,131,132) showed that increased temperatures increased both frequency and rate of mutation in Drosophila. Plough (130) has studied the frequency of lethal genes among wild populations of Drosophila from New England, Ohio, and Florida. He found that the percentage of lethals in Florida populations was 16 to 26 percent higher than that in northern stocks. He ascribed this to the fact that the mean temperature in Florida is substantially higher than that in the North. Plough (130)

suggested that increased mutation rates at higher temperatures produce a marked increase in evolutionary diversification. Moreover, Cowles (33) suggested that this relationship may explain the occurrence of the great number of species in tropical areas.

Mechanisms Employed to Escape Temperature Induced Sterility

It was pointed out earlier in this discussion that temperatures encountered seasonably, occasionally, or continuously in homeotherms, which are not detrimental to somatic tissues, may cause male sterility in a variety of organisms (3,10,33,34,59,80,85,123,133,135,138,155,171, 179). Therefore, it is logical to conclude that organisms evolved mechanisms to escape heat-induced sterility (32,33,136). In general, poikilotherms and plants evolved a pattern in which spermatogenesis is highest during the cooler months of the reproductive season (33,59,171). Birds show a diurnal rhythm of spermatogenic mitoses (32,135,136) in which peak spermatogenic activity occurs when the birds are least active. This activity is correlated with a body temperature that is $5^{o}C$ cooler than during periods of peak activity in the birds. Wolfson (176) and Salt (139) described a condition in passerine birds suggesting that avian spermatozoa are not immune to the high body temperature of birds. They found that during the breeding season, the enlarged and convoluted portion of the bird's vas deferens (which serves as a bird's seminal glomus for storage for mature spermatozos) formed a cloacal protuberance located just beneath the skin. This swollen portion of the vas deferens is hidden beneath the feathers and is at a lower temperature than the body temperature of the birds.

It appears that in the evolution of mammals the descent of the testes from the abdominal cavity into the scrotum was an adaptive consequence of the sensitivity of spermatogenesis to higher body temperatures evolved in the development of homeothermy (32,33,136,163). The scrotum is a well developed outpouching of inguinal skin containing an evagination of the abdominal peritoneum called the tunica vaginalis (163). Thermoregulatory function of the scrotum is inferred from the fact that the body temperature of most mammals prevents spermatogenesis and that the scrotum is at a lower temperature than the body (32,33,45, 110,136,163).

A number of orders of Eutherian mammals do not have a scrotum. Some have their testes completely inside the abdomen, e.g. Edentata (sloths, armadillos), some Insectivora (shrews), Cetacea (whales, dolphins), Proboscoida (elephants), Hydracoida (conies), and Sirenia (manatees and dugong). In others, the testes partially descend to lie peripherally covered by abdominal skin or in the inguinal canal, e.g., Pinnipedia (seals, sea lions and walrus), Pholida (scaly anteaters), and Tubulidentata (aardvarks) (57,66,163,169).

In other Eutherian mammals, the extent of scrotal development varies from subanal pouches in the Felidae (cats) to the extremely pendulous scrotum of some Bovidae (cattle) (111,163). In some species, the scrotum is well developed at birth, containing fully descended testes, as in some primates (44,163,170,177) and may then subsequently regress with the testes returning into the inguinal canal (174). In other animals the scrotum becomes fully developed only at puberty under the control of gonadotrophin-induced androgen secretion (6,71,163,170). In some seasonal breeding species, the testes descend into the scrotum at the start of

the breeding season, but return into the abdomen at termination of the breeding season, e.g., in most rodents, bats, and insectivores (47,163).

Regulation of Testicular Temperature in Scrotal Mammals

A balance between heat carried into the testis by the arterial blood, metabolic heat generated within the testis, and heat loss by the scrotum ultimately determine the temperature of the testis (163). The scrotum employs both active and morphological mechanisms for heat loss. More important is the close proximity of the scrotal skin and subcutaneous tissues to the surface of the testis, an arrangement that exerts an influence on the temperature of the blood within superficial testicular veins. This venous blood, when flowing through the pampiniform plexus in the spermatic cord, exchanges heat with the inflowing arterial blood. In this way changes in the temperature of the scrotum are rapidly transferred to the testis via the precooled arterial blood (163).

One of the most striking features of the vascular anatomy of scrotal testes is the convolutions of the internal spermatic artery which arises from the aorta. These convolutions of the artery in association with the pampiniform plexus form a vascular cone, described by Galen in several large domestic mammals (143). On leaving the vascular cone the artery continues on the surface of the testis with wide variations among species in its course after it has passed under the epididymis to the distal pole (143). The artery may arborize with the more or less convoluted branches entering the testis before reaching the cranial pole. The main artery in the rat does not branch, but instead takes a highly

convoluted course as it travels up the free border of the testis, branching only after it disappears into the testis near the cranial pole (143).

Veins arise within the parenchyma of the testis and run either directly to the surface or to the central vein near the mediastinum. The veins under the tunica albuginea pursue a tortuous course toward the cranial pole of the testis, where they drain into the base of the pampiniform plexus (143). The central vein follows the mediastinum to the proximal pole of the testis, where this vein also joins the pampiniform plexus (14,15). The pampiniform plexus consists of many fine veins (as many as 300 in the ram) and these lie closely applied to the coils of the internal spermatic artery (166). The number decreases gradually until the plexus reduces to a single or sometimes two intercommunicating veins within the abdomen (143). Typically, the tunica adventitia of the coiled or multiple branced internal spermatic artery merges with that of the veins, so that at many points the counterflowing blood streams are separated only by the thickness of the vessel walls (163). This arrangement of arteries and veins allows a counter-current heat exchange between blood coming into the testes resulting in precooled arterial blood (163). In addition to the increased efficiency of heat exchange between blood in the arteries and veins due to greatly increased surface area of the vessels themselves, the lengthening and coiling or branching of the vessels mean that more time is available for heat exchange.

The amount of vascular heat exchange in the spermatic cord depends solely on the magnitude of the temperature gradient between the body and the scrotum and is not in any way autoregulatory (163). Vascular heat exchange in the pampiniform plexus only serves to cool the testis

when the returning venous blood is cooler than the arterial inflow and this relationship can be maintained only if heat is being lost through the scrotum (163). When blood flow in the spermatic cord of the rat is severely reduced, the noraal rectal-testis temperature difference is still maintained by the scrotum (88,100). So the effectiveness of vascul countercurrent heat exchange depends on the thermoregulatory mechanisms of the scrotum (163). The convolutions of both arteries and veins lying on the surface of the testes, the structure of the scrotum and the close proximity of the testes to the scrotum aid in heat exchange between the two.

In previous sections it was pointed out that the scrotum helps to insure spermatogenesis by allowing testes to reside at a temperature several degrees cooler than normal body temperature (19,24,32,33,110,136, 140,163). The mechanisms allowing the scrotum to produce this microclimate for testes include: location, blood flow, sweat glands, amount of hair, the functioning of the dartos muscle in positioning the testes in response to temperature changes and nerve receptors that lead to reflex adjustments in somatic temperature (32,163).

The scrotum is influenced less by body temperature by virtue of its location some distance from the main body mass (32). This location allows the scrotum to exhibit a surface area to volume ratio more or less independent of that of the body and allows it like other extremities to function as an efficient loser of heat (75). To assist in this heat loss, scrotal skin is thin, often bare and lacks subcutaneous fat (163).

The scrotum of man and other animals has a rich blood and lymphatic vascular system (46). The volume of blood perfusing the capillaries of the scrotum doubles in rats (63,168) and in rams (58,148,163) when the

skin of the scrotum is warmed to body temperature. Arteriovenous shunts (109,163) present in the scrotum presumably alter the patterns of capillary blood flow during temperature variation and help to maintain maximum blood flow in the skin during hot conditions to insure as great a heat exchange with the environment as possible (163).

Sweating, associated with the rich vascular supply of the scrotum (46), provides for more rapid cooling by evaporative means under higher more critical temperatures as observed in Kangaroos (137), sheep (99, 165) and cattle (11). The scrotal sweat glands are capable of producing more sweat than the glands of midside skin in the Merino ram (164) and "warm receptors" were found to be more abundant in the scrotum of the rat than in skin of the legs of the rat (79). These findings indicate a greater need to sense higher temperatures and to maintain a lower temperature in the scrotum than in other body areas. Further evidence to support the importance of sweating as a means of holding scrotal temperatures below that of other body areas in shown in that scrotal sweat glands in rams (161,162,164) and cattle (11) take part in seasonal adaptation by showing a greater maximal fluid production per unit of surface area in summer than in winter. This increase is higher than for other areas of the body.

Waites (161,162) showed that warming the scrota of rams invokes mechanisms for cooling the scrotum and the entire body. Panting, a mechanism for lowering the body temperature, was induced even in the paradoxical situation of stimulating simultaneously cutaneous "warm receptors" in the scrotum and "cold receptors" on the body. Thus the hypothalamus appears to be more responsive to the need for maintaining scrotal temperature than for body temperature. This finding further

emphasizes the importance of testicular temperature regulation and scrotal involvement in this regulation.

Cooper (31) reported that the scrotum of rams and bulls varies greatly in appearance and size. Under the influence of cold the scrotum was small, contracted and wrinkled, but under the influence of heat it was relaxed, smooth on its surface and greatly extended. Fowler (58) reported that the fully relaxed scrotum of Merino rams exhibited 20 percent greater surface area than an unrelaxed scrotum. A similar degree of extension was observed in a bull standing in a hot environment (134). These observations indicate that the scrotum actively makes adjustments to conserve heat in the cold by decreasing surface area. In contrast, upon exposure to increased temperatures, scrotal adjustments favor heat losses by increasing surface area.

The tunica dartos muscle is a sheet of smooth muscle underlying and attached to the scrotum (163). Its state of contraction is generally agreed to be responsible for the appearance of the scrotum during cold and heat (58,94,128,163,166). The receptors initiating these responses are probably those found in scrotal skin and connected to the sensory fibers in the superficial perineal nerves (89). Development and maintenance of the tunica dartos muscle is under the control of androgen secretion and becomes fully functional in the rat at sexual maturity (6).

In conclusion, it is evident that the mammalian testis is maintained at a temperature a few degrees lower than body temperature by residing in the scrotum. The mechanism involves primary regulation of of scrotal temperature, and secondary heat exchanges between the two to regulate testicular temperature. In addition, precooling of arterial

blood coming to the testes is accomplished by a vascular heat exchange mechanism operative between the spermatic artery and the veins of the pampiniform plexus. This heat exchange reduces entrance of body heat by the blood-vascular system.

Histology of the Testis

Elevated temperatures have a differential effect upon specific testicular cell types. This concept will be discussed in more detail in a later section. In order to fathom why differential effects occur and how they result in a cessation of spermatogenesis, it is pertinent to review the specific microanatomy of the testis and the roles specific cell types play in testicular function.

Bloom and Fawcett (17) describe the testis as a compound tubular gland enclosed in a thick fibrous capsule, the tunica albuginea. In most forms a thickening of the capsule on the posterior aspect of the organ projects into the gland as the mediastinum testis. The testis may be divided into lobules by thin fibrous sheets called septae, which extend radially from the mediastinum to the tunica albuginea. The testis of the rat is not divided into lobules by septae (122). In each lobule are one to four highly convoluted seminiferous tubules. These are 150 to 250 microns in diameter, 30 to 70 cm long and extremely tortuous. The tubules pass into the tubuli recti, the first segment of the excretory ducts (17).

A loose connective tissue called the interstitium, extends inward from the vasculosa testis to fill the spaces among the seminiferous tubules. Among its cell types are fibroblasts, macrophages, mast cells,

perivascular mesenchymal cells and interstitial or Leydig cells. These latter cells are the endocrine tissue of the testis (17).

The vasculature of the testis is of interest to this study in that no component of the blood vascular system penetrates the seminiferous tubules. Two types of capillaries have been described; the interlobular (118) which lie in the interstices among the tubules and the peritubular (143) which closely surround the seminiferous tubules. The latter type provides the principal means for nutrient supply and waste disposal for the seminiferous tubules (17,143,160) but even with their close proximity some of the more centrally located cells of the tubules are a considerable distance from their blood supply. The significance of the peritubular capillaries to seminiferous tubule function is further emphasized by the fact that they do not develop until the time for puberty in the rat (87).

Bloom and Fawcett (17) indicate that the seminiferous tubules are lined by a complex stratified epithelium composed of two major categories of cells; supporting cells and spermatogenic cells. The supporting elements are of a single kind, the Sertoli cell, while the spermatogenic cells include several morphologically distinguishable types: spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids. The spermatogenic cells are not ontogenetically distinct cell types, but are clearly distinguishable successive stages in the continuous process of differentiation of the male germ cells.

Sertoli cells appear to be involved in the secretion of rete testis fluid (39,55,74,95,96,143,158) into the lumen of the seminiferous tubules, and may serve a nutritive role to spermatids which fit into

numerous ultramicroscopic infoldings on the membrane surface of the Sertoli cell (17,67,120).

Clermont and Bustos-Obregon (26) described in the rat distinct generations of spermatogonia: five of type A, one of the intermediate type, and one of type B. Clermont and Bustos-Obregon (26) and Clermont and Perey (29) related these types to fourteen stages of cell associations found in the cycle of the seminiferous epithelium. Clermont and Bustos-Obregon (26) observed that one type A cell (A_0) seldom divided, so they tentatively considered it to be a "reserve stem cell". They considered the remaining type A spermatogonia to be "renewing stem cells", and postulated that these cells arose from each other through successive mitotic cell divisions. They further stated that the type A_4 cells divided mitotically to form two intermediate type spermatogonia, which in turn by mitotic division give rise to type B spermatogonia. Finally, type B spermatogonia divide mitotically to form primary spermatocytes. During meiotic prophase of primary spermatocytes tetrad formation occurs. Completion of this meiotic division yields diploid secondary spermatocytes which then divide immediately to give haploid spermatids. Spermatids then differentiate into spermatozoa without further cell division.

The earliest of the germ cells, the spermatogonia, rest upon or near the basal lamina of the seminiferous tubule. Progressively later stages are found at successively greater distances from the basal lamina so that the most highly differentiated spermatids come to border directly upon the lumen of the tubule (17).

Four cycles of 12 days each are required for the development of spermatogonia into epididymal spermatozoa in the rat (90). Similarly,

four cycles of the seminiferous epithelium are required to produce epididymal spermatozoa in mice (124), rams (126), and bulls (126). In contrast, monkeys require six complete cycles of the seminiferous epithelium to produce epididymal spermatozoa (28).

The Effects of Heat on the Testes

Application of heat to the testes from several sources, including hot air, infrared radiation, immersion in hot water (43^{o}C) and experimental cryptorchidism, all produce testicular degeneration and infertility (160). Regardless of the source or means of the application, the effect of the heat seems to be similar. However, exposing the testes to body temperature was not as effective as was the direct application of local heat (73). It also appears that with a fixed time of exposure, the higher the temperature, the more effective the treatment in producing degenerative effects (30,152,160). In addition, with a fixed temperature of exposure, the longer the period of exposure the greater the degree of degeneration (30).

Depending on the degree of degeneration induced by either cryptorchidism or direct heat application to the testes, there is an accompanying decrease in testis weight (30,115,149,160) and decrease in diameter of the seminiferous tubules (82,115,149).

An animal rendered sterile by heat application or by a period of artificial cryptorchidism may regain fertility in some instances. Collins and Lacy (30) found total recovery had apparently occurred by three weeks in some animals whose testes had been exposed to a single 15 minute immersion in a 43^{o}C water bath. Animals similarly treated, but for twenty minutes, did not recover fertility until after six weeks.

A 25-minute treatment group had progressed toward recovery at six weeks but they indicated that total recovery would require additional time. At six weeks following a single 30-minute treatment at 43°C very little sign of recovery was seen by these two investigators (30). If the testes are insulated or transferred to the abdominal cavity for one or two days, and then allowed to return to the scrotum, complete recovery of the germinal epithelium may occur within 45 days (112,119). Permanent damage to the germinal elements of the seminiferous tubules and complete loss of fertility may ensue with exposure of the testis to abdominal temperatures for periods of 30 days or longer (19). Bowler (19) suggested a mean recovery time of 60 days for rats whose testes had been immersed in 43.5°C water for 20 minutes. This duration of recovery time corresponds well with the reported 52-day duration of spermatogenesis in rats (25,27).

The Effects of Heat on Testicular Blood Flow

Investigations involving the rat and the ram indicate that elevated temperatures do not affect testicular blood flow. Glover (64) showed that 38°C has a negligible effect on rat testicular blood flow. Waites et al. (168) obtained similar results in the ram at 37°C. Other workers obtained similar results in the rat (147) and in the ram (167).

The Effects of Heat on Specific Cell Types of the Seminiferous Tubules

The effects of heat treatments on specific cell populations in the seminiferous tubules appear to be dependent upon the duration of

exposure, the temperature of exposure, and the relative thermal resistance of testicular cell types (160). Morris and Collins (115) studied the histological appearance of rat testes after 3½, 7, and 14 days of experimental cryptorchidism. They found a considerable reduction in the number of spermatids and spermatogonia after 3½ days. Cellular necrosis was indicated at this time by the appearance of large, polynucleated cells and debris. After 7 days there was a further reduction in spermatocytes and after 14 days, the testes had lost all cell types associated with normal spermatogenesis except a few spermatogonia. Sertoli cells did not appear to be affected by these treatments. Ewing and Schanbacher (49) found signs of degeneration among spermatocytes associated with stages IX through XIII as early as 24 hours after translocating the testes to the abdominal cavity in rats. This finding could explain the reduction in spermatids noted by Morris and Collins (115) after 3½ days of cryptorchidism since spermatocytes give rise to the spermatids (17).

The cells of the germinal epithelium that are most sensitive to heat appear to be the primary spermatocytes in stages IX through XIV of the cycle of the seminiferous epithelium (23,30,49,154,160). The spermatids in steps 1 and 2 of spermiogenesis appear to possess a sensitivity nearly equal that of the primary spermatocytes (23,30,152,160). Spermatozoa are more heat resistant than spermatocytes or spermatids, but are damaged during the latter stages of development or in the caput portion of the epididymis (4,160). Spermatogonia are the most resistant of the germinal cells in the seminiferous epithelium (23,24,30,152,160,178).

Elevating the temperature of the testes to temperatures above that of the abdomen were equally effective in producing testicular

degeneration but required a shorter interval of time than cryptorchidism. In general, the higher the temperature the less time required to produce a similar degeneration (30,152,160). The pattern of degeneration was similar to cryptorchidism in that it reflected the relative heat stability of the cell types (30).

The Effects of Heat on the Interstitium

There is disagreement among investigators as to whether there is a heat-induced hyperplasia among the Leydig cells, but none report a reduction in their number (16,30,78,115).

To this point, the importance of and the mechanisms for maintaining testicular temperatures at or below a given maximum have been reviewed. It has been pointed out that subjecting the testes to higher temperatures results in loss of testis weight with accompanying loss of fertility. The seminiferous tubules with increasing duration of elevated temperatures display a decreasing diameter accompanied by an increasing loss of cell types with duration until only a few spermatogonia and Sertoli cells remain. No apparent change in testicular blood flow or interstitial cell number was induced by elevation in temperature of the testes.

The Effects of Heat on Testis Metabolism

Since the objective of this investigation was to demonstrate early metabolic changes which precede heat induced testicular degeneration and sterility, it is logical to review any change that reflects or leads to an altered metabolism of the testes.

Effects of Heat on Metabolism of the Interstitium

Interstitial cell-stimulating hormone (ICSH) from the anterior pituitary stimulates the interstitial cells of Leydig to produce androgens, which in turn regulate spermatogenesis (70). Since androgens from the Leydig cells are required for complete spermatogenesis, it is logical that physiological changes in Leydig cells induced by heat may affect the germinal epithelium via alterations in androgen production. Although there is disagreement among investigators as to whether there is a heat induced hyperplasia among Leydig cells or not (16,30,78,115), investigators are in general agreement that there are physiological changes among the Leydig cells in reponse to elevated temperatures (42,78,93, 97,167). Under elevated temperatures, the Leydig cells show evidences for reduction in testosterone synthesis *in vivo* (42,167) and *in vitro* (97). Consistent with these findings is a reduction in steroid-3B-ol dehydrogenase, an enzyme which converts dehydroepiandrosterone to testosterone in Leydig cells (16). In addition, there is a rise in ICSH (16,43,78,153), which is evidence for a reduced testosterone level in the peripheral circulation.

Effects of Heat on Sertoli Cell Metabolism

The Sertoli cells do not form a functional part of the germinal epithelium of the seminiferous tubules in that they do not participate directly in sperm formation. However, Sertoli cells appear to show some physiological response to heat (30,115,160). Collins and Lacy (30) noted that Sertoli cells of heat damaged seminiferous tubules accumulated

nonacidic lipids and unsaturated sterols. Furthermore, during the process of recovery of heat damaged seminiferous tubules the lipids and sterols are reduced to normal, notably during the maturation division of the primary and secondary spermatocytes corresponding to stages IX-XIV of the cycle of the seminiferous epithelium (30).

In addition to the accumulation of lipids in the Sertoli cells, they show a decrease in the synthesis of inositol (115) and a decrease in the formation of rete testis fluid (9,95,142,143,146). The reduction in synthesis of inositol occurs prior to observed damage to the germinal epithelium (115) while the reduction in rete testis fluid is evident only during the actual heat application (9,95,142,143,146). The formation of rete testis fluid is an active process (143) and its return to normal rates of secretion after the cessation of heat suggests that energy sources are again available to the Sertoli cells for its formation.

If the metabolic environment of Sertoli cells has indeed been changed by heat treatment, it is logical to assume that the metabolic environment of the germinal epithelium has also changed since both are a part of the seminiferous tubules. That this change occurs prior to any degenerative changes in the germinal epithelium is suggested by the rapidity with which the reduction in inositol synthesis occurs following application of heat to the testes.

Effects of Heat on Testicular Oxygen Consumption

As indicated in the preceding section, Sertoli cells and Leydig cells display altered physiological response when testes are subjected to increased temperatures. These findings suggest metabolic changes in these testes. Another criterion used to assess changes in metabolic

activity of a tissue is the rate of oxygen consumption by the tissue in question. Waites and Setchell (167) observed a 70% increase in the oxygen uptake by testes of conscious rams as a result of a $3^{o}C$ rise in testicular temperature over a two hour period. In contrast, oxygen consumption of rat testes *in vitro* after several days of cryptorchidism was decreased (60,65,106). Ewing and VanDemark (50) showed that *in vitro* oxygen consumption was increased at 2, 4, and 8 hours, but decreased after 24 hours of experimental cryptorchidism in rabbit testis. Thus, both *in vivo* and *in vitro* experiments show that oxygen consumption is increased following short periods of heat application. However, *in vitro* methods indicate that longer periods of heat application by cryptorchidism are followed by decreased oxygen consumption. It is significant that Ewing and VanDemark (50) found that oxygen consumption by heat treated testis started to decline at the same time that Ewing and Schanbacher (49) first noted changes in histological appearance of cryptorchid testes. This suggests that later declines in oxygen consumption by cryptorchid testes in probably due to the demise of certain cell types.

The respiratory quotient (R.Q.) is an indicator of the type of substrate being oxidized (62). The R.Q. for carbohydrate is 1.0, for protein 0.8, and for fat 0.7-0.8 depending on the chain length of the fatty acid present in the lipids. Tepperman et al. (156) observed a R.Q. of 0.9 to 1.0 in the scrotal testes of rats compared to 0.5 in cryptorchid testes. This suggests that scrotal testes oxidize carbohydrate for energy while those cells remaining in the cryptorchid testes oxidize lipid. A R.Q. of 0.5 is unusually low but could be explained by the conversion of lipid into carbohydrate (56).

In summary, elevated temperature leads to an increase in oxygen consumption by testicular tissue within two hours. This increase is apparently followed by a decline after 24 hours. A shift in requirement for energy substrate from carbohydrate to lipid is indicated by a decrease in R.Q. The initial rise in oxygen consumption is probably due to the effect of temperature on the rate of chemical reactions in cells. The loss of temperature sensitive cells after 24 hours of cryptorchidism might explain the reduction in oxygen consumption.

Effects of Heat on Testicular Protein Metabolism

Although not all of the heat labile cells of the testes synthesize protein to any large extent, some of them do and their demise should be expected to alter protein synthesis. However, with the rise in temperature and circulating gonadotrophins, certain cell types e.g., the Leydig cells, may assume a higher rate of protein synthesis. In this event a transitory decrease in protein synthesis may be expected followed by a gonadotrophin induced rise in protein synthesis.

Incubation temperatures providing maximum incorporation of radioactive amino acids into trichloroacetic acid (TCA) precipitable material occurred at temperatures similar to scrotal temperature in rat (35,36, 92), rabbit (29,69), mouse (21), hamster (21), and guinea pig (21). Somatic tissues exhibited temperature optima similar to body temperature in these species (35,160). In addition, incorporation of labeled carbons from glucose-U-^{14}C into protein by rat testis slices *in vitro* is optimal at scrotal temperature ($32^{o}C$) (92).

In 30-day cryptorchid testes the temperature resulting in maximum incorporation of radioactive amino acid into protein was body temperature in the rat (38). This suggests that the sensitivity of protein synthesis to temperatures above scrotal temperature in normal testes of rats may be specific to heat sensitive cell types.

Ewing et al. (48) found that incorporation of labeled arginine into TCA precipitable material by rat testis slices *in vitro* was reduced by 48 hours of cryptorchidism. In view of these results, it is logical to expect a decrease in protein content. Toward this end Schanbacher and Ewing (141) showed that protein of rat testis remained unchanged through 24 hours but fell significantly ($p<0.05$) after 48 hours of experimental cryptorchidism.

The adverse effect of temperatures higher than scrotal temperature on testicular protein synthesis appears to be mediated via some mechanism involving glucose metabolism (35). In support of this view, Davis (35) observed that normal rat testis slices incubated in .009 M glucose increased the rate of radioactive amino acid incorporation ino TCA precipitable material when the incubation temperature was elevated from $32^{o}C$ to $36^{o}C$, as opposed to his observation that such a temperature change in the absence of glucose decreased incorporation. Means and Hall (108) concluded that stimulation of protein synthesis by glucose *in vitro* was the result of increased ATP synthesis since they found that ATP concentration was approximately one-third lower when exogenous glucose was excluded from the incubation media. Other observations by Means and Hall (108) indicate a close relationship between ATP and protein synthesis in rat testis tissues. For instance, they found that incubation conditions such as anaerobiosis and janus green B which lower ATP concentration

invariably display low rates of protein synthesis. In contrast, incubation of immature and mature rat testis tissues in the presence of .009 M glucose caused no change and increased ATP concentrations respectively with corresponding observations of protein synthesis.

Davis (35) suggested that temperatures exceeding 32°C in the rat may lead to the rapid utilization of glucose, leading to rapid depletion of available glucose in the avascular seminiferous tubules. The resulting hypoglycemic condition might result in reduced ATP concentrations required for biosynthetic reactions in specific heat sensitive cell types.

Research for this thesis will attempt to assess early effects of artificial cryptorchidism on protein synthesis *in vitro* and then will attempt to correlate any changes observed in protein synthesis with changes in energy metabolism and ATP concentration.

Effects of Heat on Testicular Lipid Metabolism

Elevated temperature appears to have an early effect of increasing synthesis of glycerides and possibly increasing catabolism of lipids (160). No effects on phospholipid levels were reported until cell losses had occurred (160). With longer periods of heat application total amounts of most lipid components fall except esterified cholesterol which may increase or remain unchanged (83,160). However, with longer periods of heat application most lipid components increase in concentration in the degenerating testis (52,53,82,84,107). This difference in total amounts and concentrations of lipids suggest surviving cells have an

inherent higher concentration of lipids, an increase in lipid synthesis, or lipids normally used by heat labile cells are accumulating in the surviving cells.

Experimental evidence indicates that much of this rise in lipid concentration in heat treated testes was due to an accumulation of cholesterol esters principally in the Sertoli cells (30,81,127). The observations of Collins and Lacy (30) indicate that this accumulation of lipid in the Sertoli cells is closely associated with damage to the germinal epithelium. They found that lipid accumulation in the Sertoli cells of the rat was progressive with damage to the germinal epithelium and was reduced to normal levels with the recovery of the germinal epithelium, notably during the maturation division of the primary and secondary spermatocytes corresponding to stages IX-XIV of the cycle of the seminiferous epithelium. Collins and Lacy (30) suggest that lipid accumulates in the Sertoli cells not because of an increase in synthesis, but because the germinal elements which use it in the course of their development have been reduced in number. Supporting this view is the close correlation of this increase in lipid content of Sertoli cells with a fall in the concentration of phospholipid, which coincided with cell losses in the rat (52,53). This correlation between increases in Sertoli cell lipid and decreases in phospholipid concentration would lend additional significance to the hypothesis of Collins and Lacy (30) if the phospholipids involved are related to cell structure as suggested by VanDemark and Free (160).

In summary, elevated temperatures in testes lead to reduced phospholipid concentration and accumulation of other lipids. Lipid accumulation, which occurs in the Sertoli cells, may be due to either increased

synthesis or a loss in cell types which use lipid. However, these experiments were all performed upon animals cryptorchid for 2 to 30 days or in animals where disruption of cellular morphology was in progress or well advanced. Moreover, very few experiments were designed to demonstrate the de novo biosynthesis of specific lipid classes from isotopic acetate. Research for this thesis was designed to answer questions specifically concerned with the early effects of cryptorchidism on the de novo biosynthesis of specific lipid classes from isotopic acetate by rat testis in vitro and thus may assist in accounting for the increased lipid concentration observed in cryptorchid testes.

The Importance of Glucose in Testis and the Effects of Heat on Testicular Carbohydrate Metabolism

The importance of glucose oxidation in testis has been demonstrated experimentally in vitro by incubating tissues in the absence of glucose. These experiments indicated a fall in oxygen uptake (50,51,60), a fall in R.Q. (40), and a fall in ATP concentration (108). In addition, rapid conversion of glucose-U-^{14}C to $^{14}CO_2$ (35,61) and the importance of glucose in stimulating protein biosynthesis in vitro stress a role for this carbohydrate in testis metabolism. In other tissues glucose is not only involved in cellular respiration but metabolic pathways utilizing glucose serve as a source of needed cofactors and small molecules for the biosynthesis of lipids, nucleic acids and proteins (62). Examples of such involvement are: the supply of NADPH for lipid synthesis by the pentose shunt pathway, the supply of ribose-5-phosphate for the synthesis of nucleotides by the pentose shunt pathway and the formation of some

amino acids from intermediates of the Krebs cycle. It is logical to expect a similar metabolic involvement for glucose in testes.

Experimental evidence indicates the pentose shunt pathway is operative in testis but uses only a small fraction of the total glucose consumed by the testes (60). Activity of the pentose shunt in testis has been demonstrated by incubations *in vitro* by comparing the amount of $^{14}CO_2$ evolved when glucose-6-^{14}C and glucose-1-^{14}C, respectively, were added to the incubation media (61,144). In addition, the rate of $^{14}CO_2$ evolution from gluconate-1-^{14}C by testis *in vitro* (61) and total activities of pentose shunt enzymes (2,7) in testis suggest activity of this pathway in the testes.

Most of the glucose (5/6) which enters the pentose shunt pathway is cycled back to reform hexose monophosphate members of the glycolytic pathway (62,101). The glucose used by the pentose shunt pathway gives rise to CO_2 and to NADPH, a cofactor needed for *de novo* synthesis of lipids (101). Ribose-5-phosphate, an intermediate of the pentose shunt pathway, may be used by the testes to synthesize the nitrogenous bases needed for nucleic acid synthesis (101).

The glycolytic pathway is the usual route taken by glucose or glycogen to enter cellular respiration (62) and thus is another biochemical pathway utilizing glucose in testis. In some other tissues, particularly skeletal muscle, this pathway under anaerobic conditions becomes hyperactive (62,101). This pathway does not need oxygen to produce the high energy compound ATP. Therefore, hyperactivity of this pathway compensates for reduction in ATP production by the energy yielding pathways requiring oxygen. However, only those tissues having stored carbohydrate can experience elevated glycolysis to compensate for anoxic

conditions (62). Since stored carbohydrate reserves are usually low in mammalian testis tissues (22), elevated glycolysis cannot compensate for reduced ATP synthesis under anaerobic conditions in vivo (60). Thus lactic acid, a product of anaerobic glycolysis, should not be expected to increase as much in testis during hypoxia in vivo as it may in other tissues where more glucose and glycogen are available.

Most of the trioses produced by glycolysis appear to be oxidized via pyruvate dehydrogenase and citric acid cycle enzymes (60,144), but some dihydroxyacetone phosphate is converted to α-glycerophosphate (101). Since α-glycerophospate is involved in the synthesis of triglycerides in other tissues (101), it may also represent another way in which glucose is involved in lipid synthesis in testis.

Fatty acids and pyruvate enter the citric acid cycle as Acetyl coenzyme A following β oxidation and the action of pyruvate dehydrogenase respectively (62). Thus the citric acid cycle can involve both carbohydrate and certain lipids in energy production. Since Acetyl coenzyme A may be used to synthesize squalene and fatty acids, glucose via Acetyl coenzyme A may be used for synthesis of a variety of lipid classes in addition to energy release (101).

Experimental evidence indicates that glucose via citric acid cycle intermediates may support protein synthesis in a way in addition to ATP synthesis (77,116,145) and suggests another mechanism by which glucose may stimulate or how a deficiency in glucose may lead to decreased protein synthesis in testicular tissue. Hollinger and Davis (77) found that considerable drain is placed on citric acid cycle intermediates to form simple amino acids in rat testis in vitro. They observed that aspartate, glutamate and glutamine together accounted for almost as much

label from glucose-U-^{14}C (18.9 percent) as did lactate (21.3 percent). Moreover, these amino acids were labeled to a much greater extent than any perchloric acid-soluble intermediate of the citric acid cycle and this suggests that glucose metabolized via the citric acid cycle is strongly committed to synthesize amino acids and subsequent biosynthesis of protein. Furthermore, this commitment of citric acid cycle intermediates is not confined to rat nor to the in vitro environment since Mounib (49) made similar observations in rabbit and cod, and Setchell et al. (145) observed such in the testis of the conscious ram.

The continued oxidation of Acetyl coenzyme A by means of the citric acid cycle requires the simultaneous presence of oxaloacetate. This is ordinarily provided by the cyclical nature of the process, but it also means that if there should be any drain on the cycle or its members for synthetic processes e.g., protein synthesis, a means must be provided for its replenishment. In animals, these anaplerotic sequences are provided by carboxylation reactions, which interconvert pyruvate to malate by action of malic enzyme or to oxaloacetate by action of an ATP-dependent pyruvate carboxylase (60,101). It follows that any drain of citric acid cycle intermediates should be accompanied by carboxylation of pyruvate and an increase in this latter reaction could be accepted logically as evidence for a drain on the citric acid cycle intermediates and related amino acid synthesis. Thus additional evidence indicating involvement of citric acid cycle intermediates in testicular amino acid synthesis was shown by the labeling of citric acid cycle intermediates by $^{14}CO_2$ in testis of rabbit (61,116), cod (116), and rat and chicken (116).

Experimental evidence suggests that there are both quantitative and qualitative differences in energy metabolism between the germinal and non-germinal elements of the testes. Non-germinal cells of the rat testes have a higher oxygen uptake per unit weight than normal testis tissue during in vitro incubations in the absence of glucose (72,156). This indicates that the basic metabolic rate of the interstitial tissue may be higher than that of the seminiferous tubules. In contrast to the results obtained with intact germinal epithelium glucose added to the incubation media does not stimulate oxygen uptake in aspermatogenic rat testis tissue (60,65,156). These data suggest that non-germinal cells may rely upon endogenous carbohydrate (72) or upon lipid (60,65) in the cryptorchid rat testes. It should be pointed out that endogenous carbohydrate concentrations in intact rat testes are lower (less than 30%) than those of 42-day cryptorchid rat testes (72). Gomes (65) and Free (60) suggested that the tubular elements of the testis oxidize glucose but that the elements which survive cryptorchidism oxidize lipids.

In previous sections the effects of temperature on the in vitro interrelationships of oxygen uptake, CO_2 production and the metabolism of carbohydrate with lipid and protein synthesis have been discussed. These studies are important in that they indicate changes in metabolic capabilities due to temperature treatment but they may not accurately represent the in vivo condition. The following sections will be concerned primarily with in vivo changes in respect to concentration of carbohydrate substrates and intermediates and the enzymes involved in their metabolism. This information would logically give more conclusive evidence for elucidating metabolic changes involving carbohydrate which

may contribute to the demise of temperature sensitive cells. Studies in this area are few and were usually made after a comparatively long period of cryptorchidism.

Ewing and VanDemark (50) found 12% and 27% less glucose and lactic acid respectively in rabbit testis rendered cryptorchid for 24 hours than in control testis. Zogg et al. (180) obtained similar changes after 48 hours cryptorchidism in the same species. After 2 - 2½ hours of local heating of the ram testes at 39°C, Waites and Setchell (167) found a 67% increase in oxygen uptake, variable glucose uptake, and little change in lactate production.

Ewing and Schanbacher (49) found that hexokinase activity of the rat testis was reduced significantly ($p<0.01$) within 24 hours of cryptorchidism and phosphofructokinase enzyme activity was significantly ($p<0.01$) reduced within 4 hours. These same investigators found no significant changes in the activity of glucose-6-phosphate dehydrogenase, pyruvate kinase or lactate dehydrogenase within 48 hours of cryptochidism. These *in vitro* observations suggest that alterations in carbohydrate metabolism of testis occur *in vivo* soon after induction of cryptorchidism and that these changes may be induced by interference with activity of some key enzymes.

Mechanisms by Which Temperature Inhibits Spermatogenesis

Glucose appears to be involved in heat induced cessation of spermatogenesis in scrotal animals as indicated by the protection of testis tissue against elevated temperature when glucose is in the incubation media (35). Means and Hall (108) observed that the effect of glucose on

testicular protein synthesis in vitro was related to ATP concentration. They found that glucose prevents the decline in testicular ATP seen during incubation in vitro and increases the rate of protein biosynthesis during the same period of time. In general, Means and Hall (108) found that any situation that decreased testicular protein synthesis was associated with decreased ATP concentration e.g., anaerobiosis and janus green B. However, Hollinger and Davis (77) suggested that glucose was also involved in amino acid production. Davis (35) suggested that hypoglycemia induced by elevated temperature led to cessation of spermatogenesis and resulting infertility. He suggested the possibility that temperatures exceeding 32°C in the rat may lead to rapid utilization of glucose and subsequent depletion of available glucose, particularly to the cells of the avascular tubules. This resulting hypoglycemic condition might lead to the demise of cell types having a high dependency on glucose for energy.

Baldwin and Ewing (7), LeVier (92), and Ewing and Schanbacher (49) concluded that disturbance in the activity of enzymes involved in glucose metabolism might conceivably be responsible for heat sterilization. Baldwin and Ewing (7) working with rabbit testes and LeVier (92) experimenting with rat testes concluded that elevated temperatures inhibited the hexosemonophosphate shunt pathway with subsequent stimulation of glycolysis via reduced inhibition of phosphofructokinase enzyme. This favoring of glycolysis may eventually lead to hypoglycemia with resulting sterilization. In a later work, Ewing and Schanbacher (49) found that phosphofructokinase activity was significantly ($p<0.01$) reduced after 4 hours of cryptorchidism. Since this enzyme regulates glycolysis,

these authors speculated that cells containing reduced phosphofructokinase activity could not metabolize glucose at a normal rate. This implies that temperatures higher than scrotal temperature may result in reduced glucose catabolism in certain cell types leading to their death.

Others contend that temperature induced sterility of mammalian males is brought about by hypoxia or accumulation of CO_2. Waites and Setchell (167) and Waits and Moule (166) have concluded from their experiments that testicular hypoxia resulting from increased testicular temperature is the causative factor for spermatogenic damage. In contrast, Fleeger et al. (54) suggest that carbon dioxide accumulation might account for spermatogenic arrest. Baldwin and Ewing (7) agreed that higher temperatures probably induced hypoxia in testes, but that a glucose deficiency was also involved.

Summary

Higher temperatures are detrimental to male gametogenesis in most animals resulting in increased mutagenesis in some species.

Numerous mechanisms have evolved among organisms to evade the deleterious effects of high temperature during the reproductive season. The scrotum evolved as an evolutionary defense mechanism protecting against heat damage to spermatogenic tissue in most terrestrial mammals. Location of the testes in the scrotum plus the vasculature of the scrotum and testes permit the testes to reside at a temperature several degrees cooler than body temperature. Should this defense mechanism be overwhelmed by scrotal insulation, extreme ambient temperature, direct heat application or cryptorchidism impaired fertility results. This is manifested by an eventual loss of all spermatogenic elements of the

seminiferous tubules except for a few spermatogonia. These changes in cellular content are accompanied by an accumulation of lipid and decrease in protein synthesis *in vitro*.

Alterations in carbohydrate metabolism are manifested by decreased conversion of glucose and pyruvate to CO_2 *in vitro* and accumulation of glucose, glucose-6-phosphate and glycogen *in vivo*. Associated with this apparent decrease in carbohydrate utilization is a decrease in R.Q. which suggests a possible shift to a greater dependence on lipids for energy. It has been postulated that this apparent shift occurs because surviving cells normally use lipid for energy whereas the heat labile cells use carbohydrate. The observation that cryptochidism decreases ATP concentration and the close association among glucose, ATP concentration and protein synthesis in testis noted by Means and Hall (108) indicate a possible relationship between glucose and heat sterility. In general, investigators agree that the mechanism of heat induced sterilization involves glucose, but disagree concerning the nature of this mechanism.

The present research was designed to elucidate how soon after temperature application changes in lipid and protein synthesis occur *in vitro*. Early changes in the conversion of glucose-U-^{14}C and pyruvate-2-^{14}C to $^{14}CO_2$ *in vitro* will be used to determine changes in the known major metabolic pathways utilizing these metabolites in testis. In addition to these pathways, the relative availability of oxygen and the relative energy charge will be assessed by measuring *in vivo* lactate, ATP, NADH, NADPH, and several metabolites of the Embden-Meyerhof pathway and Krebs cycle in cryptorchid testes. Furthermore, changes in

concentrations of metabolites of the Embden-Meyerhof and Krebs cycle may indicate if suppression of enzyme activity is involved in heat sterilization.

CHAPTER III

MATERIALS AND METHODS

Materials

Animals

Sexually mature male Sprague-Dawley rats, 70 to 80 days of age, weighing 250 ± 10 grams were acquired from the Holtzman Company, Madison, Wisconsin.

Radioactive Isotopes

A total of five different radioactive isotopes was obtained from the New England Nuclear Corporation, Boston, Massachusetts. They included: Sodium acetate-1-^{14}C, lysine-U-^{14}C, sodium pyruvate-2-^{14}C, 2-deoxy-D-glucose-1-^{14}C, and D-glucose-U-^{14}C. Prior to use, the isotopes were stored as suggested by the manufacturer. On the day of use, the isotopes were evaporated under nitrogen, if necessary, and Krebs-Ringer solution added to acquire the desired radioactivity per unit volume of solution. A listing of radioactive substances and their range of specific activities can be found in Appendix A. Each of these solutions containing specific isotopes was prepared further by adding amounts of the nonradioactive forms of the isotopes to bring the solutions to the desired concentration.

Scintillation Counting Material

Two scintillation fluids were used in this study: 1) toluene scintillation fluid containing 12 g of 2,5-diphenyloxazole (POP) and 0.12 g of 1,4-bis-1-(5 phenoxazolyl)-benzene (POPOP) in 3 liters spectro quality toluene; 2) Bray's Solution (20) containing 180 g of Naphthalene, 12 g of POP, 0.6 g POPOP, 300 ml of methyl alcohol, 60 ml of ethylene glycol and sufficient p-dioxane (spectro quality) to bring the solution to a 3,000 ml final volume. These solutions were allowed to equilibrate for one day prior to use and were stored in the dark.

Enzymes

Enzymes were acquired from the Sigma Company, St. Louis, Missouri, then stored as suggested on the labels. These enzymes or the working dilutions of them were monitored under experimental conditions prior to use by spectrophotometrically assaying cuvettes containing known amounts of the substrates to be measured rather than extracts from experimental animals. Completion of the reaction within a time specific for each enzyme was used to determine enzymatic activity. All enzymes purchased and their origin, type, etc. may be found in Appendix A.

Stock enzyme solutions from the company were diluted in some instances to obtain desired rates of activity. A listing of these dilutions may be found in Appendix B.

Cofactors and Substrates

Substrates needed for incubation media for the investigations *in vitro* and the preparation of solutions for standardization of

spectrophotometric and spectrophotofluorometric measurements were obtained from the Sigma Company. Cofactors needed for enzymatic determinations of tissue substrate levels were obtained from Sigma Company. Precautions were taken to store these chemicals as suggested by the manufacturer. A listing of these substances along with technical data concerning them may be found in Appendix A. The procedures for the preparation of working solutions of the cofactors and substrates used in this experiment are listed in Appendix B.

Methods

Animal Housing and Preparation

The rats used in this experiment were housed in stainless steel cages in an animal room maintained at a temperature of $21^{o}C \pm 1^{o}$ with a regimen of 14 hours of light and 10 hours of darkness daily. The animals were provided with water and Purina Laboratory Chow *ad libitum*. All animals were allowed to adjust to these conditions for at least one week before being used in the present study.

Surgical Procedures

Anesthesia was induced by diethyl ether (anesthesia grade), and the hair was clipped from the scrotal and pelvic area. The surgical field was swabbed with 70% ethanol and a longitudinal incision approximately one centimeter long was made through the skin about one-half centimeter lateral to and slightly posteriorad to the penal orifice on both sides to expose the inguinal canals. The testes and attached epididymides were forced into the abdominal cavity and the inguinal canals gently

teased free from surrounding connective tissue. Surgical thread (size 00 silk) was passed under each inguinal canal posterior to the position of the tail of the epididymis and tied securely enough to prevent return of the testes and epididymides to the scrotum, but not so tight as to cause tissue ischemia. The incisions through the skin were closed by use of surgical clamps or surgical thread. The surgical procedure for sham operations was the same, except the inguinal canals were not tied off and the testes were forced back into the scrotum prior to closure of the incision.

Incubations

Tissue Preparation. Each animal was sacrificed by cervical dislocation of the spinal cord. The testes were quickly removed and placed in either ice cold 0.154 M KCl or Krebs-Ringer bicarbonate solution. The testes were quickly freed of extraneous tissue, blotted with soft tissue paper and weighed to the nearest milligram. Following removal of the tunica albugenia, testis tubules were teased on a watchglass by using small curved stainless steel forceps. The watchglasses were kept on ice and contained a small amount of 0.154 M KCl to facilitate separation of the tubules.

Tissue Extractions

Tissue Preparation. Each animal was sacrificed by cervical dislocation of the spinal cord. The testes were quickly removed and dropped into liquid nitrogen. After freezing in the liquid nitrogen, the testes were removed for brief intervals (30 seconds maximum) and freed

of extraneous frozen tissues by use of a sharp wood chisel, cooled in liquid nitrogen. The frozen testicular tissues were then powdered with a stainless steel mortar and pestle precooled and embedded in dry ice. The powder was removed from the mortar with a stainless steel spatula (precooled in liquid nitrogen) and placed into pyrex glass test tubes which were held in a liquid nitrogen bath.

Alkaline Extraction. A weighed aliquot (200 to 300 mg) of the frozen powder was used to prepare an alkaline testis tissue extract by the method of Williamson (172). This extract was used for the assay of NADH and NADPH in vivo. Two ml of 1.5 N ethanolic KCl was placed into a 10 ml beaker. The beaker and contents were weighed. The frozen powder was placed into the beaker and the beaker and contents quickly weighed a second time to determine exact weight of powder to the nearest 0.1 mg. The contents were mixed with a glass stirring rod for 60 seconds while held in a 55^{o}C water bath. The clear digest was cooled and 2 ml of cold 0.5 M triethanolamiane hydrochloride (pH 6.5) were added slowly with mixing. The extracts were carefully neutralized to pH 8.0 with 2 N HCl during vigorous mixing. The contents of the beaker were then transferred to a clean plastic centrifuge tube and centrifuged at 49,000 x g for 10 minutes. The clear supernatant was finally filtered with a .22 μ Millipore filter by using a 10 ml syringe and Millipore syringe adapted filter holder. The extract was measured and kept cold until assayed for NADH and NADPH.

Acid Extraction. A weighed aliquot of the frozen power was used to prepare a perchloric acid extract by a modification of the method of Williamson (172). The modification encompassed the use of 10 M KOH to

neutralize the acid extract to a pH of 7.0-7.5 rather than using 3 M K_2CO_3 to neutralize to pH 6.0. Six ml of 6% perchloric acid were placed into a 15 ml Ten Broeck glass homogenizer (No. 7727). The homogenizer and contents were then weighed and placed on ice. Frozen testis powder (2-3 g) was added to the homogenizer and the contents quickly weighed to determine weight to the nearest milligram. The contents of the homogenizer were homogenized on ice, placed in 12 ml plastic centrifuge tubes and centrifuged in the cold at 49,000 x g for 10 minutes. The supernatant was poured into 10 ml glass beakers and neutralized in the cold with 2 N KOH to pH 7.0-7.5. The resultant precipitated potassium perchlorate was removed by centrifugation in the cold at 49,000 x g for 10 minutes. The supernatant was measured, filtered through a .22 μ Millipore filter, and kept cold or frozen until assayed for ATP and intermediates of glycolysis and the Krebs cycle. The least stable intermediates, such as pyruvate were analyzed in the fresh extracts as soon as possible.

Determination of Percentage Dry Weight

Percentage dry weights of testis tissues were determined in order to express metabolites in terms of micromoles per gram of testis dry weight. Aliquots of the frozen powders (100-150 mg) were placed on tared aluminum planchets. The planchets and powders were quickly weighed and then placed in a 100°C oven to dry overnight. The following day the planchets were weighed again. The following formula was used to determene percentage dry weight:

$$\frac{\text{weight of dry tissue}}{\text{weight of wet tissue}} \times 100 = \text{percentage dry weight}$$

Spectrophotometric Assays

The procedures found in Methods of Enzymatic Analysis (13) were followed in most of these analyses. In general, these means were used to determine metabolites measurable in terms of micromoles per gram of dry weight and included α-Keloglutaric acid, lactic acid, and malic acid.

Blank cuvettes contained either distilled water or the buffer found in the experimental cuvettes. The experimental cuvettes contained buffer, substrates, ions, cofactors, coupling enzymes, and acid extract. The absorbancy at 340 nm were used to calculate the amounts of the compounds being measured per ml of extract. These values were used to determine concentrations of the compounds in terms of micromoles per gram of testis dry weight. See pages 64, 65 and 66 for detailed descriptions of each assay.

Spectrophotofluorometric Assays

Some metabolites are found in concentrations so low that they must be measured via spectrophotofluorometric assays. Spectrophotofluorometry affords at least a 100 fold increase in sensitivity when compared to conventional spectrophotometric methods.

Metabolites measured in this manner included fructose-6-phosphate, fructose-1,6-diphosphate, 2-phosphoglyceric acid, pyruvate, NADH, and NADPH.

A metabolite fluorometer designed and constructed at the Johnson Research Foundation, and a matched set of low fluorescence glass cuvettes were used in these assays. Tissue extract, buffered solution and required solutions of ions and cofactors to make a total of 2 ml were

added to the cuvettes. The sequence for pipetting reagents was to add buffer solution first, then solutions of ions and cofactors and finally the tissue extract. The contents of cuvettes were thoroughly mixed with a plastic stirring rod and the cuvettes were placed in a warming chamber maintained at 28°C. The sensitivity of the fluorometer was set to give 70 to 90% of full scale deflection with one nanamole of cofactor e.g., NADH, in 2 ml of buffer. After a cuvette had reached control temperature in the warming chamber it was transferred to the recording chamber which was also temperature controlled at 28°C. The record button was then depressed and the compensating voltage dial turned until the recording pen remained steady on center scale. The compensating voltage at this time was recorded as the initial reading. Usually ten microliters of the appropriate enzyme were then added to the cuvette and thoroughly mixed with its contents to start the reaction. The compensating voltage control knob was adjusted during the course of the reaction to keep the recording pen on the recording scale. The reaction within the cuvette was considered complete when the recording pen remained at a constant setting. The pen was set at center scale and the compensating voltage recorded. The change in compensating voltage was determined and recorded. To determine how much of the change in compensating voltage was due to the fluorescence of the added enzyme (enzyme blank), an additional equal amount of the enzyme was added to the cuvette with mixing and the change in compensating voltage determined as before. The actual change in compensating voltage due to the change in fluorescence attributable to the chemical reaction involving that particular metabolite being measured was then calculated and recorded for that metabolite.

In order to determine how much of the metabolite in question was present in the experimental cuvette, an internal standard was determined. The precedure for this involved the addition of a nanomole of a standardized 0.1 mM solution of the metabolite to the cuvette after determination of the enzyme blank. The change in compensating voltage was determined as described previously and the change in compensating voltage per nanomole of metabolite calculated. This information was used to determine the amount of metabolite that was in the cuvette originally and subsequently the amount of the metabolite per gram of testis dry weight. The standard 0.1 mM solution of the metabolite was standardized by spectrophotometric means by using the same regimen of buffer and ions in a quartz cuvette, but 1/2 ml of the standard and sufficient cofactor to assume consumption of all the metabolite in the standard.

In several instances, additional coupling enzymes were added to determine other metabolites that were linked in sequence along the metabolic pathways.

Metabolites of glycolysis were determined using modifications of the methods as described by Maitra and Estabrook (102). NADH and NADPH were determined using modifications of the method of Williamson and Corkey (173). See pages 61, 62, 63 and 64 for detailed description of each assay.

Experimental Design

The primary objective of this investigation was to determine early effects of elevated temperature on some anabolic and catabolic phases of testicular carbohydrate metabolism and to gain insight into the metabolic basis of heat sterilization.

Experiment 1: Effects of Artificial Cryptorchidism on Incorporation of Lysine-U-^{14}C Into TCA Precipitable Material by Rat Testis

The objective of this experiment was to determine the early effects of cryptorchidism on the synthesis of protein *in vitro*. This objective was accomplished by incubating testis tubules from cryptorchid rats under appropriate conditions in a medium containing a specified isotopic substrate. The relative rate of protein synthesis by a tissue *in vitro* can be measured by incubating the tissue for a time in an appropriate medium containing labeled amino acid and then measuring the incorporation of label into TCA precipitable proteinaceous material. The effects of glucose on this protein synthesis can be investigated by comparing incubations with and without exogenous glucose. Details of these incubations and methods of chemical analysis will be given in the following sections.

Rats were rendered cryptorchid prior to sacrifice for periods of 2, 4, 8, 16, 32, 64, and 128 hours. Control animals for this experiment were normal animals which had not been subjected to a sham operation. The experiment was arranged in a completely randomized block design consisting of seven treatment groups and a control with 5 replicates per treatment to make a total of 40 rats used. All data were subjected to analysis of variance (see Appendix C) and when found to be significant, differences in treatment means were detected by Duncan's New Multiple-Range Test (151).

The mechanics of this experiment involved placing 200 mg of teased testis tubules in an ice-cold 25 ml Erlenmeyer flask prior to adding

3 ml of Krebs-Ringer bicarbonate buffer containing lysine-U-^{14}C (0.25μCi; 0.1 mM) with and without glucose (10 mM). Flasks were flushed with 95% O_2:5% CO_2 for 15 seconds, stoppered and incubated at 37°C in a shaking water bath for one hour. At the end of the incubation period the reactions were stopped by adding 0.3 ml of 5 N $HClO_4$. The contents of the flasks were then homogenized in 2 ml of 0.5 N $HClO_4$ and centrifuged at 35,000 x g for 15 minutes. The supernatant was discarded and the resulting pellet extracted to isolate the protein. The protein isolation procedure consisted of subjecting the $HClO_4$ precipitable material to a series of solvents in order to remove substances other than protein. The solvents were added in 5 ml quantities. This was followed subsequently by: thorough mixing, centrifugation at 35,000 x g for 15 minutes, and discarding of the supernatant. The sequence of solvents used consisted of the following: 1.5N TCA at 70°C for 15 minutes, 95% ethyl alcohol, chloroform:methanol (2:1), benzene and diethyl ether.

Following the last extraction, the centrifuge tubes containing the resulting proteinaceous pellets were placed in a hood to evaporate any remaining diethyl ether. Five ml of 0.5 N NaCl were added to the tubes containing the protein pellets and the contents heated for 2 hours in a 90°C water bath to digest the protein. Incorporation of radioactive lysine into protein was then determined by adding 0.2 ml of the NaOH digest and 12 ml of Bray's solution to a glass scintillation vial. The contents of the vials were mixed thoroughly by vigorous shaking. The vials were cooled to 5°C and then counted in a Packard Ti-Carb Liquid Scintillation Spectrometer, Model 3003, using the Automatic External Standard to determine the percent efficiency of the counting procedure.

The dpm's were calculated per milligram of protein after total protein in each digest had been determined by the method of Lowry et al. (98).

Experiment 2: Early Effects of Cryptorchidism on Lipid Synthesis

In order to determine if hyperthermia affected testis synthesis in general or was specific only to proteins, this experiment was designed to investigate possible changes in the synthesis of several classes of lipids by measuring the rate of incorporation of labeled acetate into lipid by testis tubules *in vitro*. In this procedure 200 mg of testis tubules from each animal used in the previous experiment were placed in ice-cold 25 ml Erlenmeyer flasks prior to adding 3 ml of Krebs-Ringer bicarbonate buffer containing acetate-1-^{14}C (25μCI;2.5 mM) with or without glucose (10 mM). Flasks were flushed with 95% O_2:5% CO_2 for 15 seconds, stoppered and incubated at 37°C in a shaking water bath for one hour. After incubation, the flasks were placed on ice and the contents of each flask were homogenized on ice in a glass grinding vessel with a teflon pestle until pieces of tissue were no longer visible.

Homogenates were placed in beakers containing 35 ml of chloroform: methanol (2:1,v/v) to extract total lipids. Aliquots of the total lipid fraction were placed on scintillation vials, evaporated and 12 ml of toluene scintillation fluid were added for radioactivity determination. In some experiments total lipids were separated into various lipid classes (mono-, di, and triglycerides, free fatty acids, phospholipids, sterols and sterol esters) by chromatographing an aliquot of the total lipid fraction on thin layer plates. The plates were prepared with silica gel, activated (110°C for 1 hour), divided into nine lanes

(2 cm wide) and developed in an ascending manner in sealed tanks containing petroleum ether:ether:acetic acid (90:10:1,v/v). After the solvent front had migrated 16 cm from the origin, the plates were air-dried and re-chromatographed in another sealed tank containing ether: petroleum ether:acetic acid (70:30:1,v/v) up to 9 cm from the origin. A mixture of standard lipids corresponding to the lipid classes noted above was spotted on the end of each thin layer plate and chromatographed as outlined. This lane was sprayed with a 0.2% solution of 2',7'-dichlorofluorescin in isopropyl alcohol, visualized under ultra-violet light and material with the same chromatographic mobility as mono-, di-, and tripalmatin, sphingomylin, oleic acid, cholesterol and cholesterol acetate was aspirated into a disposable Pasteur pipette (4 3/4" long) containing a glass wool plug. Lipids were eluted directly into scintillation vials with chloroform:methanol (2:1,v/v), the contents of the vials were evaporated and 12 ml of toluene scintillation fluid were added for radioactivity determination. Further identification of the lipid classes isolated on thin-layer plates was made by using antimony trichloride in chloroform or solutions of Rhodamine B, diphenylamine, 2,4-dinitrophenyl hydrazine or ninhydrin as described by Mangold (103). Bromocresol green solution (86) was used to identify free fatty acids. Molybdic acid solution followed by exposure of the plate to ultra-violet light was utilized to detect phosphate-containing lipids (8).

Methyl esters of non-volatile fatty acids present in an aliquot of the total lipid extract were prepared with diazomethane (12). The esters were separated and identified on a Barber-Coleman, Model 5000, gas chromatograph equipped with an ionization detector. The radioactive

effluent gas was trapped in glass capsules containing anthracene (12). The vials were capped, placed in specially prepared holders and radioactivity was counted directly. Collection of the effluent was facilitated by using a Packard gas chromatograph fraction collector. With this method 60-70% of the radioactivity placed on the column was recovered.

Radioactivity was measured in a Packard Tri-Carb, Model 3365, liquid scintillation spectrometer equipped with automatic external standardization to determine the percent efficiency of counting. The dpm's were calculated from the efficiency of the counter per 100 mg of tissue (wet weight) for total lipids and for each class of lipid isolated.

Experiment 3: Early Effects of Cryptorchidism on Glucose Transport

This experiment was designed to investigate the possibility that the hyperthermia of cryptorchidism may induce changes in the rate of glucose transport by testis tissue. The procedure involved incubating testis tubules from cryptorchid and control rats in an appropriate medium containing 2-deoxyglucose-1-^{14}C, isolation of 2-deoxyglucose-6-phosphate from the tissue and counting the radioactivity of 2-deoxyglucose-1-^{14}C-6-phosphate to assess relative rate of glucose transport per 100 mg of tissue (wet weight).

Rats were rendered cryptorchid prior to sacrifice for periods of 2 and 8 hours. Control animals for this experiment had been sham operated equivalent periods of time prior to sacrifice. The experiment was arranged in a completely randomized block design consisting of two treatment groups and two sham operated controls with 8 replicates per treatment to make a total of 32 rats used. All data were subjected to

analysis of variance (see Appendix C) and when found to be significant, differences in treatment means were detected by Duncan's New Multiple-Range Test (151).

According to a modification of the methods of Smith and Gorski (150), approximately 100 mg of teased testis tubules from each rat were placed in ice-cold 25 ml Erlenmeyer flasks prior to the addition of 2 ml of Krebs-Ringer bicarbonate containing 2-deoxyglucose-1-^{14}C (0.8μCi;10 mM). The vessels were gassed for 15 seconds with 95% O_2:5% CO_2, capped and incubated in a shaking water bath at 37°C for one hour. Reactions were stopped by placing the flasks on ice. The tubules were removed from the incubating media and washed three times in 5 ml portions of ice-cold Krebs-Ringer bicarbonate buffer to free the tissues of most adhering 2-deoxyglucose-1-^{14}C. The tissues were placed into a glass, motor-driven homogenizer and homogenized in one ml of ice-cold 5% TCA containing one mg each of 2-deoxyglucose and 2-deoxyglucose-6-phosphate as carriers. Homogenization was followed by: centrifugation at 1500 x g for 10 minutes, washing the aqueous supernatant three times in 5 ml portions of diethyl ether and lyophilization of the aqueous portion of the washes. The lyophilized powder was dissolved in glass distilled water and stripped on one-inch wide strips of Whatman No. 1 chromatography paper. The strips were developed by descending chromatography with a solvent system of butanal-1:acetic acid:water (2:1:1,v/v). Separate standard strips spotted with unlabeled 2-deoxyglucose and 2-deoxyglucose-6-phosphate respectively, were chromatographed simultaneously and after development were dried, sprayed with 0.5% benzidine in ethanol:acetic acid (4:1,v/v) and heated at 120°C for 10 minutes to visualize sugars for determination of Rf values of the sugars.

After drying of the experimental chromatograms, radioactivity was located on them by means of a Packard, Model 7201, strip scanner. Two widely separated peaks of radioactivity were located on each strip and identified as 2-deoxyglucose-1-^{14}C or 2-deoxyglucose-1-^{14}C-6-phosphate by comparing their Rf values with the standards. The strip sections containing the phosphorylated form of the glucose were shredded with scissors into separate counting vials. After adding 10 ml of toluene scintillation fluid, the vials were capped, cooled to 5°C and counted with a Packard Tri-Carb, Model 3365, liquid scintillation spectrometer equipped with automatic external standard for determination of efficiency of counting. Dpm's per 100 mg of teased testis tubules (wet weight) were calculated and recorded for each rat.

Experiment 4: Effects of Artificial Cryptorchidism on the Conversion of Glucose-U-^{14}C Into $^{14}CO_2$ by Incubated Rat Testis

The objective of this experiment was to determine early changes in the metabolism of carbohydrate along the combined Glycolytic Krebs cycle pathway. This objective was accomplished by incubating testis tubules from the cryptorchid rats used in Experiment 3 under appropriate conditions in a medium containing a specific isotopic substate. Relative activity was assessed by capturing evolved $^{14}CO_2$, determining its radioactivity and then calculating dpm's per 100 mg of tissue (wet weight). Details of incubation and methods of chemical analysis will be given in the following sections.

Approximately 100 mg of teased testis tubules from cryptorchid rats were placed in the main chamber of ice-cold Warburg flasks prior to the

addition of 3 ml of Krebs-Ringer biocarbonate buffer containing glucose-U-^{14}C (0.50μCi; 10 mM). The flasks were gassed for 15 seconds with 95% O_2:5% CO_2, capped with serological caps, placed on a shaking water bath at 37°C and allowed to incubate for two hours.

After incubation, the reactions were stopped by injecting 0.25 ml of 1 N H_2SO_4 into the main compartment of the flasks. To capture evolved $^{14}CO_2$, 0.25 ml of hyamine hydroxide was injected into the center well of each flask. Injections were made by using a 1 ml. tuberculin syringe equipped with a (1½" long) 27 gauge needle. The flasks were allowed to shake an additional hour to complete capture of $^{14}CO_2$ by the hyamine hydroxide in the center wells.

Following capture of the $^{14}CO_2$, the serological caps were removed from the flasks and the contents of the center wells transferred to glass scintillation vials by using one ml of methanol in 1/3 ml aliquots. Transfers were made with a (4½" long) Pasteur pipette. After adding 12 ml of Bray's scintillation fluid to each vial, they were capped, shaken vigorously, cooled to 5°C and counted by using a Packard Tri-Carb, Model 3365, liquid scintillation spectrometer equipped with automatic external standardization to determine the percent efficiency of counting. After calculating the dpm's from the percent efficiency and cpm's, the dpm's per 100 mg of tissue (wet weight) were determined and recorded for each incubation.

Experiment 5: The Effects of 2 and 8 Hours of Cryptorchidism Upon the Conversion of Pyruvate-2-^{14}C to $^{14}CO_2$ by Incubated Rat Testis

The objective of this experiment was to determine the early effects of hyperthermia induced by artificial cryptorchidism on the citric acid cycle independent of glycolysis in rat testis *in vitro*. This objective was accomplished by incubating testis tubules from the cryptorchid rats under appropriate conditions in a medium containing a specific isotopic substrate. The procedure involved incubating testis tubules in an appropriate medium containing pyruvate-2-^{14}C. Relative activity was assessed by capturing evolved $^{14}CO_2$, determining its radioactivity and then calculating dpm's per 100 mg of tissue (wet weight). Details of incubation and methods of chemical analysis are given in the following sections.

Experimental design and methods of statistical analysis were the same as for experiment 3.

Approximately 100 mg of teased testis tubules from cryptorchid rats were placed in the main chamber of ice-cold Warburg flasks prior to the addition of 3 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing pyruvate-2-^{14}C (0.15μCi; 16.7 mM). The flasks were gassed for 15 seconds with 95% O_2:5% CO_2, capped with serological caps, placed on a shaking water bath and allowed to incubate for one hour.

The methods of stopping the reactions, trapping evolved $^{14}CO_2$, counting and assessing metabolic activity of the testis tubules were the same as in Experiment 4 for glucose-U-^{14}C.

Experiment 6: The Effects of Artificial Cryptorchidism for 2 Hours on Some Metabolites and Cofactors of Glucose Metabolism in Rat Testes in vivo

The objectives of this experiment were: 1) measure in vivo the early effects of temperature on the concentration of intermediates of glucose metabolism in the testes; 2) measure in vivo changes in NADH and NADPH concentrations that may accompany changes in glucose metabolism; and 3) measure in vivo early change in ATP that may be associated with elevated temperature in the testes. These objectives were accomplished by quickly removing testes from cryptorchid rats, stopping further chemical reactions by rapidly freezing the testes with liquid nitrogen, powdering the frozen testes after removing extraneous tissues, extracting the frozen powder, and finally measuring the amount of each metabolite in the extracts and calculating its concentration per gram of testis (dry weight). The procedures for preparation of the extracts and the general procedures for measuring the amounts of metabolites in the extracts by spectrophotometric and spectrophotofluorometric methods have been described earlier tn this chapter. The specific details for the measurement of each metabolic will be given later in this section.

Experimental design and the methods of statistical analysis for the above experiment were the same as for Experiment 3.

Measurement of Fructose-6-phosphate. The procedures for measurement of this compound were those of Maitra and Estrabrook (102).

The reaction mixture (pH 7.4) for the assay contained in a final volume of 2 ml of the following components: 1.0 ml of acid extract, 0.65 ml of 0.1 M triethanolamine buffer (pH 7.4), 0.1 ml of 0.4 M KCl, 0.1 ml of 0.2 M $MgCl_2$, 0.1 ml of 0.04 M ATP (pH 7.0), 0.05 ml of 0.01 M NADP, and 10 microliters (.2 mg/ml) of glucose-6-phosphate dehydrogenase to act as a coupling enzyme. After making the initial reading on the metabilte fluorometer, 10 microliters of phosphoglucoisomerase (1 mg/ml) were added to determine fructose-6-phosphate. Then the enzyme blank and the internal standard were determined. Calculations for fructose-6-phosphate were based on the change in compensating voltage of the fluorometer brought about by the addition of one nanomole of glucose-6-phosphate. After the amount of fructose-6-phosphate was determined in one ml of the acid extract, the amount per gram of testis (dry weight) was determined by using grams of testis (wet weight) represented per ml of extract and the percentage dry weight.

Measurement of Fructose-1,6-diphosphate. The procedures for measurement of this compound were similar to the methods of Maitra and Extrabrook (102) for determination by fluorometry.

The reaction mixture (pH 7.4) for the assay contained in a final volume of 2 ml the following components: 0.5 ml of acid extract, 1.3 ml of 0.1 M triethanolamine buffer (pH 7.4), 0.1 ml of 0.4 M KCl, 0.05 ml of 0.08 M $MgCl_2$, 0.05 ml of 0.4 mM NADH and 10 microliters each of the enzymes, α-glycerophosphate dehydrogenase (1 mg/ml) and triose-phosphate isomerase (1 mg/ml) to act as coupling enzymes. After making the initial compensating voltage reading, fructose-1,6-diphosphate was determined in the extract by adding 10 microliters of aldolase

(2.5 mg/ml). After adding an equal additional amount of the aldolase enzyme to determine the enzyme blank, the actual change in compensating voltage due to the reactions involving fructose-1,6-diphosphate was determined. After adding to the cuvette 10 microliters of a standard solution containing one nanomole of fructose-1,6-diphosphate to determine the internal standard, the amount of fructose-1,6-diphosphate in the extract was determined using the change in compensating voltage for this metabolite and for the internal standard. Then by using the weight of testis tissue (wet weight) represented per 0.5 ml of extract and the percentage dry weight, the amount of fructose-1,6-diphosphate was calculated and expressed in terms of nanomoles per gram of testis (dry weight).

Measurement of Pyruvate and 2-Phosphoglyceric Acid. The procedures for the determination of these two compounds also used an adaptation of the fluorometric methods of Maitra and Estrabrook (102).

The reaction mixture (pH 7.4) for this assay contained in a final volume of 2 ml the following components: 0.5 ml of acid extract, 1.2 ml of 0.1 M triethanolamine buffer (pH 7.4), 0.1 ml of 0.4 M KCl, 0.1 ml of 0.2 M $MgCl_2$, 0.05 ml of 0.1 M ADP and 0.05 ml of 0.4 mM NADH. After making the initial reading of the compensating voltage, pyruvate was determined by the addition of 10 microliters of lactic dehydrogenase (2.5 mg/ml). Ten microliters of pyruvate kinase (2 mg/ml) were added as a coupling enzyme. After the metabolite fluorometer had stabilized, 10 microliters of enolase (10 mg/ml) were added to determine the 2-phosphoglyceric acid. Additional 10 microliter portions of each enzyme were added to the cuvettes to determine the enzyme blanks and one

nanomole (10 microliters of a 0.1 mM solution) of 2-phospoglycerate was added to determine the internal standard. Calculations for determining the amount of each metabolite in a gram of testis (dry weight) were based on the change in compensating voltage brought about by the internal standard, change in compensating voltage due to reactions involving that particular metabolite, grams of testis (wet weight) represented per 0.5 ml of acid extract and the percentage dry weight of the testes. Final results were thus expressed as nanomoles of metabolite per gram of testis (dry weight).

Determination of NADH and NADPH. Determination of NADH and NADPH was based on the fluorometric procedures of Williamson and Corkey (173). The reaction mixture (pH 7.4) consisted of 2 ml of the following components: 0.5 ml of alkaline extract, 1.49 ml of 0.1 M triethanolamine buffer (pH 7.4) and 0.01 ml of a substrate solution prepared by mixing 0.1 ml portions of 0.3 M pyruvate, 0.3 M α-ketoglutarate and 3.0 M $(NH_4)_2SO_4$. After mixing and warming the contents of the cuvettes, the initial compensating voltage reading was established. Relative amounts of NADH and NADPH in the cuvettes were then determined by adding 5 microliter portions of lactic dehydrogenase (0.2 mg/ml) and glutamic dehydrogenase (4 mg/ml) respectively. To determine the internal enzyme blanks additional 5 microliter portions of each enzyme were added to the cuvettes. Internal standardizaiton for NADH and NADPH was determined on cuvettes made up as the experimental cuvettes but containing an additional nanomole each of NADH and NADPH. Difference in the compensating voltage determinations between the two cuvettes for each compound being assayed were established as the change in compensating voltage due to

reactions involving one nanomole of NADH or NADPH. After determining the internal standard, this information along with the change in compensating voltage due to reactions in the experimental cuvettes, the grams of testis represented per 0.5 ml of alkaline extract and the percentage dry weights were used to calculate the amount of each compound in terms of nanomoles per gram of testis (dry weight).

Determination of α-Ketoglutaric Acid. The procedure for the determination of α-ketoglutaric acid was a modification of the spectrophotometric methods of Bergmeyer and Bernt (13). The modification was introduced to conserve acid extract and consisted of using a smaller volume of extract and a shorter (1 cm) light path.

The reaction mixture consisted of 1.5 ml of acid extract and 0.02 ml of NADH solution (1 mg/ml of 0.1 M TRA buffer; pH 8.2) in a quartz cuvette. The blank was glass distilled water. After placing the cuvettes in a Hitachi Perkin-Elmer Coleman, Model 124, double beam spectrophotometer and making the initial reading at 340 mm, 10 microliters of glutamic dehydrogenase (20 mg/ml) were added to the experimental cuvette. The reaction was followed by the change in optical density brought about by the reduction in NADH concentration. After the reaction was completed, the change in optical density due to the oxidation of NADH was used to calculate the amount of α-ketoglutaric acid present in the cuvette on the basis of a molar extinction coefficient of 6.22×10^3 for NADH. The calculated amount of α-ketoglutaric acid present in 1.5 ml of acid extract, the amount of testis (wet weight) represented by 1.5 ml of acid extract and the percentage dry weight of the testis were used to calculate micromoles of α-ketoglutaric acid per gram of testis (dry weight).

Determination of Malic Acid and Lactic Acid. The methods employed to measure these two compounds were the same as described by Hohorst (76) with the exception that larger volumes were used. The reaction mixture was made up to 3 ml in a quartz cuvette (1 cm light path) by the following additions: 0.5 ml of acid extract, 10. ml glass distilled water, 1.35 ml of hydrazine-glycine buffer (pH 9.5) and 0.15 ml of NAD solution (80 mg/ml). Measurements were based on changes in optical density due to reduction of NAD which was followed at 340 mm with a Zeiss, Model M4 AIII, spectrophotometer. After zeroing the instrument with a blank consisting of 3 ml of buffer, malic dehydrogenase (5 mg/ml) and lactic dehydrogenase were added sequentially to determine amounts of malate and lactic acid, respectively, in the acid extract. After the addition of each enzyme, the reactions involving it were allowed to go to completion before adding another enzyme. The amount of each compound in the cuvette was caluclated on the basis of a molar extinction coefficient of 6.22×10^3 for NADH and the amount of change in optical density ascribed to that compound. Then using these values, the amount of testis tissue represented by 0.5 ml of extract and the percentage dry weight, the amount of each compound was calculated in terms of micromoles per gram of testis (dry weight).

Determination of ATP. The methods for analysis of ATP were an adaptation of the methods of Addanki et al. (1). This method allows the rapid determination of picamole quantities of ATP by using a liquid scintillation counter to measure the intensity of luminescence produced when extract from the firefly (Photinus pyralis) is added to a solution containing ATP. Firefly lantern extract (FLE) (Luciferin-Luciferase,

Sigma FLE-50) was prepared according to the instructions of the manufacturer. Then it was filtered in a temperature-controlled room at $4^{o}C$ and diluted to 10 ml with buffer (0.05 M sodium arsenate, 0.02 M $MgSO_4$; pH 7.4). Standard ATP solutions were prepared from a 0.1 M sodium arsenate buffer (pH 7.4) and an aqueous ATP solution which had been standardized by measuring the absorption at 259 mm with a Carey, Model 15, recording spectrophotometer and then calculating its concentration from the known extinction coefficient (15.5×10^3) of ATP at the wavelength. A Packard Tri-Carb liquid scintillation spectrometer, Model 3365, was employed to measure the luminescence rate (LR) at optimum tritium settings (gain 52%, discriminator 50-1000) and one second repeat counting. Standards were prepared by pipetting 1.7 ml of glass distilled water and 0.1 ml of 0.1 M arsenate buffer containing known amounts (10-50 picamoles) of ATP to glass counting vials. After mixing the contents of the vials they were placed in the scintillation counter to cool. The procedure followed in making the counts was: vigorously inject 0.2 ml of FLE into the uncapped vials just before they descended into the counting chamber. After obtaining the first one-second count (11 seconds after FLE addition) at least two subsequent counts were obtained to insure that the luminescence rate was in a rapid decay stage. By using the counts per second (cps) of the initial reading for each of the ATP standards, a standard curve was constructed with counts per second (cps) versus picamoles of ATP along the Y axis and X axis respectively. After making adequate dilutions of acid extracts with 0.1 M sodium arsenate (pH 7.4) 0.1 ml aliquots of these dilutions were assayed as were the standards. The amount of ATP per ml of each acid extract was calculated using the cps for the diluted acid extract, the standard

curve and the dilution factor. Then by using the grams of testis tissue (wet weight) represented by one ml of acid extract and the percentage dry weight, the ATP concentration was calculated and expressed as micromoles per gram of testis (dry weight).

CHAPTER IV

RESULTS

Review of the literature reveals that raising the temperature of the mammalian testes by artificial cryptorchidism leads to infertility. Most attempts to elucidate the biochemical mechanism (s) have centered on anabolic and catabolic changes observed *in vitro* well after the beginning of histological deterioration of the seminiferous tubules. The primary objective of this investigation was to determine early changes in testicular metabolism, thus defining biochemical correlates preceding germ cell degradation due to heat.

A series of six experiments was designed to accomplish this objective. The first experiment was to determine the effect of translocation of rat testes into the abdominal cavity for 2, 4, 8, 16, 32, 64, and 128 hours on the incorporation of lysine-U-^{14}C into TCA precipitable material by rat testis *in vitro*. The results were used to predict possible early changes in protein synthesis by cryptorchid testes.

The second experiment was designed to investigate the effect of experimental cryptorchidism for 2, 4, 8, 16, 32, 64, and 128 hours on the incorporation of acetate-1-^{14}C into several lipid classes by rat testis *in vitro*. The results were interpreted as indicative of early changes of *de novo* lipid synthesis induced by abdominal temperature and thus related to changes in the accumulation of lipid in cryptorchid testes.

The third experiment was to determine if cryptorchidism for 2 and 8 hours was accompanied by changes in phosphorylation of 2-deoxyglucose by testis *in vitro*. The results were used to indicate possible changes in glucose transport which might account for changes in glucose metabolism.

The fourth experiment was designed to detect changes induced by abdominal temperatures for 2 and 8 hours on conversion of glucose-U-^{14}C to $^{14}CO_2$ by rat testicular tissue *in vitro*. The results were used to relate possible changes in energy metabolism involving glucose to changes in glucose transport and to changes in the biosynthesis of protein and lipid.

The fifth experiment was to determine the effects of artificial cryptorchidism for 2 and 8 hours on the conversion of pyruvate-2-^{14}C to $^{14}CO_2$ by rat testis *in vitro*. The observations of this investigation were used to assess the effects of cryptorchidism on pyruvate dehydrogenase and enzymes of the citric acid cycle independent of the glycolytic and pentose shunt pathways.

The sixth experiment was designed to determine the effects of abdominal temperature for 2 hours on the concentrations of ATP, NADH, NADPH, lactate, fructose-6-phosphate, fructose-1,6-diphosphate, 2-phosphoglyceric acid, pyruvate, α-ketoglutarate and malate in rat testes *in vivo*. The results of this experiment were used to assess possible heat induced shifts in reactions important in energy metabolism.

Preliminary Experiment: Effects of Abdominal Temperature on the Weight of Rat Testes

Artificial cryptorchidism is accompanied by a reduction in testicular weight (115,125,149,160). To assure that the method employed in producing artificial cryptorchidism was an effective means of heat treatment, the testes from animals in the first series of experiments were weighed. The results (Table III, Appendix C) are shown in Figure 1. Placing testes in the abdomen for 64 hours did not result in a significant change in testis weight. However, between 64 and 128 hours, testis weight declined significantly ($p<0.05$) from an average of 2.86 to 2.05 grams. This observed decrease in testis weight indicated that the method employed in rendering the rats cryptorchid was causing degeneration of the germinal epithelium in the testes.

Preliminary Experiment: Effect of Sham Operations on Testis

Sham operations were conducted to investigate the possibility that surgical stress might introduce changes in testis metabolism *in vivo*. Testes of the sham operated animals were analyzed for metabolites in the same fashion as cryptorchid testes. Analysis of variance indicated that there were no significant differences ($p>0.10$) in the concentrations of any of the metabolites. This observation assured that differences observed among treatment groups were due to the effect of heat. In addition, these results were interpreted to mean that testes of the two-hour sham-operated rats served as a valid control for this series of experiments.

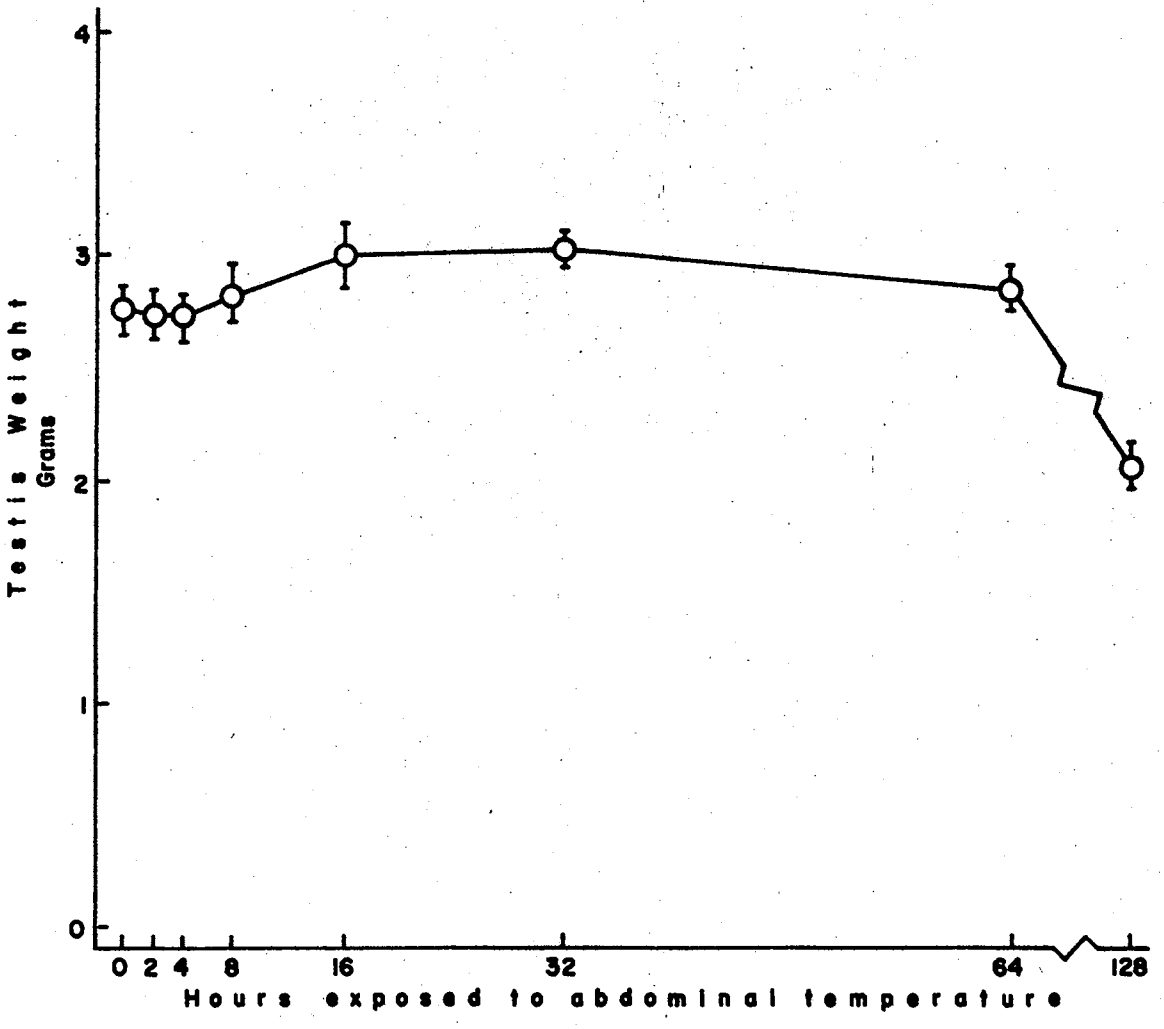

Figure 1. Effect of Abdominal Temperature on Testis Weight.

Experiment 1: Effects of Artificial Cryptorchidism on Incorporation of Lysine-U-^{14}C Into TCA Precipitable Material by Rat Testis

Results of this experiment (Table IV, Appendix C) are shown in Figure 2. In the absence of glucose, the incorporation of lysine-U-^{14}C into TCA precipitable material declined significantly ($p<0.01$) from the control value at 2 hours of artificial cryptorchidism. The rate of lysine-U-^{14}C incorporation remained essentially the same through the 16th hour of artificial cryptorchidism, but then began to increase. At 128 hours incorporation of lysine-U-^{14}C was significantly ($p<0.01$) greater than scrotal testes.

As seen in Figure 2, lysine-U-^{14}C incorporation into TCA precipitable material by testis tissue in vitro in the presence of exogenous glucose (10 mM) was greatly enhanced over that observed in the absence of glucose. This observation was in agreement with the finding of Davis (35) and Means and Hall (108) in the rat. The reduction in lysine-U-^{14}C incorporation in the presence of exogenous glucose was significant ($p< 0.01$) from 4 through 128 hours of experimental cryptorchidism.

Results of this experiment indicated that translocating the testes into the abdominal cavity rapidly reduces the incorporation of lysine-U-^{14}C into TCA precipitable material by rat testis in vitro within 2 hours in the absence of exogenous glucose and within 4 hours in the presence of exogenous glucose (10 mM).

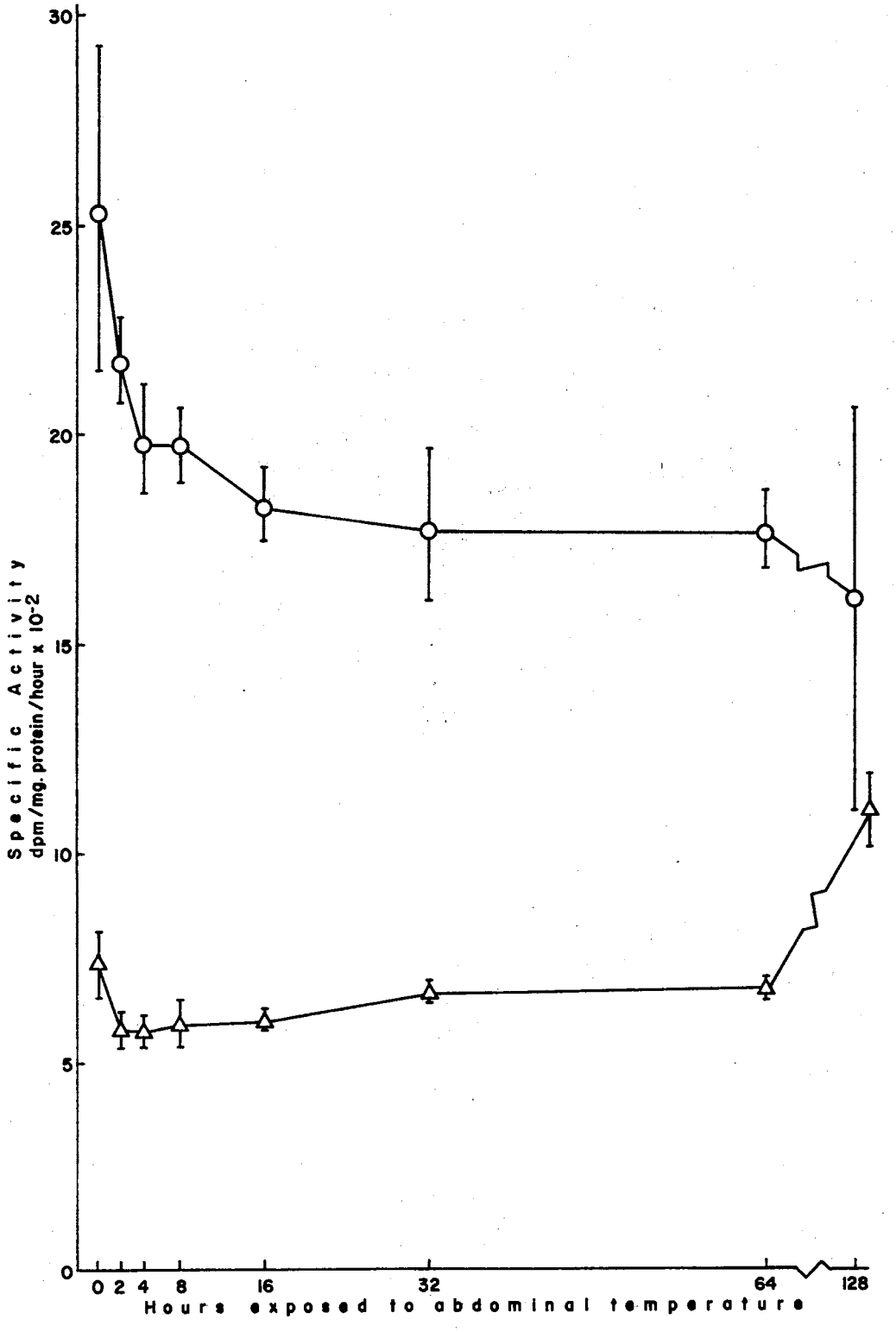

Figure 2. Effect of Abdominal Temperature on the Incorporation of Lysine-U-^{14}C Into TCA Precipitable Material by Cryptorchid Testis Tissue in the (O)Presence and (Δ)Absence of Glucose.

Experiment 2: Effects of Artificial Cryptorchidism on the Incorporation of Acetate-1-^{14}C Into Lipid by Incubated Rat Testis

Results of this experiment (Table V, Appendix C) are shown in Figures 3-10. Total testicular lipid synthesis from acetate-1-^{14}C in the presence and absence of glucose declined significantly ($p<0.01$) from scrotal testes within 2 hours of confinement to the abdomen. Additional significant ($p<0.01$) decreases in total lipid synthesis in the presence of glucose occurred at 16, 32, and 64 hours. In contrast, no additional decreases in total lipid synthesis occurred in tissues incubated in the absence of glucose. This trend appeared to prevail for most of the classes of lipid investigated.

Results of Experiment 2 indicated an even greater stimulation of *in vitro* lipid synthesis (5-10 fold) by glucose than was observed for protein synthesis (1.5-3.5 fold) and like protein synthesis, stimulation of lipid synthesis by glucose in the culture media lessened with time of cryptorchidism.

In summary lipid synthesis was greatly enhanced by glucose in the culture media. Total testicular lipid synthesis *in vitro* declined significantly ($p<0.01$) within the first two hours of artificial cryptorchidism. Additional reductions were noted with increasing periods of cryptorchidism up to 128 hours. Cholesterol esters, which have been shown to accumulate in cryptorchid testes (30,81,127) exhibited the slowest rate of *de novo* synthesis from acetate-1-^{14}C among the lipid classes investigated.

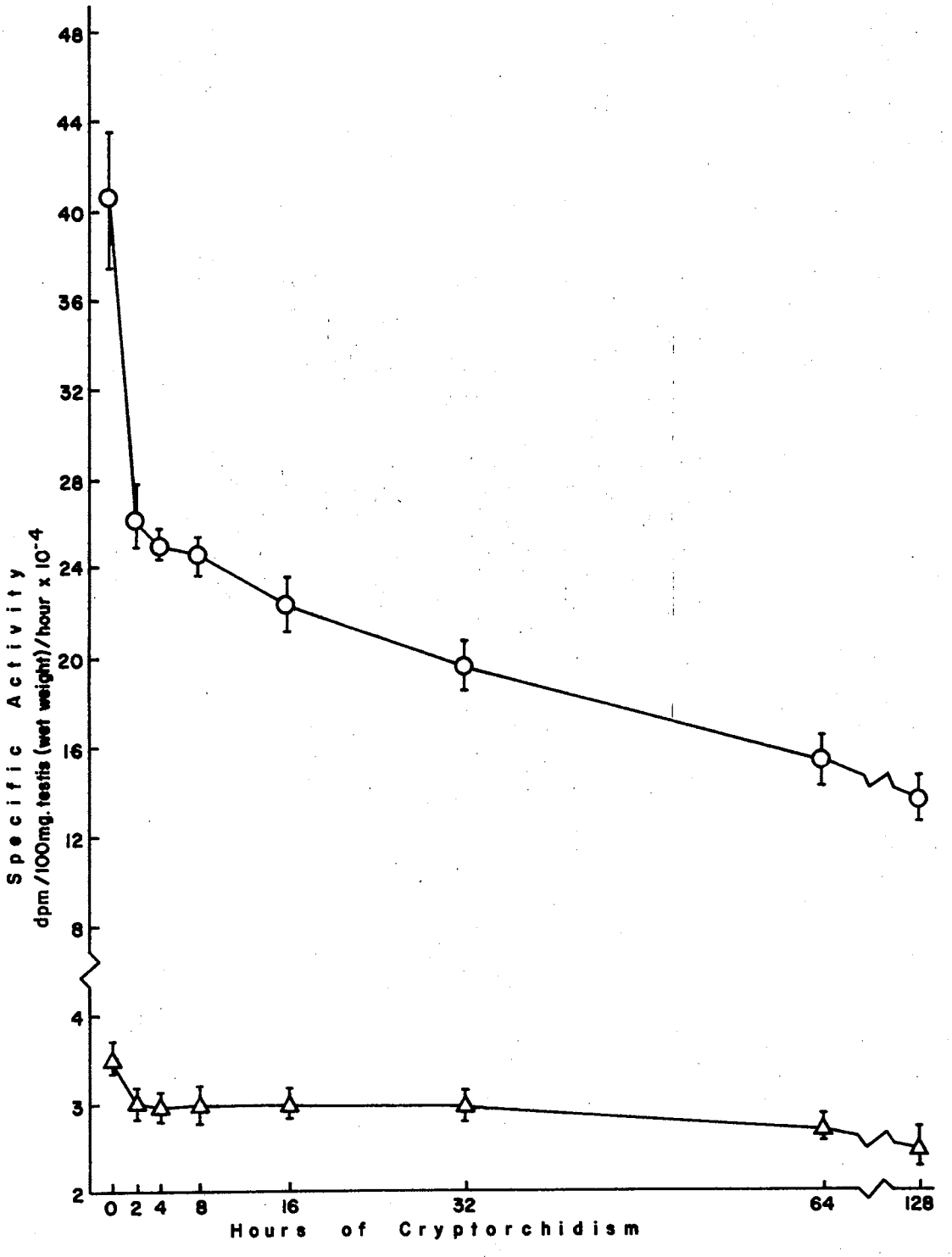

Figure 3. Incorporation of Acetate-1-^{14}C Into Total Lipid by Cryptorchid Testis Tissue <u>in vitro</u> in the (O)Presence and (Δ)Absence of Glucose.

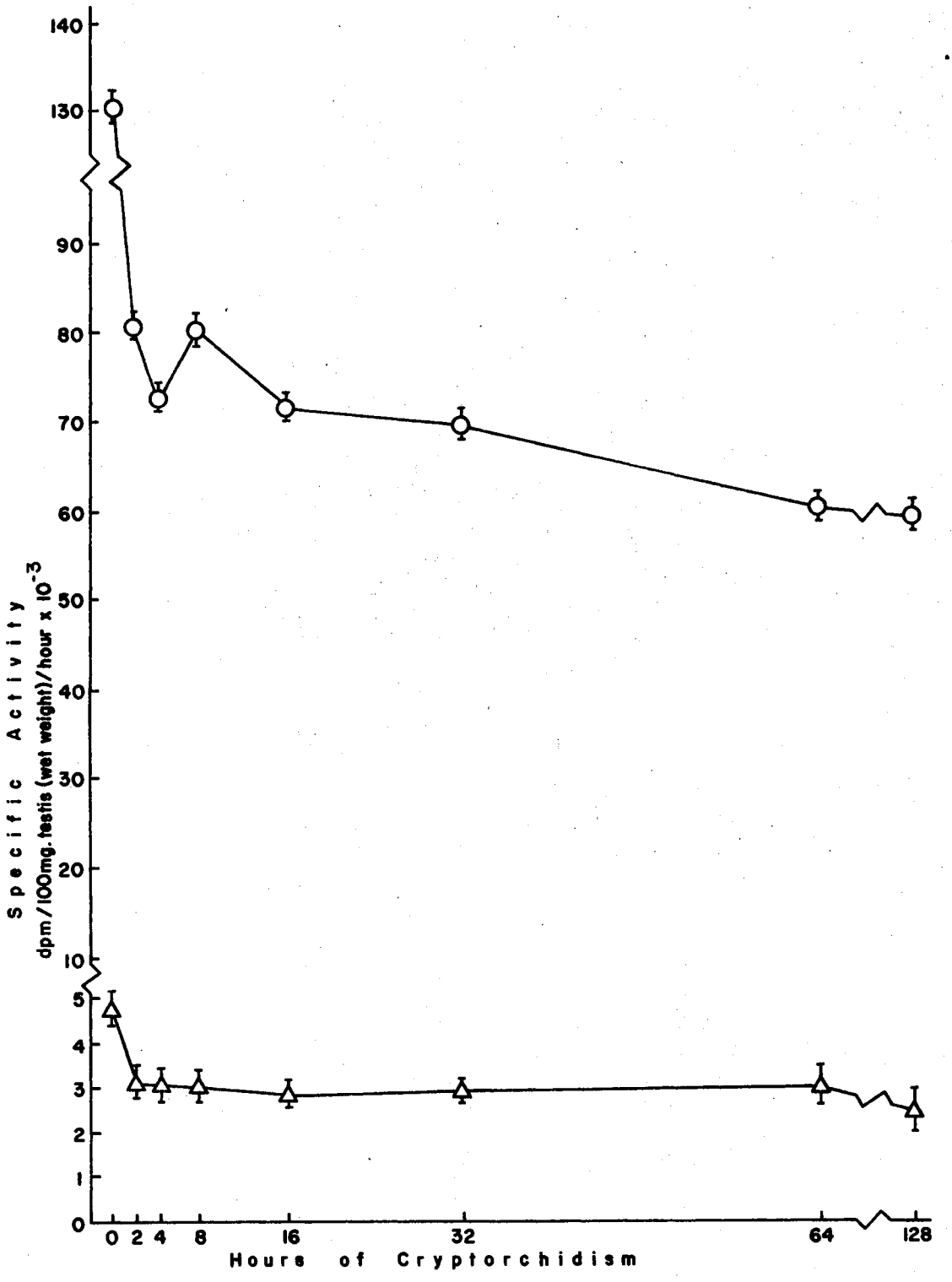

Figure 4. Incorporation of Acetate-1-^{14}C Into Triglycerides by Cryptorchid Testis Tissue <u>in vitro</u> in the (O)Presence and (Δ)Absence of Glucose.

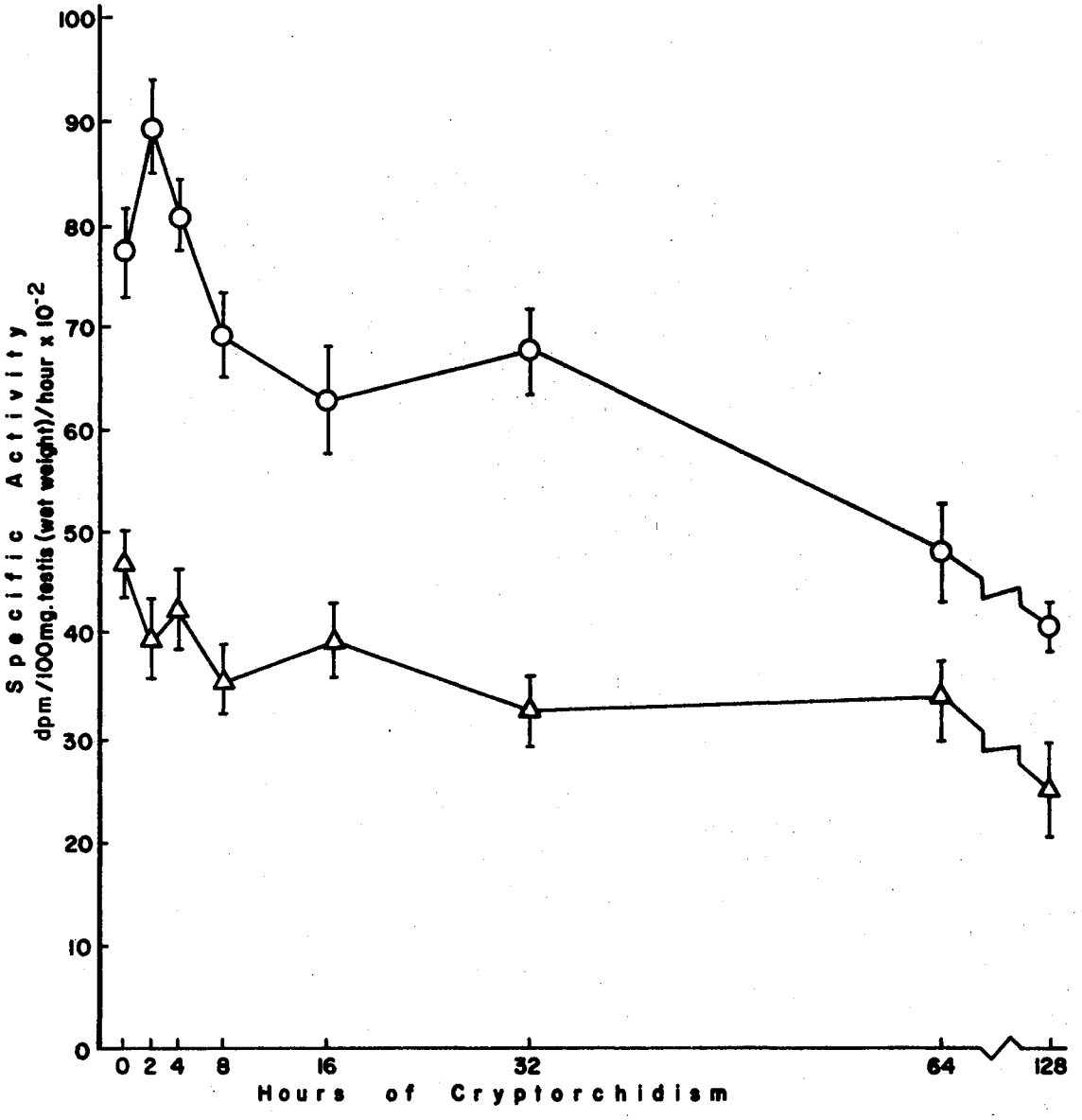

Figure 5. Incorporation of Acetate-1-^{14}C Into Diglycerides by Cryptorchid Testis Tissue *in vitro* in the (O)Presence and (Δ)Absence of Glucose.

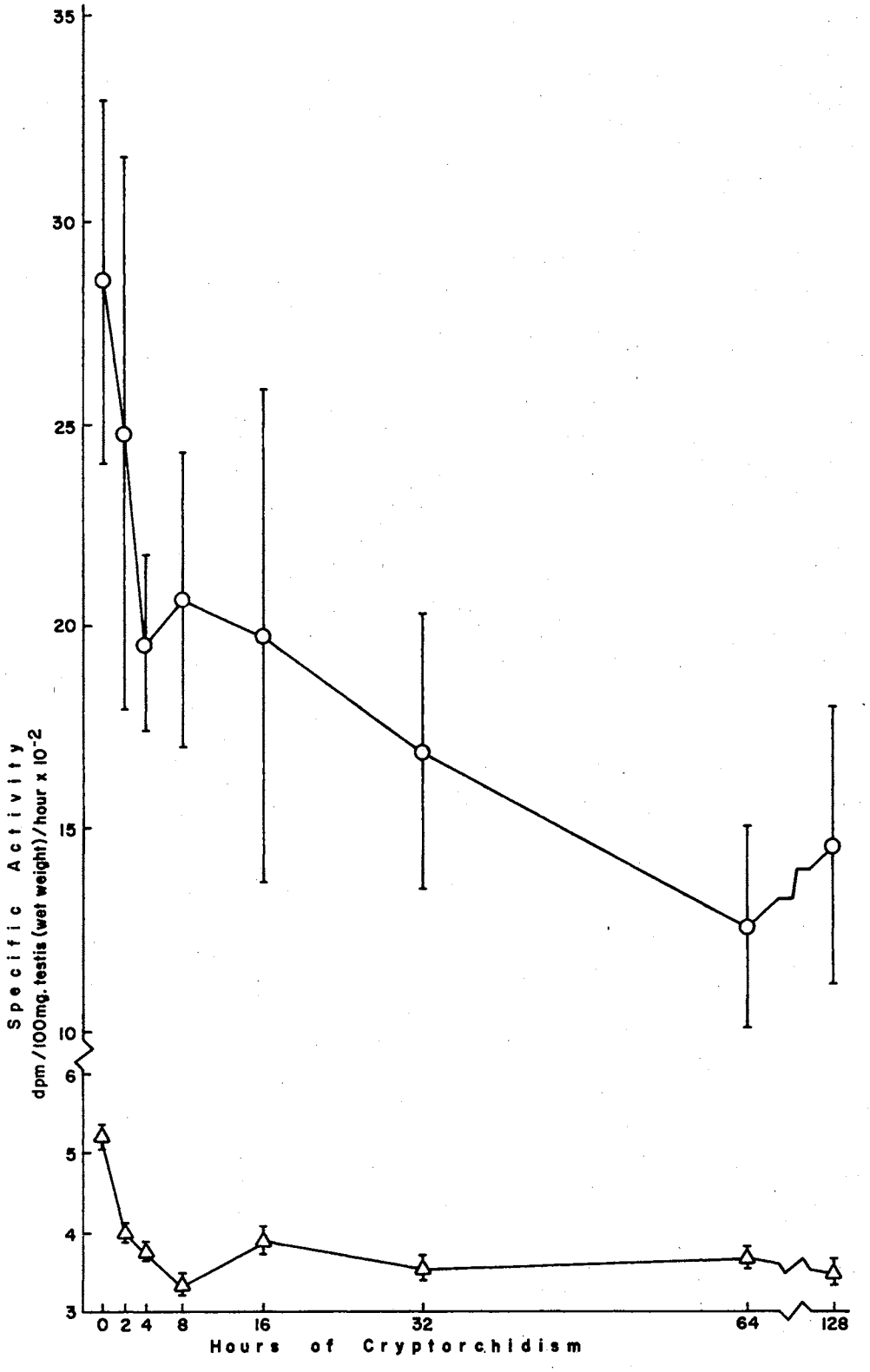

Figure 6. Incorporation of Acetate-1-^{14}C Into Monoglycerides by Cryptorchid Testis Tissue in vitro in the (O)Presence and (Δ)Absence of Glucose.

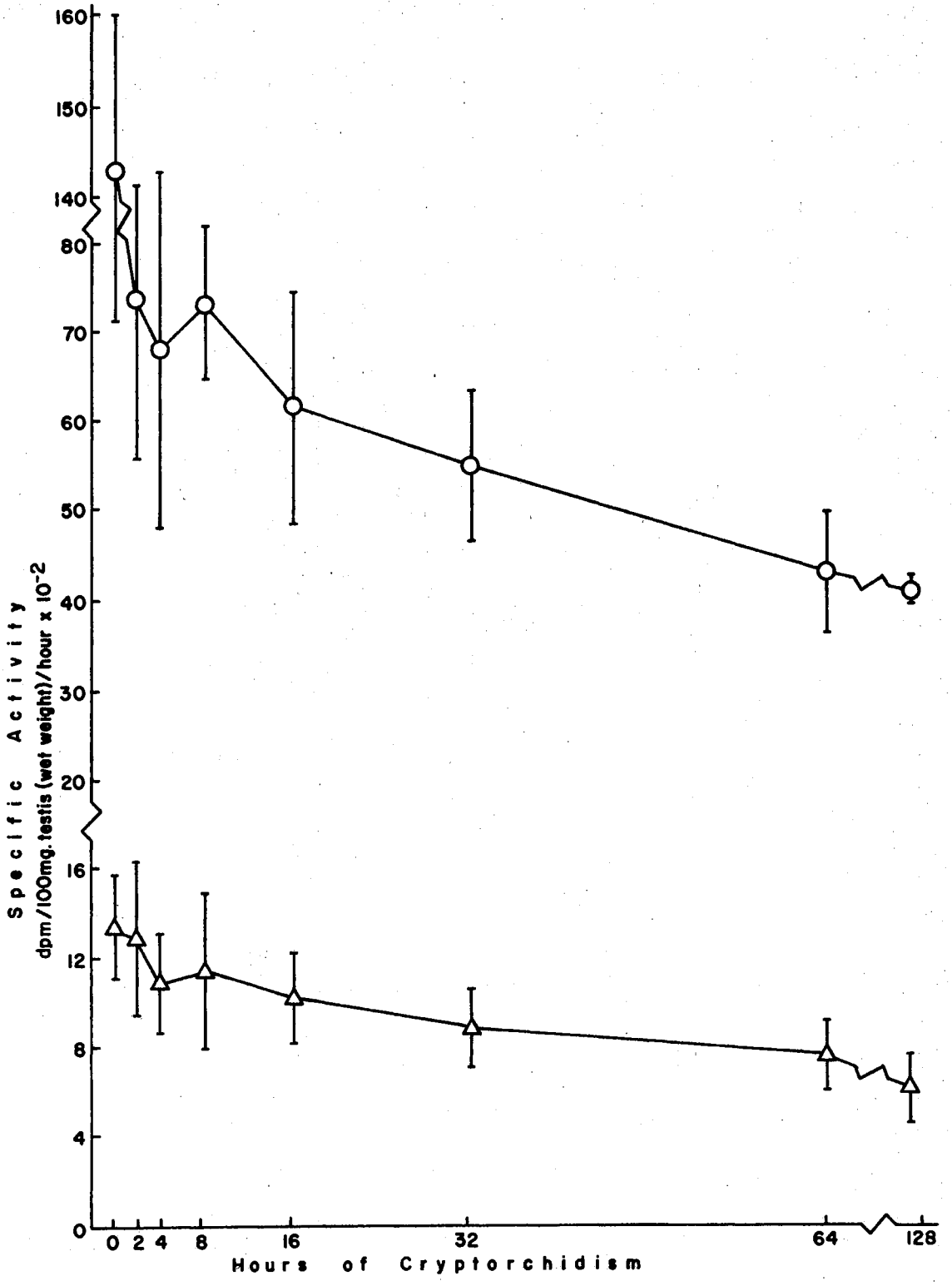

Figure 7. Incorporation of Acetate-1-^{14}C Into Sterols by Cryptorchid Testis Tissue <u>in vitro</u> in the (O)Presence and (Δ)Absence of Glucose.

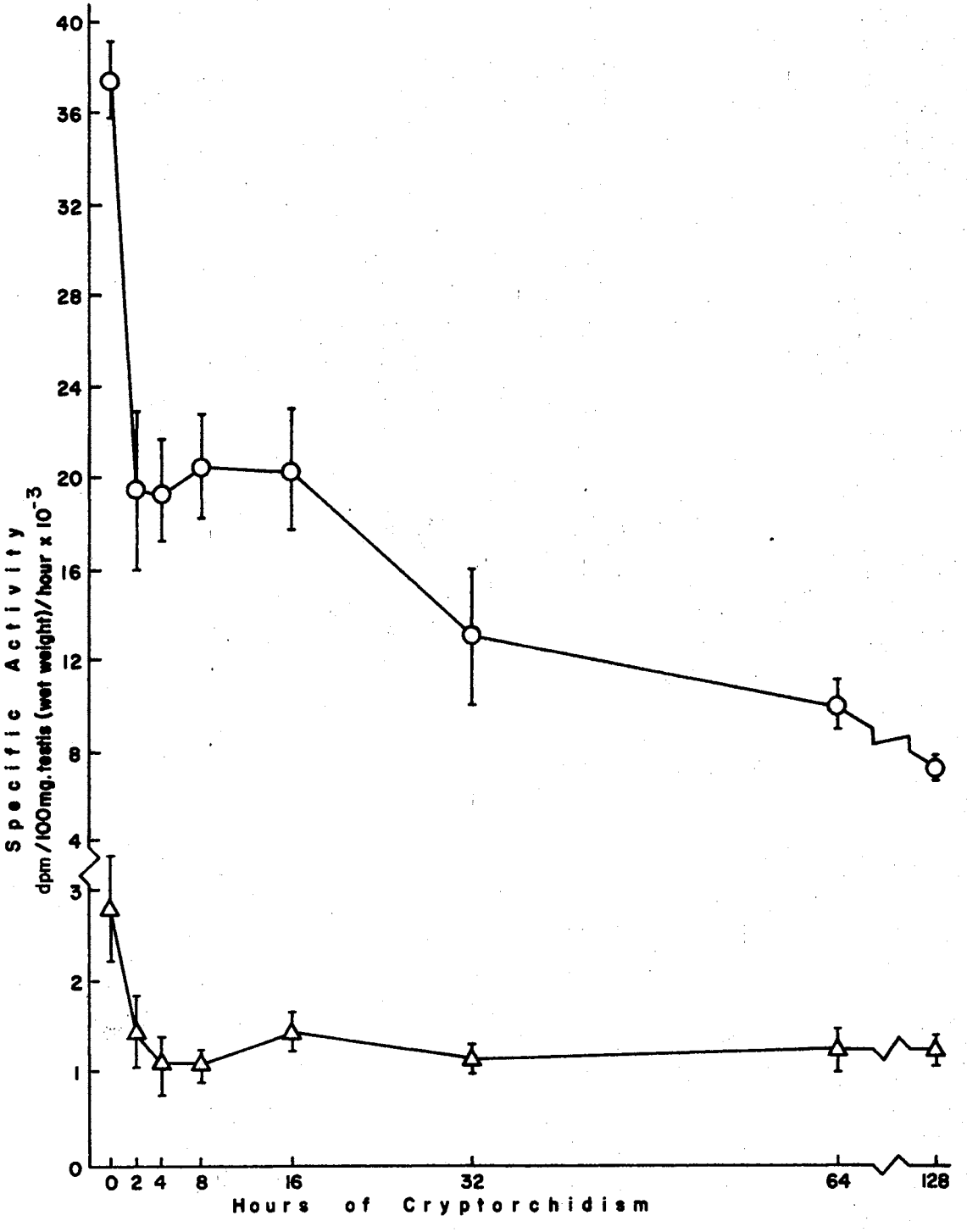

Figure 8. Incorporation of Acetate-1-^{14}C Into Sterol Esters by Cryptorchid Testis Tissue *in vitro* in the (O)Presence and (Δ)Absence of Glucose.

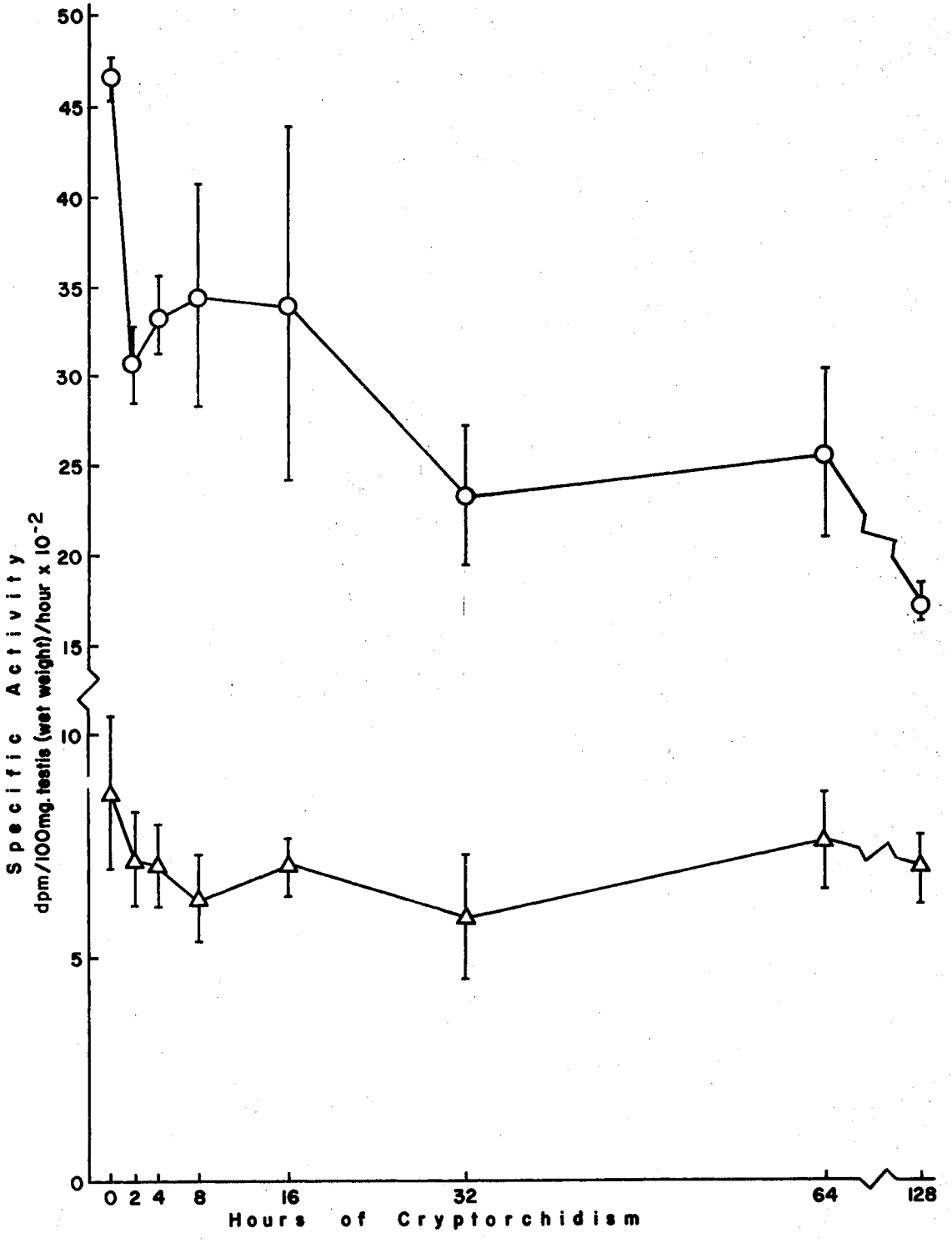

Figure 9. Incorporation of Acetate-1-^{14}C Into Phospholipids by Cryptorchid Testis Tissue <u>in vitro</u> in the (O)Presence and (Δ)Absence of Glucose.

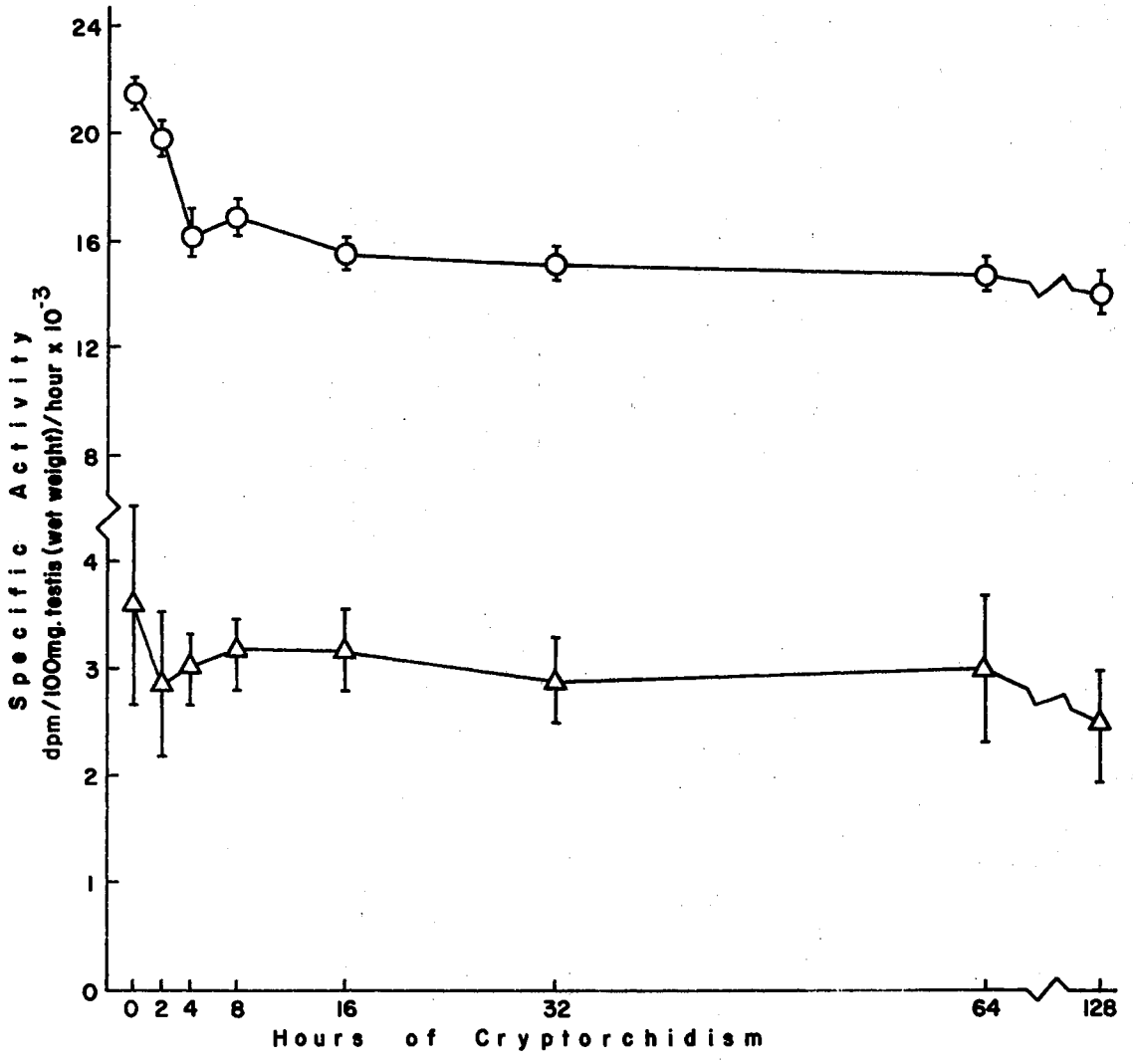

Figure 10. Incorporation of Acetate-1-^{14}C Into Non Volatile Fatty Acids by Cryptorchid Testis Tissue *in vitro* in the (O)Presence and (Δ)Absence of Glucose.

Experiment 3: The Effects of Cryptorchidism for 2 and 8 Hours Upon Glucose Transport by Rat Testis *in vitro*

This experiment was designed to determine if decreased protein and lipid synthesis in cryptorchid testis might be caused by concomitant decreases in glucose transport. Glucose transport was measured during incubation *in vitro* by phosphorylation of the non-utilizable sugar, 2-deoxyglucose-1-^{14}C. This process appears to be analogous to transport and the phosphorylated compound is not metabolized further, and therefore, its recovery from tissues served to measure glucose transport quantitatively (150).

Results of this experiment (Table VI, Appendix C) are shown in Figure 11. Glucose transport as indicated by the phosphorylation of 2-deoxyglucose-1-^{14}C showed almost no change by eight hours of cryptorchidism. Consequently, glucose transport did not appear to be responsible for any change in glucose dependent biosynthesis of protein and lipid.

Experiment 4: Effects of Artificial Cryptorchidism on the Conversion of Glucose-U-^{14}C into $^{14}CO_2$ by Incubated Rat Testis

This experiment was designed to determine if impaired cellular respiration involving glucose catabolism to CO_2 might account for the observed decrease in glucose dependent synthesis of protein and lipid by cryptorchid testis. The results (Table VII, Appendix C) are shown in Figure 12. Conversion of glucose to CO_2 by cryptorchid testis was not significantly different ($p>0.10$) from testis of sham operated animals

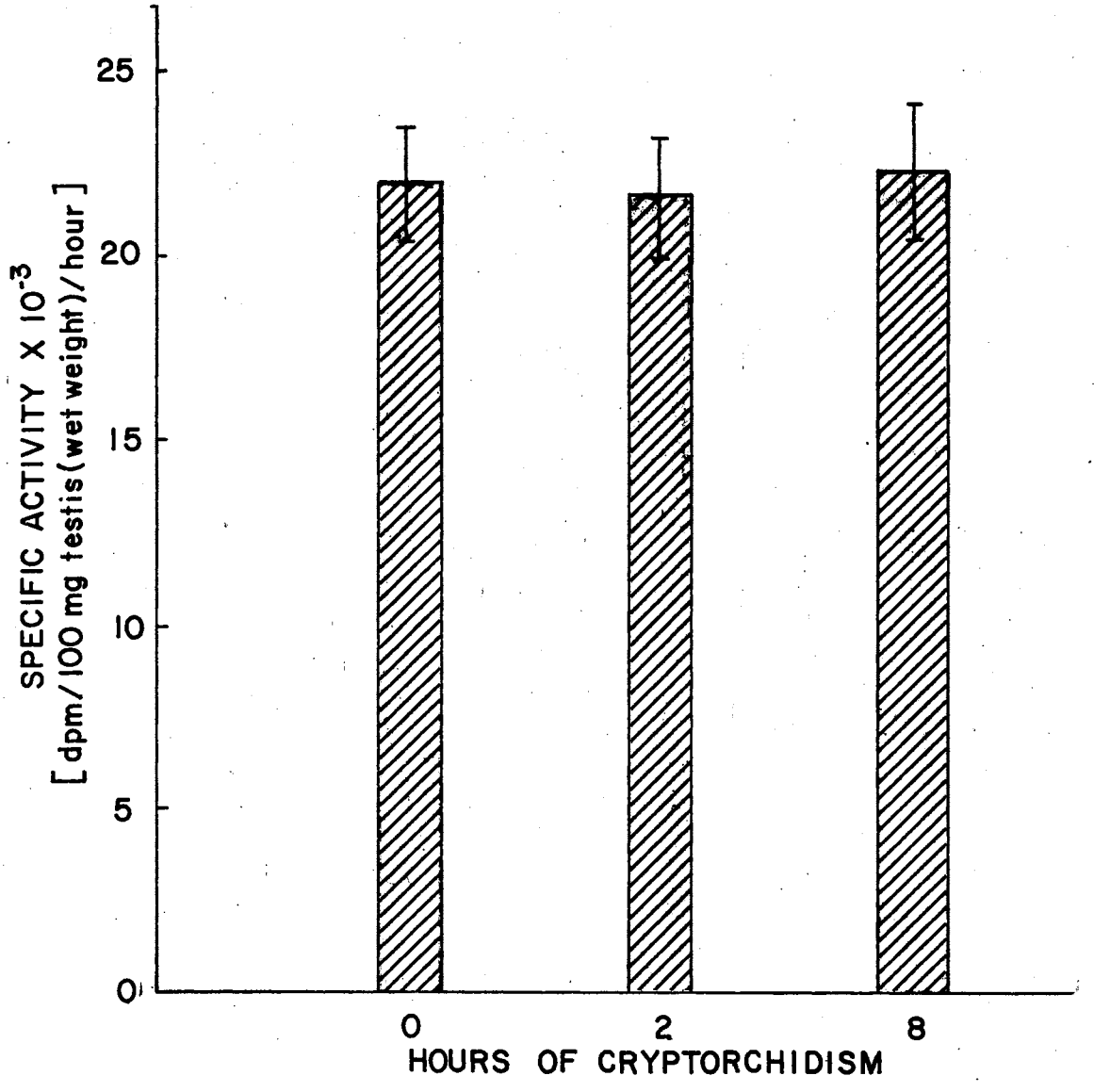

Figure 11. Glucose Transport by Cryptorchid Testis Tissue as Measured by the Phosphorylation of 2-Deoxyglucose-1-^{14}C *in vitro*.

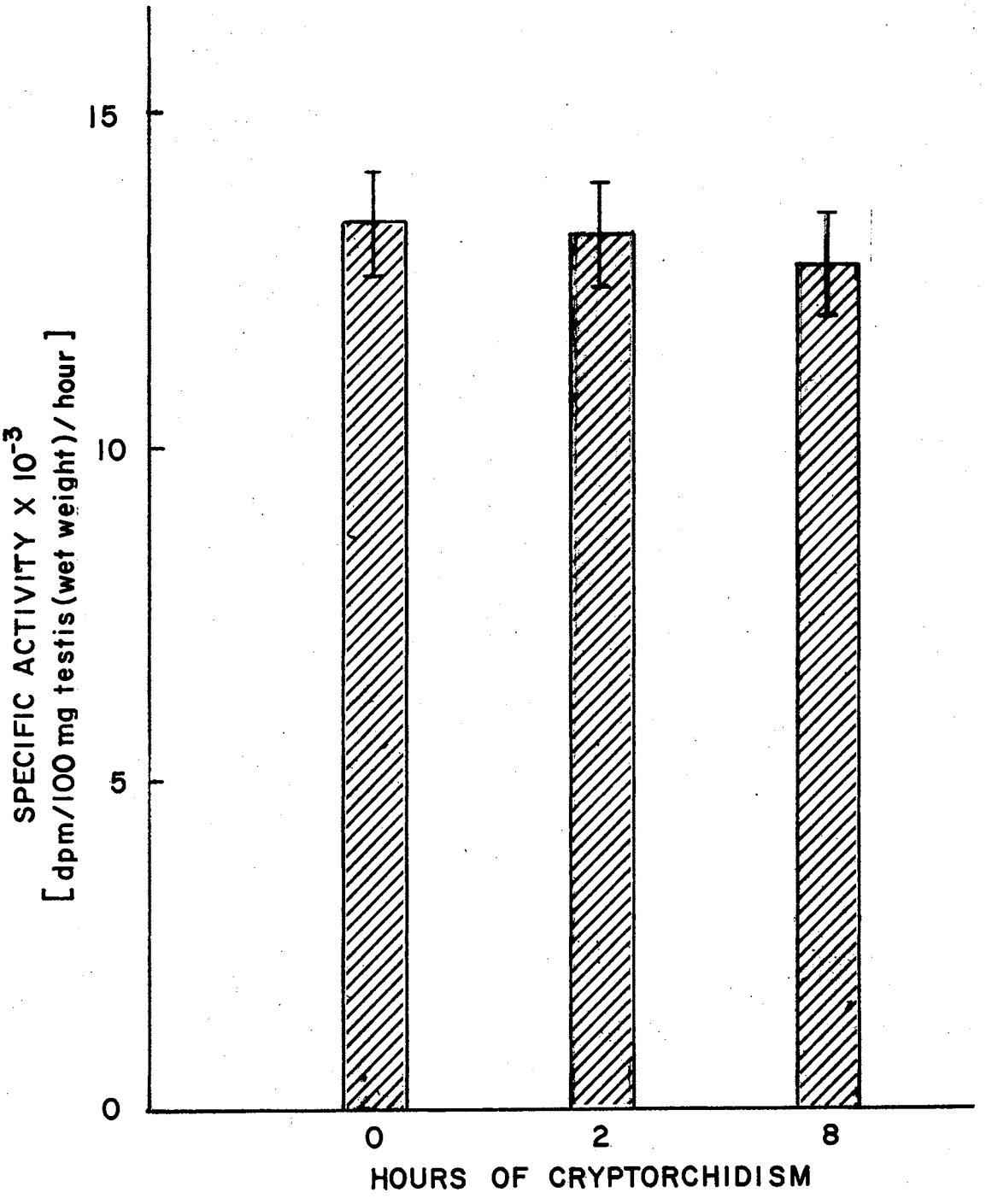

Figure 12. The Conversion of Glucose-U-^{14}C Into $^{14}CO_2$ by Cryptorchid Testis Tissue in vitro.

2 and 8 hours after experimental cryptorchidism. These decreases were far short of the 45% reduction in CO_2 formation from glucose observed by Hollinger and Davis (77) in 30-day cryptorchid testes of the rat. The rate of conversion of glucose to CO_2 by rat testis in vitro at 2 and 8 hours of cryptorchidism decreased much less than the observed biosynthesis of protein and lipid in similar tissues. Consequently, changes in testicular energy metabolism involving glucose conversion to CO_2 did not account for the decreased biosynthesis of protein and lipid materials observed in cryptorchid rat testis in vitro.

Experiment 5: The Effects of 2 and 8 Hours of Cryptorchidism Upon the Conversion of Pyruvate-2-^{14}C to $^{14}CO_2$ by Incubated Rat Testis

The purpose of this experiment was to determine if cryptorchidism preferentially affected activity of pyruvate dehydrogenase and the enzymes of the citric acid cycle. This was done by measuring the amount of $^{14}CO_2$ evolved from incubations of testis tissue in the presence of pyruvate-2-^{14}C.

Results (Table VIII, Appendix C) of this experiment are shown in Figure 13. There was no significant difference ($p>0.10$) in oxidation of pyruvate-2-^{14}C to $^{14}CO_2$ between the sham-operated control and cryptorchid testis. This suggests that testicular energy metabolism involving pyruvate was not materially affected by short intervals (8 hours) of cryptorchidism.

These experiments involving glucose catabolism and transport suggested that heat induced changes in these aspects of testicular

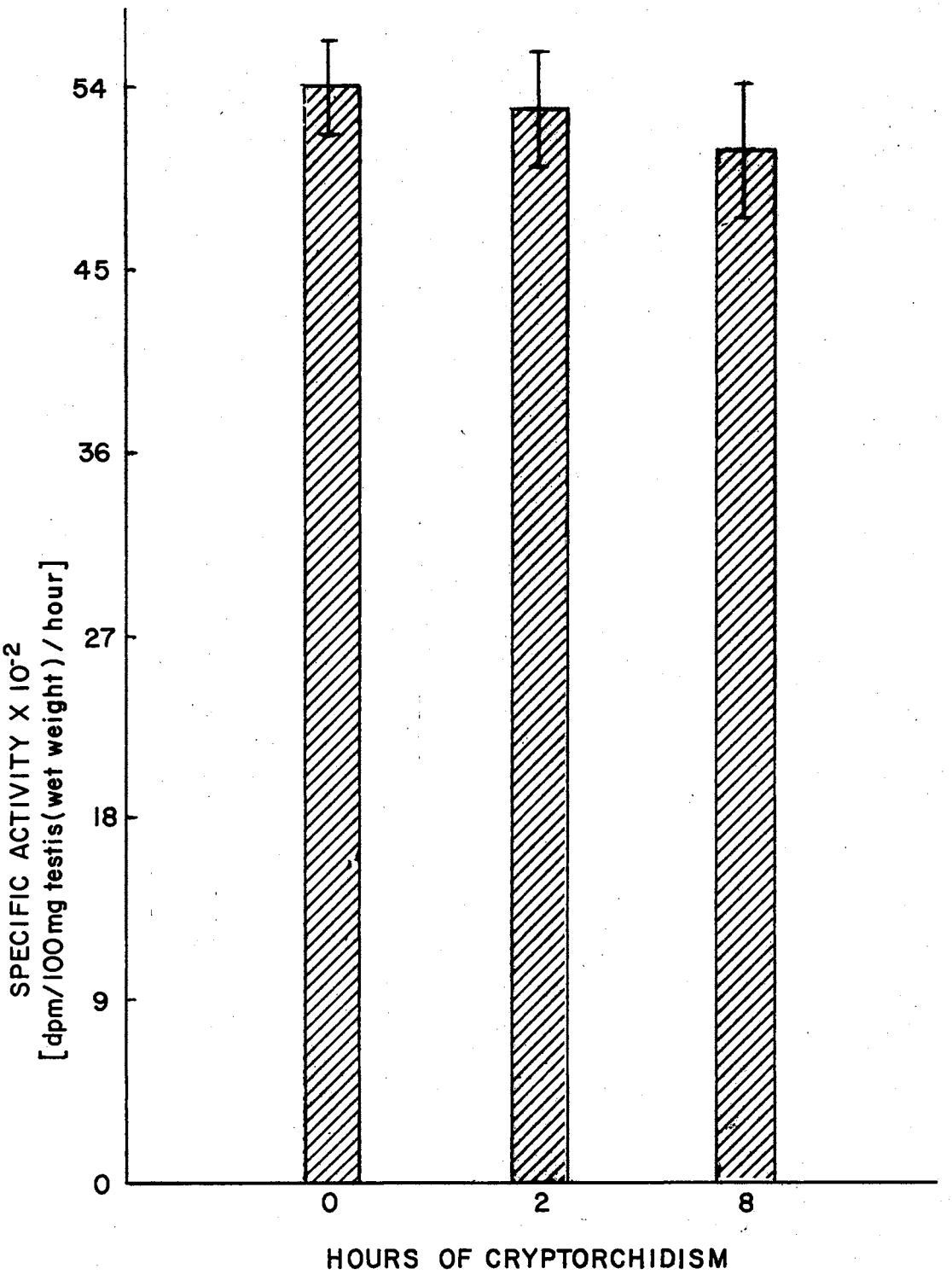

Figure 13. The Effects of Artificial Cryptorchidism on the Conversion of Pyruvate-2-^{14}C to $^{14}CO_2$ by Teased Testis Tissue *in vitro*.

metabolism were not implicated in decreased biosynthesis of protein and lipid materials in cryptorchid testes. However, these experiments were gross determinations of total metabolism and did not differentiate metabolic activity among tissue compartments in testis. It was possible that glucose-U-^{14}C and pyruvate-2-^{14}C were sequestered in tissue compartments not affected adversely by heat. Cells in these compartments may have expressed increased conversion of glucose and pyruvate to CO_2 and masked a reduction in the oxidation of these compounds in other compartments. Furthermore, the lack of correlation between total testicular tissue glucose oxidation and biosynthesis of lipid and protein give additional credence to this hypothesis. This view would resolve the dilemma between biosynthesis and total glucose oxidation, particularly, if the compartment where lipid and protein synthesis took place were a compartment that had experienced reduced glucose oxidation. Histological organization of the testes (17) suggest that the seminiferous tubules, the compartment where most protein and lipid synthesis occurs (35), is the one most likely to experience difficulty in obtaining adequate amounts of glucose.

Experiment 6: The Effects of Artificial Cryptorchidism for 2 Hours on Some Metabolites and Cofactors of Glucose Metabolism in Rat Testes *in vivo*

The observation that lipid and protein biosynthesis in rat testis had been adversely affected by 2 hours of artificial cryptorchidism led to the decision to investigate some testicular glucose metabolite concentrations *in vivo* at this interval of cryptorchidism. It was

conceivable that changes in glucose metabolite concentrations should have been concomitant with these perturbations of lipid or protein biosynthetic ability. Decline in oxidation of glucose and pyruvate to CO_2 *in vitro* was insignificant in cryptorchid testis and did not appear to account for decreased protein and lipid biosynthesis observed in this tissue. However, *in vitro* incubation experiments may not have reflected *in vivo* testicular conditions. Consequently this experiment was designed to measure *in vivo* some of the metabolites and cofactors of glucose oxidation, namely: fructose-6-phosphate, fructose-1,6-diposphate, 2-phosphoglyceric acid, NADPH, α-ketoglutaric acid, malic acid, lactic acid, ATP, and NADH.

Effects of Cryptorchidism Upon Testicular Hexoses

This part of Experiment 6 involved the measurement of fructose-6-phosphate and fructose-1,6-diphosphate concentrations in cryptorchid rat testis *in vivo*. Evaluation of these compounds (Table IX, Appendix C) are shown in Figure 14. These slight increases of fructose-6-phosphate (4%) and fructose-1,6-diphosphate (1%) concentrations were not significantly ($p>0.10$) different from controls. These concentrations at 2 hours of cryptorchidism suggested no impairment in the activity of phosphofructokinase, the principal regulatory enzyme of glycolysis. This observation did not necessarily conflict with the observation of Ewing and Schanbacher (49) who did not find a significant ($p<0.05$) decrease in activity of this enzyme until 8 hours of cryptorchidism in the rat.

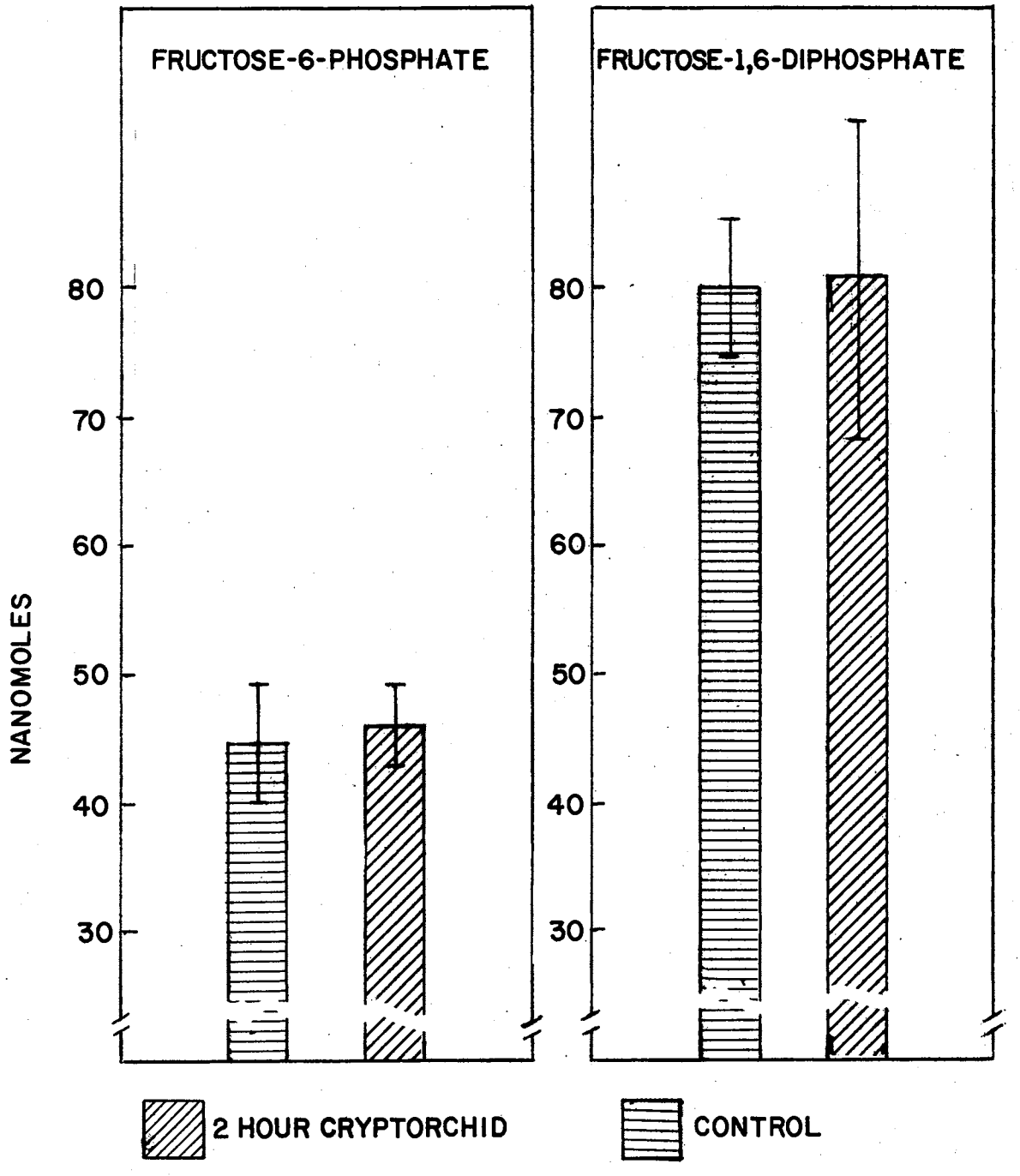

Figure 14. Effects of Artificial Cryptorchidism on Concentrations of Testicular Fructose-6-Phosphate and Fructose-1,6-Diphosphate in vivo. Values are expressed as nanomoles/gram of testis (dry weight).

Effects of Artificial Cryptorchidism on the Concentrations of Testicular Trioses in vivo

This part of Experiment 6 involved determining concentrations in vivo of some trioses of the Embden-Meyerhof glycolytic pathway in order to determine if trioses in this metabolic route of glucose metabolism were affected by 2 hours of artificial cryptorchidism. Results (Table IX, Appendix C) are shown in Figure 15. All three of the trioses measured showed small increases in concentration that were not significantly ($p>0.10$) greater than control concentration of these metabolities. This observation indicated no preferential effect of cryptorchidism on the lower end of the Embden-Meyerhof glycolytic pathway and was in agreement with the results of the hexose section of this experiment.

Effects of Artificial Cryptorchidism on the Concentrations of Testicular Citric Acid Cycle Intermediates in vivo

This section of Experiment 6 was to investigate the effect of artificial cryptorchidism on concentrations in vivo of α-Ketoglutarate and malate in rat testis. Evaluation of these intermediates of the citric acid cycle should aid in correlating glycolytic activity in cryptorchid testis with activity of the citric acid cycle.

Results (Table IX, Appendix C) of this experiment are shown in Figure 16. Concentrations of α-Ketoglutarate and malate were not significantly ($p>0.10$) different from control values. However, they showed slight increases above control values to a level comparable to those observed for the glycolytic metabolites.

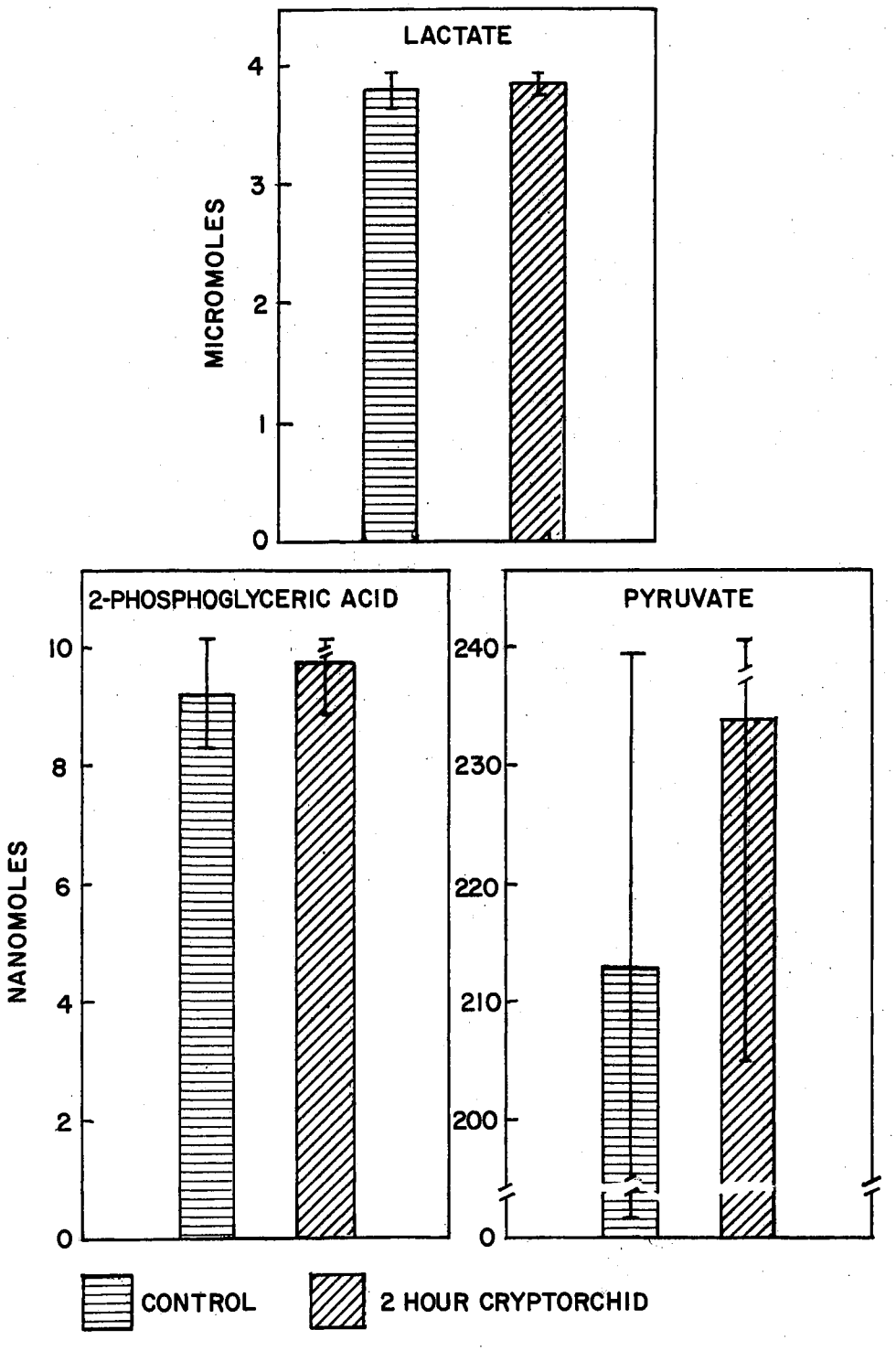

Figure 15. The Effects of Artificial Cryptorchidism on Concentrations of Testicular Lactate, 2-Phosphoglyceric Acid, and Pyruvate in vivo. Values are expressed as micromoles or nanomoles/gram of testis (dry weight).

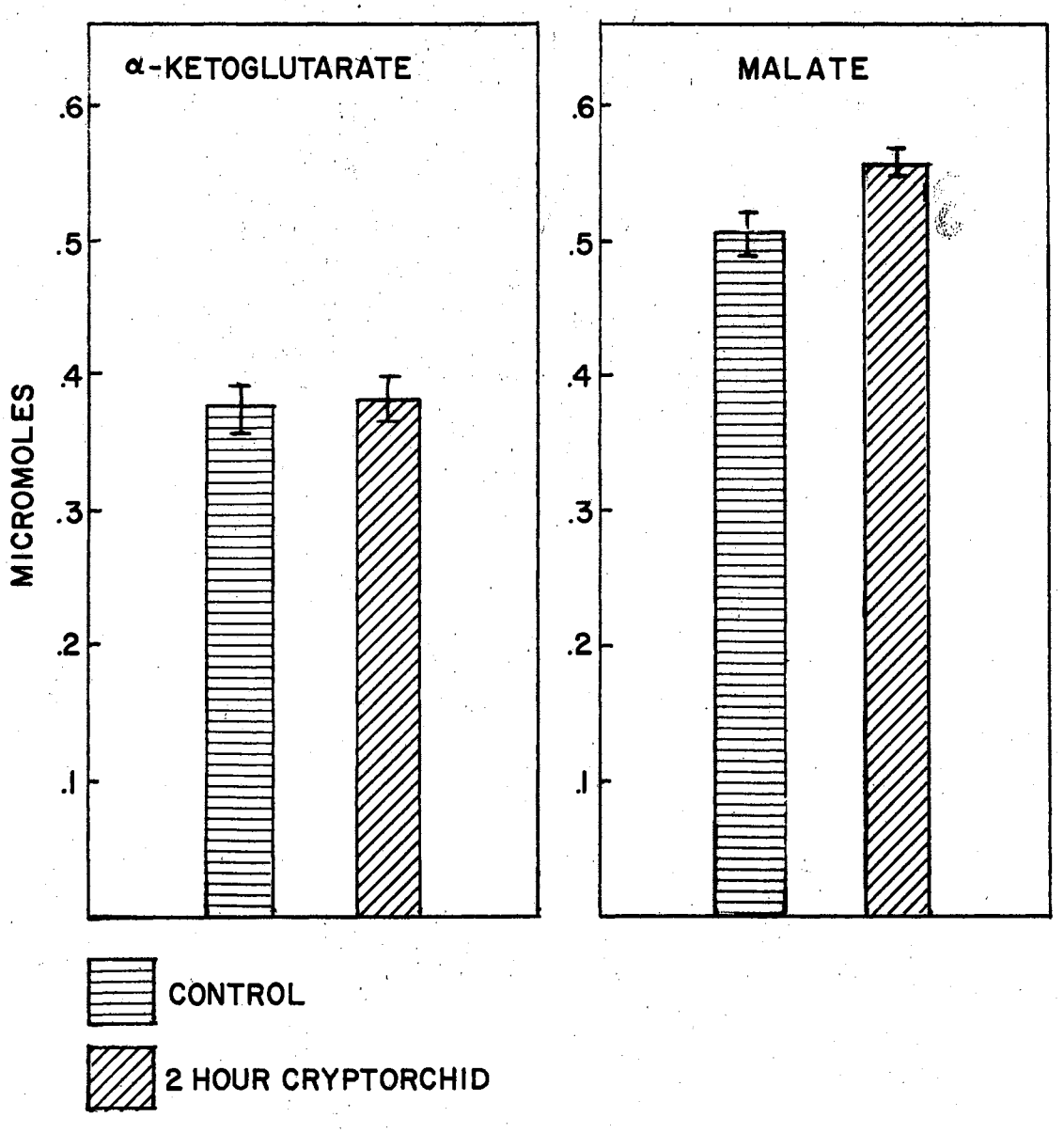

Figure 16. The Effects of Artificial Cryptorchidism on Concentrations of Testicular α-Ketoglutarate and Malate in vivo. Values are expressed as micromoles/gram of testis (dry weight).

In summary, the results of this experiment suggested that total energy metabolism of cryptorchid testis *in vivo* was not different from control testis.

The Effects of Artificial Cryptorchidism on Concentrations of NADH and ATP in vivo in Rat Testicular Tissue

Although concentrations of measured metabolites of the Embden-Meyerhof glycolytic pathway and citric acid cycle were essentially the same as the control concentrations, the consistant small increases in concentrations among all of the metabolites suggest a mild suppression of energy metabolism. Furthermore, the small decreases in $^{14}CO_2$ formation from glucose-U-^{14}C and pyruvate-2-^{14}C *in vitro* also suggest this possibility. This section of Experiment 6 was designed to investigate NADH and ATP concentrations in cryptorchid testis *in vivo* as a test for this hypothesis.

Results (Table IX, Appendix C) are shown in Figure 17. AThough concentrations of these two compounds were not significantly ($p>0.10$) different from controls, a 6% decrease in ATP concentration and a 14% increase in NADH concentration from control levels gave token support to the hypothesis, that at 2 hours of artificial cryptorchidism, a form of mild suppression of total energy metabolism was prevalent. It is not possible at this stage to make any positive statements concerning the nature of this suppression.

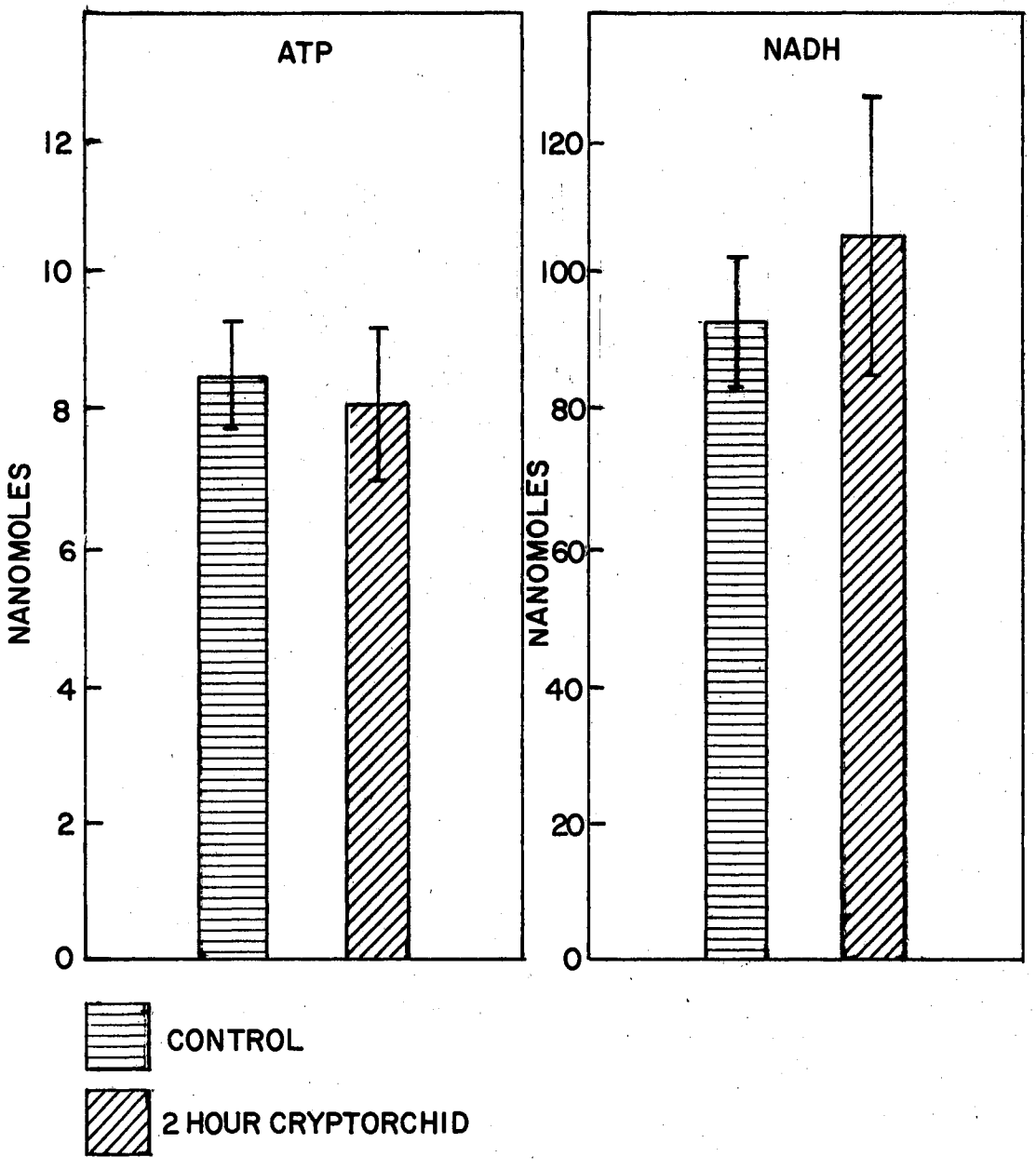

Figure 17. The Effects of Artificial Cryptorchidism on Concentrations of Testicular ATP and NADH in vivo. Values are expressed as micromoles or nanomoles/gram of testis (dry weight).

The Effects of Artificial Cryptorchidism on the Concentration of NADPH in Rat Testicular Tissue in vivo

The literature revealed reports of increased levels of lipid in cryptorchid testes (30,81,127). Since NADPH is a necessary cofactor in lipid synthesis (101), this section of Experiment 6 was designed to investigate the effects of 2 hours of artificial cryptorchidism on NADPH concentration in these testes *in vivo* and relate changes to lipid synthesis.

Results (Table IX, Appendix C) of this experiment are shown in Figure 18. Mean values for NADPH concentration in both tissues were almost identical, although a large variance among replicates may have masked some level of real difference. As a consequence, this observation was of little worth in relating to lipid synthesis *in vivo*.

In conclusion, no changes could be measured in the *in vivo* concentrations of metabolites and cofactors between control and cryptorchid testes. Results expressed as mean $\pm$ standard error of means are given in Table IX of Appendix C. Results expressed as percent difference ($\pm$) from control are given in the same table. Variance among replicates was considerable as shown by analysis of variance in Table LI-LX of Appendix C. This variance among replicates and between treatments in a replicate made it difficult to resolve any absolute real differences between treated and control testes. The best obtainable results indicated a possible small decrease in testicular energy metabolism at 2 hours of artificial cryptorchidism. However, this approach was not fruitful in revealing the effects of cryptorchidism on total testicular energy metabolism.

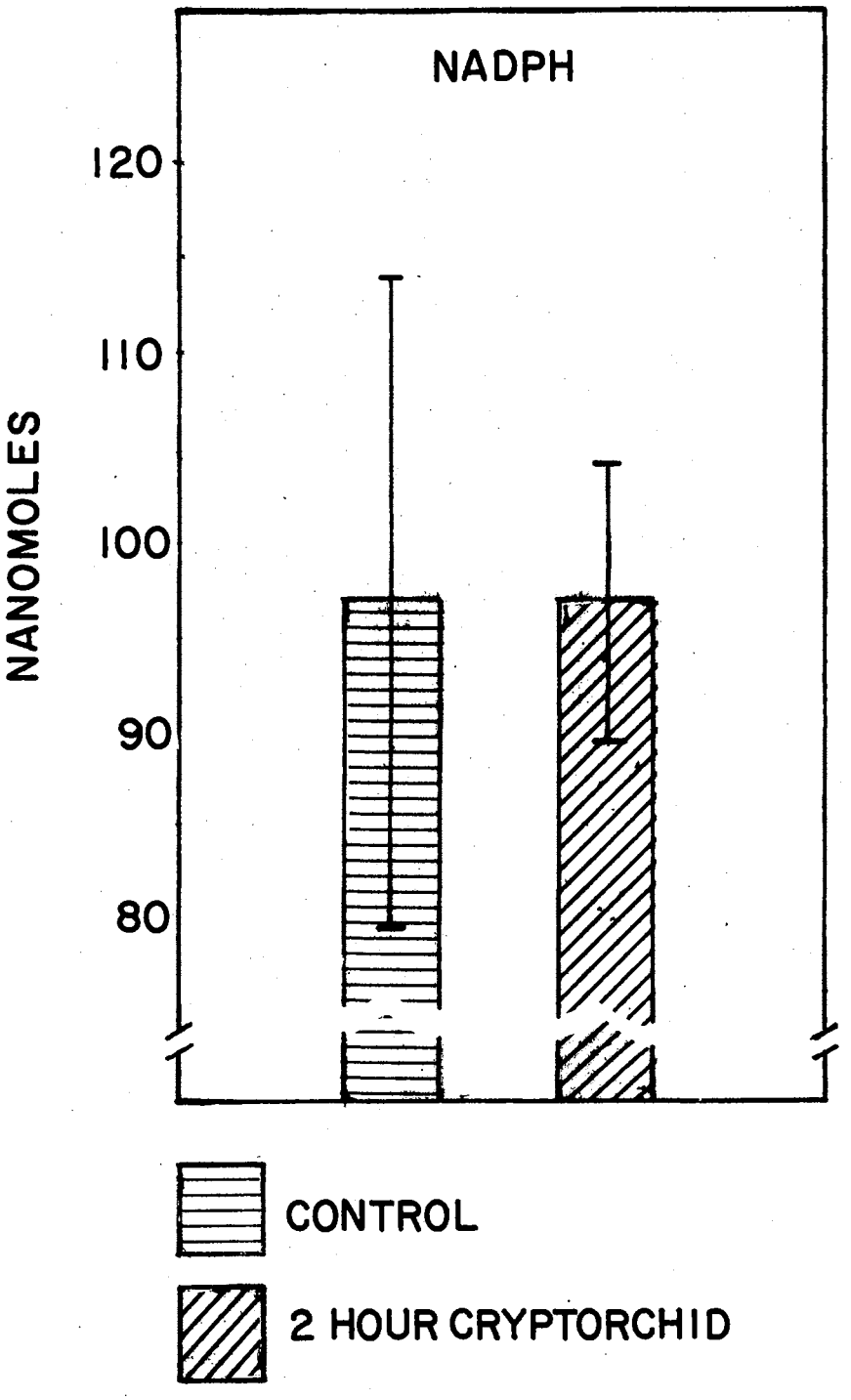

Figure 18. The Effect of Artificial Cryptorchidism on the Concentration of Testicular NADPH in vivo. Values are expressed as nanomoles/gram of testis (dry weight).

Possibly the major weakness of this approach resided in its failure to differentrate energy metabolism among the cellular compartments of testicular tissue. It is logical that increased energy metabolism *in vivo* in some testicular compartments may have masked a decreased energy metabolism in other compartments.

CHAPTER V

DISCUSSION

Numerous investigations have shown that artificial cryptorchidism cuases sterility in a variety of mammals (19,24,110,140). Other investigations have shown that histological changes in cryptorchid testes accompany the loss of fertility (30,49,115,152,160). Additionally, metabolic changes accompany these histological alterations as evidenced by changes in: oxygen uptake (50,60,65,106,167), R.Q. (156), synthesis of protein (35,38,48,108), lipid concentration (30,81,127), ATP concentration (77,108), rate of conversion of glucose to CO_2 (35,61), and in the concentration of endogenous carbohydrates (50,72,180). Unfortunately, most of these investigations were conducted on testes suffering advanced tissue degradation due to the heat treatment. Thus, measurements were made on testes with vastly different cellular makeup from normal testes. Such studies were valuable in that they showed metabolic capabilities of residual cells but were not productive in explaining why certain cell types did not survive.

Most of the cell types which fail to survive heat stress by artificial cryptorchidism are spermatogenic cells. These cells, by a succession of mitotic and meiotic divisions, are involved in renewal of the seminiferous epithelium. Such renewal obviously requires biosynthesis of molecules needed for the new cells. Consequently, it is logical that heat treatment interfering with biosynthetic processes could lead to a

cessation of germ cell renewal. Ultimately, this could lead to the disappearance of all spermatogenic cells except the undifferentiated cells involved in the initial mitotic division, namely, the "reserve stem cells" or type A_0 cells described by Clermont and Bustos-Obregon (26). How soon after translocating the testes to the abdominal cavity do interferences with the biosynthetic processes start? The solution to this question was the quest of the first two experiments.

Ewing et al. (48) observed that protein synthesis by rat testis *in vitro* was reduced by 48 hours of cryptorchidism. However, Ewing and Schanbacher (49) found decreased enzyme activity in rat testis by 4 hours of cryptorchidism. This latter finding suggested that protein biosynthetic reactions may be impaired as early as 4 hours. In fact, results of Experiment 1 (Figure 2) indicated that *in vitro* protein biosynthesis by rat testis in the absence of exogenous glucose was significantly ($p<0.01$) decreased from controls by 2 hours of experimental cryptorchidism. It was quite probable that reduction in protein synthesis occurred prior to this time in these testes. The fact that protein synthesis by these testes in the presence of exogenous glucose was not significantly different from controls at 2 hours but was significantly ($p<0.01$) decreased by 4 hours suggested that some cells after 2 hours of cryptorchidism were capable of continued protein biosynthesis in the presence of glucose but not in its absence. This suggested the survival of some specific cells was contingent upon the presence of some factor which could be provided by or derived from glucose molecules.

Lysine-U-^{14}C incorporation into TCA precipitable material by testis tissue *in vitro* in the presence of glucose was greatly enhanced over that

observed in the absence of glucose (Figure 2). Davis (35) and Means and Hall (108) also made this observation in rat testis.

Testis tissue from 128-hour cryptorchid rats, when incubated in the absence of exogenous glucose, showed a significant ($p<0.01$) increase in lysine incorporation over the controls and displayed the highest lysine-U-^{14}C incorporation rate of all the tissues incubated in the absence of exogenous glucose. Davis et al. (38) reported similar increases over scrotal testis in the testis of 30-day cryptorchid rats. Harkonen and Kormano (72) found 3 and 5 times as much glycogen and glucose, respectively, in 42-day cryptorchid rat testis as in normal mature scrotal testis. This latter observation may explain the decreased dependence on exogenous glucose with increasing interval of cryptorchidism.

In summary, it appears that artificial cryptorchidism induces a major reduction in the biosynthesis of protein *in vitro* in rat testis by 2 hours.

The second experiment in this study was designed to answer the questions: 1) Does artificial cryptorchidism affect the biosynthesis of lipids as well as proteins? 2) How soon after translocating the testes to the abdomen does such an effect of lipid synthesis begin? 3) Does this lipid synthesis account for lipid accumulation or must it be accounted for by some other mechanism? and, 4) Does glucose stimulate lipid synthesis in testis as it does protein synthesis?

Figures 3 through 10 provide the answers to these questions. *De novo* biosynthesis of total lipids and, in most instances, the various lipid classes from acetate-1-^{14}C by cryptorchid rat testis tissue *in vitro* was significantly ($p<0.01$) decreased from scrotal testis by 2 to 4 hours. In addition, exogenous glucose stimulated lipid synthesis

(5-10 fold) more than it did protein synthesis (1.5-3.5 fold). This observation tended to indicate that rat testis lipid synthesis may have a greater dependency on glucose than does protein synthesis. As in protein synthesis this dependency seemed to be less in cryptorchid than in scrotal testis.

The increased lipid concentration observed in cryptorchid testis by several experimenters (52,53,82,84,107) was obviously not due to an increase in the rate of lipid synthesis but conceivably must have been due primarily to decreased utilization. It is logical that part of this decreased utilization was representative of decreased incorporation of lipids into cellular components normally needed for renewal of the cells of the germinal epithelium. Experiments 1 and 2 indicated that *in vitro* protein and lipid biosynthesis decreased in rat testis within 2 hours of artificial cryptorchidism. Furthermore, these biosynthetic processes appeared to be strongly dependent upon the presence of glucose. Experiments 3, 4, and 5 were designed to investigate the nature of this relationship between glucose transport and metabolism and protein and lipid biosynthesis in rat testicular tissue *in vitro*. It is logical that a disturbance of glucose transport and/or metabolism in cryptorchid testes could account for reduced biosynthesis and subsequent sterility.

Results of Experiment 3 (Figure 11) showed no difference ($p>0.10$) in glucose transport *in vitro* as measured by the phosphorylation of 2-deoxyglucose-1-^{14}C at 2 and 8 hours of experimental cryptorchidism. Results of Experiment 3 and 4 (Figures 12 and 13) showed only small decreases in conversion of glucose and pyruvate to CO_2 *in vitro* by testicular tissue from rats cryptorchid for 2 and 8 hours. These three experiments suggested that the observed decreased biosynthetic ability

of cryptorchid testis tissue was not due to reduced glucose transport or oxidation of glucose or pyruvate to CO_2.

Experimental evidence indicates that biosynthetic processes in testis tissue are connected with carbohydrate metabolism and possibly ATP production (35,37,77,108). It may have been that these investigations of carbohydrate metabolism in vitro failed to link a decreased biosynthetic capability with glucose metabolism in cryptorchid testis because in vitro incubation conditions were totally dissimilar to those conditions existing in vivo. The last experiment of this series (Experiment 6) was designed to measure in vivo concentrations of ATP and some metabolites and cofactors of glucose metabolism at 2 hours of experimental cryptorchidism. Logically, this information might reveal some aspects of carbohydrate metabolism not obtainable by in vitro occurred within 2 hours of heat treatment, it was logical to center this part of the investigation on this interval of experimental cryptorchidism.

Results of this experiment (Figures 14-18) showed only small changes in tissue concentrations of ATP and metabolites and cofactors of glucose metabolism. This suggested that glucose utilizing metabolic pathways were essentially not altered by 2 hours of artificial cryptorchidism. However, these small changes in metabolite concentrations in vivo along with the small decreases in conversion of glucose and pyruvate to CO_2 observed in in vitro both suggested that there was a small reduction in total glucose metabolism in cryptorchid testis tissue.

It may be argued that an insignificant decrease in ATP and increase in NADH, respectively, coupled with no alterations in glucose metabolites

rule out the possibility that cryptorchidism alters glucose metabolism, thus resulting in death and dissolution of specific germ cell types. However, these measurements were for all the combined testis tissue cells as an average. Under the conditions presented by the histological organization of the testes, it was highly probable that the tubular tissue compartment of these cryptorchid testes experienced concentrations of these metabolites that were considerably different from concentrations within the interstitial compartment. If such variations exist between these compartments, it would permit masking of perturbations from normal concentrations in one compartment, provided the departures from normal were reversed in the other compartment, e.g., a higher than normal concentration of ATP in the interstitium would mask a lower than normal concentration of ATP in the tubular compartment.

It is evident from this experiment that measuring glucose metabolism of all tissues in cryptorchid rat testes did not elucidate definite mechanisms responsible for the loss of sterility due to heat. The difficulty may reside in the distinct compartmentalization of the testes and the lack of means to distinguish metabolic activity in one compartment from similar activity or the lack of it in another compartment.

In summary, the current research indicated that decreases in lipid and protein biosynthesis were pronounced by two hours of artificial cryptorchidism. Definite changes in carbohydrate metabolism <u>in vivo</u> and <u>in vitro</u> as measured in all tissue types of cryptorchid rat testis in this research did not reveal a definite role for glucose in decreased biosynthesis of lipid and protein in these testes.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Temperatures higher than scrotal temperature have been shown to disrupt spermatogenic processes in the testes of a variety of mammals including man. Degenerative changes in the histological organization of the testes and decreased reproductive potency follow prolonged hyperthermia. The decreased biosynthesis of protein shown to attend this testicular disruption has been associated with glucose metabolism and subsequent ATP production. Other disruptive changes in these testes are associated with lipid metabolism. Experiments were designed to establish the early effects of abdominal temperature in the rat on the biosynthesis of proteinaceous and lipid materials that one might expect to precede gross degenerative changes in testis tissue. Other experiments were designed to discover changes in glucose transport and metabolism that might be responsible for any change in biosynthesis of protein and lipid. The treatments consisted of either sham-operation or exposure of the testes to abdominal temperatures for 0, 2, 4, 8, 16, 32, 64, or 128 hours.

The results of these experiments indicated that the sham operation had no significant ($p>0.10$) influence on metabolism of rat testicular tissue.

Biosynthesis of protein and lipid materials <u>in vitro</u> by testis tissue from rats cryptorchid for 2 or 4 hours was significantly ($p<0.01$) decreased from controls with and without the presence of exogenous

glucose in the culture media. However, in all instances, biosynthesis in the presence of exogenous glucose was at least one and a half times greater than in the absence of exogenous glucose. This demonstrated the dependence of both protein and lipid biosynthesis on glucose.

Glucose transport and the conversion of glucose and pyruvate to CO_2 in vitro by rat testis from testes exposed to abdominal temperatures for 2 and 8 hours showed insignificant reductions from control testis. Measurements in vivo of concentrations of ATP and selected metabolites and cofactors of metabolic pathways utilizing glucose for energy also showed insignificant differences from controls at 2 hours of exposure to abdominal temperature. These evaluations suggested that glucose metabolism was not involved in decreased biosynthesis of protein and lipid. However, determinations of metabolism were made on total testicular tissue which logically may not be uniform in all tissue compartments. Thus it is conceivable that a disturbance in one direction among pools of metabolites in one testis tissue compartment may have masked a disturbance among pools of metabolites in the reverse direction in a different testis compartment. Verification of such an event awaits the development of techniques that can differentiate metabolic activity occurring in one testis tissue compartment from similar activity in a separate compartment.

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APPENDIX A

CHEMICALS

TABLE I

CHEMICALS

Chemicals purchased from the New England Nuclear Company, Boston, Massachusetts:

Sodium acetate-1-^{14}C (2-10 mCi/mM)

D-glucose-U-^{14}C (10-15 mCi/mM)

L-lysine-U-^{14}C (220 mCi/mM)

Sodium pyruvate-2-^{14}C (1-5 mCi/mM)

2-deoxyglucose-1-^{14}C

Chemicals purchased from the Packard Company, Downers Grove, Illinois:

Hyamine hydroxide (10X)

1,4-bis-1-(5 phenoxazolyl)-benzene (POPOP)

2,5-diphenyloxazole (POP)

Chemicals purchased from the Sigma Company, St. Louis, Missouri:

Adenosine-5'-diphosphate, sodium salt, Grade I

Adenosine-5'-triphosphate, disodium salt, Sigma Grade

Aldolase, rabbit muscle, A Grade

2-deoxy-D-glucose

2-deoxy-D-glucose-6-phosphate, sodium salt

Ethylenediaminetetra-acetate (EDTA)

Enolase, from rabbit muscle, Type I

Fructose-1, 6-diphosphate, tetrasodium salt, Sigma Grade

TABLE I (Continued)

Glucose-6-phosphate dehydrogenase, from Baker's Yeast, Type VII
Glutamate dehydrogenase, from bovine liver, Type I
α-Glycerophosphate dehydrogenase, from rabbit muscle, A Grade
Glycine
Hexokinase, from yeast, Type C-300
Hydrazine sulfate
α-Ketoglutaric acid
Lactate dehydrogenase, from rabbit muscle, Type II
Luciferin-luciferase (Sigma FLE-50)
L-lysine
Malate dehydrogenase, from pig heart
Nicotinamide adenine dinucleotide, from yeast, Grade III
Nicotinamide adenine dinucleotide, reduced form, disodium salt, from yeast, Grade III
Nicotinamide adenine dinucleotide phosphate, sodium salt, Sigma Grade
cis-Oxaloacetic acid, Grade I
Phosphoglucoisomerase, from yeast, Type III
2-phosphoglyceric acid, sodium salt
Pyruvate, potassium salt, Type III
Pyruvate kinase, from rabbit muscle, Type II
Sodium arsenate
Triethanolamine hydrochloride
Triose phosphate isomerase, from rabbit muscle, Type III
Tris(hydroxymethyl) aminomethane, Sigma 7-9

APPENDIX B

SOLUTION PREPARATION

TABLE II

PROCEDURE FOR PREPARATION OF REAGENTS USED

Buffers

Triethanolamine-hydrochloride - (mol wt = 185.6)
Magnesium sulfate - (mol wt = 123.2)
EDTA - (mol wt = 744.4)
50 mM triethanolamine-HCL = 2.32 g/250 ml DDW*
10 mM magnesium sulfate = .308 g to above solution
5 mM EDTA = .9306 g to above solution and
adjusted to pH 7.0 or 7.4

Triethanolamine-hydrochloride - (mol wt = 185.6)
0.1 M triethanolamine-HCL = 4.64 g/250 ml DDW
adjusted to pH 7.4 or 8.2

Triethanolamine-hydrochloride - (mol wt = 185.6)
0.5 M triethanolamine-HCl = 18.56 g/200 ml DDW
adjusted to pH 6.5

Hydrazine sulfate - (mol wt = 187.5)
Glycine - (mol wt = 52.0)
EDTA - (mol wt -744.4)
0.4 M hydrazine sulfate = 5.2 g/40 ml DDW
1.0 M glycine - 7.5 g to above solution
to the above solution add 0.2 g EDTA and
51 ml of 2 N NaCH and bring to 100 ml
with DDW, adjusted to pH 9.5

Krebs-Ringer bicarbonate buffer
20.0 ml of 0.77 M NaCl
0.8 ml of 0.77 M KCl
0.6 ml of 0.55 M $CaCl_2$
0.2 ml of 0.77 KH_2PO_4
0.2 ml of 0.77 M $MgSO_4 \cdot 7\ H_2O$
4.2 ml of 0.77 M $NaHCO_3$
103.0 ml of DDW
Gas with 95% O_2:5% CO_2 for 10 minutes
Adjusted to pH 7.4 before use

TABLE II (Continued)

Buffers (Continued)

Sodium arsenate - (mol wt = 312)
Magnesium sulfate·7 H_2O = .4926 g to the above solution
50 mM soldium arsenate = 1.56 g/100 ml DDW
20 mM $MgSO_4$·7 H_2O = .4926 g to the above solution
adjusted to pH 7.4

Sodium arsenate - (mol wt = 3.2)
0.1 M sodium arsenate = 31.2 g/1000 ml DDW
adjusted to pH 7.4

Metal Solutions

Calcium Chloride		(mol wt = 110.9)
0.55 M $CaCl_2$	=	61.0 g/1000 ml DDW
Magnesium Chloride		(mol wt = 95.23)
0.2 M $MgCl_2$	=	4.07 g $MgCl_2$·6 H_2O/100 ml DDW
0.08 M $MgCl_2$	=	1.63 g $MgCl_2$·6 H_2O/100 ml DDW
Magnesium Sulfate 7 H_2O	=	(mol wt = 248.05)
0.77 M $MgSO_4$ 7 H_2O	=	191.0 g/1000 ml DDW
Potassium Carbonate		(mol wt = 138.2)
3.0 M K_2CO_3	=	82.9 g/200 ml DDW
Potassium Chloride		(mol wt = 74.55)
0.4 M KCl	=	2.98 g/100 ml DDW
0.154 M KCl	=	11.48 g/1000 ml DDW
0.77 M KCl	=	57.4 g/1000 ml DDW
Potassium Dihydrogen Phosphate		(mol wt = 137.01)
0.77 M KH_2PO_4	=	105.5 g/1000 ml DDW
Potassium Hydroxide		(mol wt = 56.1)
2.0 N KOH	=	22.4 g/200 ml DDW
Sodium Bicarbonate		(mol wt = 84.0)
0.77 M $NaHCO_3$	=	65.0 g/1000 ml DDW
1% solution of $NaHCO_3$	=	1.0 g/100 ml DDW
Sodium Chloride		(mol wt = 58.44)
0.77 N NaCl	=	45.0 g/1000 ml DDW

TABLE II (Continued)

Miscellaneous Solutions

Ammonium Sulfate		(mol wt = 132.14)
2.5 M $(NH_4)_2SO_4$	=	66.07 g/200 ml DDW
3.0 M $(NH_4)_2SO_4$	=	39.6 g/100 ml DDW
2.1 M $(NH_4)_2SO_4$	=	27.7 g/100 ml DDW
Ethanolic Potassium Hydroxide solution		(mol wt(KOH) = 56.1)
1.5 N ethanolic KOH	=	16.83 g KOH/200 ml 50% ethanol
Hydrochloric Acid		(concentrated HCl = 10 N)
2.0 N HCl	=	20 ml concentrated HCl/80 ml DDW
Perchloric Acid		(mol wt = 100.4)
0.6 N $HClO_4$	=	6.02 g/100 ml DDW
Trichloroacetic Acid (TCA)		(mol wt = 163.4)
5.0% TCA	=	5.0 g/95 ml DDW
15.0% TCA	=	15.0 g/85 ml DDW

Dilution of Coupling Enzymes

Aldolase (A Grade) 10 mg protein/ml $(NH_4)_2SO_4$
1:4 dilution(DDW) = 2.5 mg protein/ml

Glucose-6-Phosphate Dehydrogenase (Type VII) 0.8 mg protein/ml
1:4 dilution(2.1 M $(NH_4)_2SO_4$) = 0.2 mg protein/ml

Glutamate Dehydrogenase (Type I) 20 mg protein/ml
1:5 gilution(DDW) = 4 mg protein/ml

-Glycerophosphate Dehydrogenase 10 mg protein/ml
1:10 dilution(DDW) = 1 mg protein/ml

Lactate Dehydrogenase (Type II) 10 mg protein/ml
1:50 dilution(DDW) = 0.2 mg protein/ml
1:4 dilution (2.1 M $(NH_4)_2SO_4$) = 2.5 mg protein/ml

Malate Dehydrogenase 10 mg protein/ml
1:20 dilution(DDW) = 0.5 mg protein/ml

Phosoglucoisomerase (Type III) 10 mg protein/ml
1:10 dilution (2.1 M $(NH_4)_2SO_4$) = 1 mg protein/ml

Pyruvate Kinase (Type II) 10 mg protein/ml
1:5 dilution(DDW) = 2 mg protein/ml

TABLE II (Continued)

Dilution of Coupling Enzymes (Continued)

Triose Phosphate Isomerase (Type III) 10 mg protein/ml
1:10 dilution(DDW) = 1 mg protein/ml

Cofactors and Subtrates

Acetate, sodium salt (mol wt = 86.0)
2.5 mM = 12.9 mg/60 ml Krebs-Ringer bicarbonate buffer

Adenosine diphosphate (mol wt = 504.8)
0.1 M ADP = 252 mg/5 ml DDW
adjusted to pH 6.8 with solid $NaHCO_3$

Adenosine triphosphate, Sigma disodium salt (mol wt = 629.43)
0.1 M = 623.0 mg/9.6 ml DDW, adjusted to pH 7.0
0.04 M = 124.6 mg/4.8 ml DDW, adjusted to pH 7.0
0.001 M = 6.29 mg/10 ml DDW, adjusted to pH 7.0

2-Deoxy-D-glucose (mol wt = 164.16)
0.01 M = 59.1 mg/36 ml Krebs-Ringer bicarbonate buffer
1.0% = 10 mg/ml 5% TCA

2-Deoxy-D-glucose-6-phosphate, sodium salt (mol wt = 288.1)
1.0% = 10 mg/ml 5% TCA

Fructose-1,6-diphosphate (mol wt = 508)
0.01 M = 25.4 mg/5 ml DDW
0.0001 M = 25.4 mg/500 ml DDW
(prepare 0.0001 M by 1:100 dilution of 0.01 M)

Glucose (mol wt = 180.16)
0.01 M = 18 mg/10 ml DDW
0.01 M = 108 mg/60 ml Krebs-Ringer bicarbonate buffer

Glucose-6-phosphate (mol wt = 261.13)
0.01 M = 26.1 mg/10 ml DDW
0.0001 M = 26.1 mg/1000 ml DDW
(prepare 0.0001 M by 1:100 dilution of 0.01 M)

-Ketoglutarate (mol wt = 146)
0.3 M = 219 mg/5 ml DDW, adjusted to pH 6.0

Lysine (mol wt = 146.19)
0.1 mM = 8.7 mg/60 ml Krebs-Ringer bicarbonate buffer

NAD (for wt = 777.26)
0.05 M(calculated) = 40 mg/ml DDW

TABLE II (Continued)

Cofactors and Subtrates (Continued)

NADH (for wt = 778.26)
10 mM = 7.8 mg/ml 0.1 M Tri-HCl buffer
0.4 mM, dilute 10 mM 1:25 with buffer
0.1 mM, dilute 10 mM 1:100 with buffer
0.5 mg/ml = 5 mg/10 ml 0.1 M Tri-HCl buffer

NADP (for wt = 862.1)
0.01 M = 8.4 mg/ml DDW

NADPH (for wt = 863.1)
0.01 mM = 1 mg/11 ml Tri-HCl buffer, pH 8.2

Oxaloacetic acid (mol wt = 132)
5.0 mM = 13.2 mg/20 ml DDW, pH 6.0
0.05 mM = 5.0 mM diluted 1:100 with DDW

2-Phosphoglyceric acid (tetrahydrazonium salt) (for wt = 360)
0.01 M = 18 mg/5 ml DDW
0.0001 M = 0.01 diluted 1:100 with DDW

*DDW = Double distilled water (glass)

APPENDIX C

RESULTS AND ANALYSIS

TABLE III

EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON TESTIS WEIGHT

Criteria	Hours after experimental cryptorchidism[1]							
	0	2	4	8	16	32	64	128
Testis weight (g)	2.79 ±0.12	2.76 ±0.12	2.72 ±0.13	2.89 ±0.13	3.01 ±0.12	3.00 ±0.05	2.86 ±0.09	2.05 ±0.06

[1]Each value represents the mean ± standard error of five rats.

TABLE IV

EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF LYSINE-U-^{14}C INTO TRICHLOROACETIC ACID PRECIPITABLE MATERIAL BY TEASED TESTIS TUBULES IN THE PRESENCE AND ABSENCE OF GLUCOSE

Incubation Criteria	Hours of experimental cryptorchidism[1]							
	0	2	4	8	16	32	64	128
No glucose	729 ± 83	565 ± 38	563 ± 33	584 ± 57	582 ± 25	650 ± 19	667 ± 13	1,099 ± 93
Plus glucose	2,511 ± 392	2,154 ± 108	1,960 ± 122	1,952 ± 78	1,807 ± 93	1,766 ± 184	1,777 ± 94	1,605 ± 462
Ratio[2]	3.44 ±0.31	3.81 ±0.28	3.48 ±0.29	3.34 ±0.25	3.10 ±0.26	2.71 ±0.20	2.66 ±0.21	1.46 ±0.16

[1]Each value represents the mean ± standard error of five rats expressed as dpm/mg of protein.

[2]Ratio of lysine-U-^{14}C incorporated into trichloroacetic acid precipitable material in the presence and absence of glucose.

TABLE V

EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO VARIOUS LIPID CLASSES BY TEASED TESTIS TUBULES INCUBATED IN THE ABSENCE OR PRESENCE OF GLUCOSE

Lipid class	Glucose[2]	Hours after experimental cryptorchidism[1] 0	2	4	8	16	32	64	128
Monoglycerides	-	520 ± 49	407 ± 29	375 ± 26	338 ± 24	386 ± 61	363 ± 40	367 ± 29	349 ± 42
	+	2,860 ± 407	2,472 ± 614	1,953 ± 192	2,071 ± 326	1,970 ± 530	1,689 ± 310	1,253 ± 226	1,458 ± 305
Diglycerides	-	4,825 ± 679	3,961 ± 775	4,289 ± 737	3,604 ± 663	3,931 ± 690	3,272 ± 672	3,345 ± 725	2,537 ± 794
	+	7,731 ± 834	8,982 ± 860	8.093 ± 654	6,829 ± 771	6,254 ± 930	6,743 ± 875	4,804 ± 966	4,168 ± 425
Triglycerides	-	4,658 ± 386	3,115 ± 416	3,043 ± 442	3,012 ± 438	2,763 ± 380	2,913 ± 264	3,041 ± 538	2,439 ± 475
	+	131,649 ± 910	82,778 ± 558	74,035 ± 479	80,719 ± 645	73,026 ± 392	68,949 ± 579	62,684 ± 283	59,424 ± 496
	-	3,659 ± 820	3,806 ± 631	3,037 ± 281	3,221 ± 313	3,223 ± 351	2,833 ± 347	3,016 ± 590	2,547 ± 439

TABLE V - CONTINUED

Lipid class	Glucose[2]	Hours after experimental cryptorchidism[1] 0	2	4	8	16	32	64	128
Non-volatile fatty acids	+	21,443 ± 740	19,849 ± 750	16,182 ± 841	16,860 ± 572	15,544 ± 576	15,344 ± 406	14,755 ± 455	14,180 ± 740
	-	2,779 ± 554	1,407 ± 333	1,178 ± 289	1,140 ± 149	1,399 ± 205	1,206 ± 155	1,242 ± 211	1,236 ± 173
Phospholipids	+	37,498 ± 1,489	19,566 ± 3,058	19,481 ± 2,130	20,489 ± 2,398	20,360 ± 2,360	13,060 ± 2,750	10,166 ± 890	7,390 ± 509
	-	1,347 ± 209	1,288 ± 349	1,067 ± 197	1,144 ± 309	1,016 ± 177	871 ± 167	762 ± 137	623 ± 133
Sterols	+	14,269 ± 1,519	7,332 ± 1,300	6,760 ± 1,730	7,324 ± 774	6,197 ± 1,135	5,494 ± 781	4,321 ± 600	4,121 ± 109
	-	867 ± 150	732 ± 90	715 ± 78	639 ± 86	720 ± 62	594 ± 120	761 ± 98	717 ± 73
Sterol esters	+	4,655 ± 102	3,096 ± 216	3,360 ± 204	3,417 ± 556	3,396 ± 931	2,343 ± 342	2,576 ± 412	1,751 ± 89
	-	35,360 ± 3,300	30,331 ± 2,300	29,248 ± 2,435	29,855 ± 2,344	30.069 ± 3,160	30,325 ± 2,341	27,140 ± 1,222	25,227 ± 1,941

TABLE V - CONTINUED

Lipid class	Glucose[2]	Hours after experimental cryptorchidism[1] 0	2	4	8	16	32	64	128
Total lipids	+	404,124 ±27,400	265,106 ±12,143	253,293 ± 6,090	248,169 ± 7,451	226,762 ± 9,670	198,363 ± 8,350	153,016 ± 7,468	140,269 ± 5,491

[1]Each value represents the mean ± standard error of five rats expressed as dpm/100 mg testis (wet weight).

[2]In the absence (-) or pressence (+) of glucose (10mM).

TABLE VI

THE EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* TRANSPORT OF GLUCOSE BY TEASED TESTIS TUBULES AS MEASURED BY THE PHOSPHORYLATION OF 2-DEOXYGLUCOSE-1-^{14}C

	Hours after experimental cryptorchidism[1]		
Criteria	0	2	8
dpm/100 mg of testis tubules (wet weight)	21,784 ±1,540	21,479 ±1,660	21,977 ±2,050

[1]Each value represents the mean ± standard error of eight rats.

TABLE VII

EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* OXIDATION OF GLUCOSE-U-^{14}C TO $^{14}CO_2$ BY TEASED TESTIS TUBULES

	Hours of experimental cryptorchidism[1]		
Criteria	0	2	8
dpm/100 mg of testis tubules (wet weight)	13,628 ± 840	13,416 ± 750	12,957 ± 820

[1]Each value represents the mean ± standard error of eight rats.

TABLE VIII

EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* OXIDATION OF PYRUVATE-2-^{14}C TO $^{14}CO_2$ BY TEASED TESTIS TUBULES

	Hours after experimental cryptorchidism[1]		
Criteria	0	2	8
dpm/100 mg of testis tubules (wet weight)	5,478 ± 225	5,336 ± 258	5,335 ± 315

[1]Each value represents the mean ± standard error of eight rats.

TABLE IX

EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATIONS OF ATP, NADH, NADPH AND SELECTED INTERMEDIATES OF GLUCOSE ENERGY METABOLISM

Compound	Percent change[2]	Hours after cryptorchidism[1]	
		0	2
Fructose-6-phosphate[3]	+ 4.17	45.760 ± 5.380	47.670 ± 3.430
Fructose-1,6-diphosphate[3]	+ 1.12	82.400 ± 5.740	83.320 ±12.560
2-Phosphoglyceric acid[3]	+ 6.40	9.660 ± 1.150	10.280 ± 1.070
Pyruvate[3]	+11.02	213.350 ±28.830	236.880 ±30.430
Lactate[4]	+ 3.05	3.940 ± 0.330	4.060 ± 0.240
α-Ketoglutarate[4]	+ 1.06	0.376 ± 0.035	0.380 ± 0.033
Malate[4]	+ 9.20	0.500 ± 0.031	0.546 ± 0.019
Adenosine triphosphate[4]	- 5.89	8.650 ± 0.850	8.146 ± 1.144
NADH[3]	+13.94	93.320 ±10.240	106.320 ±20.910
NADPH[3]	- 0.26	96.450 ±16.560	96.200 ± 7.440

[1]Each value represents the concentrations expressed as nanomoles or micromoles/gram of testis (dry weight) ± standard error of the number of rats involved in each determination.

[2]Each value represents the percent increase (+) or decrease (-) from control measurements.

[3]Values expressed in nanomoles.

[4]Values expressed in micromoles.

TABLE X

ANALYSIS OF VARIANCE OF TESTIS WEIGHT AFTER EXPOSURE OF THE TESTES TO THE ABDOMINAL CAVITY: PRELIMINARY EXPERIMENT

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	3.830	---	---
Treatment	7	3.270	.467	27.63**
Replicates	4	.087	.022	1.29
Error	28	.474	.017	---

**($p<0.01$)

TABLE XI

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO MEAN WEIGHT OF PAIRED TESTES AFTER TRANSLOCATION OF THE TESTES TO THE ABDOMINAL CAVITY: PRELIMINARY EXPERIMENT

Treatment (hours)	128	4	2	0	64	8	32	16
Mean	2.05	2.72	2.76	2.79	2.86	2.89	3.00	3.01

Value of p (d.f. = 28)	2	3	4	1% 5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	.227	.237	.243	.249	.252	.255	.257

($S_{\bar{X}}$ = .0581)

[1]Steel and Torrie (151)

TABLE XII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF LYSINE-U-^{14}C INTO TRICHLOROACETIC ACID PRECIPITABLE MATERIAL IN THE ABSENCE OF GLUCOSE: EXPERIMENT 1

Source of variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	1,250,364	---	---
Treatment	7	1,123,824	160,546.3	43.72**
Replicates	4	23,071	5,767.8	1.56
Error	28	103,469	3,695.3	---

**($p<0.01$)

TABLE XIII

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF LYSINE-U-^{14}C INTO TRICHLOROACETIC ACID PRECIPITABLE MATERIAL IN THE ABSENCE OF GLUCOSE: EXPERIMENT 1

Treatment (hours)	4	2	16	8	32	64	0	128
Mean	563	565	582	584	650	667	729	1,099

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	106.27	110.89	113.61	116.33	117.96	119.32	120.41

($S_{\bar{X}}$ = 27.18)

[1]Steel and Torrie (151)

TABLE XIV

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTROCHIDISM ON THE IN VITRO INCORPORATION OF LYSINE-U-^{14}C INTO TRICHLOROACETIC ACID PRECIPITABLE MATERIAL IN THE PRESENCE OF GLUCOSE: EXPERIMENT 1

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	5,454,598	---	---
Treatment	7	2,795,610	399,372.9	4.90**
Replicates	4	375,763	93,940.8	1.15
Error	28	2,283,225	81,543.8	---

**($p<0.01$)

TABLE XV

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF LYSINE-U-^{14}C INTO TRICHLOROACETIC ACID PRECIPITABLE MATERIAL IN THE PRESENCE OF GLUCOSE: EXPERIMENT 1

Treatment (hours)	128	32	64	16	8	4	2	0
Mean	1,605	1,766	1,777	1,807	1,952	1,960	2,154	2,511

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR ($S_{\bar{X}}$ = 127.7)	499.30	521.00	533.80	546.60	554.60	560.60	565.70

[1]Steel and Torrie (151)

TABLE XVI

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO MONOGLYCERIDES IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	188,100.4	---	---
Treatment	7	115,224.4	16.460.6	7.76**
Replicates	4	13,504.0	3,376.0	1.59
Error	28	59,372.0	2,120.4	---

**($p<0.01$)

TABLE XVII

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO MONOGLYCERIDES IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	8	128	32	64	4	16	2	0
Mean	338	349	363	367	375	386	407	520
Value of p (d.f. = 28)	2	3	4	1% 5	6	7	8	
SSR	3.91	4.08	4.18	4.28	4.34	4.37	4.43	
LSR	80.50	84.00	86.07	88.13	89.36	89.98	91.21	

($S_{\bar{X}}$ = 20.59)

[1]Steel and Torrie (151)

TABLE XVIII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO MONOGLYCERIDES IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	16,038,179	---	---
Treatment	7	9,548,217	1,364,031.0	7.66**
Replicates	4	1,503,193	375,798.3	2.11
Error	28	4,986,769	178,098.9	---

**($p<0.01$)

TABLE XIX

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO RHE EARLY EFFECT OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO MONOGLYCERIDES IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	64	128	32	4	16	8	2	0
Mean	1,253	1,458	1,689	1,953	1,970	2.071	2.472	2,860

Value of p (d.f. = 28)	2	3	4	5	6	7	8
				1%			
SSR	3.91	4.08	4.18	4.28	4.34	4.37	4.43
LSR	737.9	770.0	788.9	807.8	819.1	824.8	836.1

($S_{\bar{X}}$ = 188.73)

[1]Steel and Torrie (151)

TABLE XX

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO DIGLYCERIDES IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	38,136,572	---	---
Treatment	7	17,008,300	2,429,757.1	3.84**
Replicate	4	3,389,590	847,397.5	1,34
Error	28	17,738,682	633,524.4	---

**($p<0.01$)

TABLE XXI

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO DIGLYCERIDES IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	32	64	8	16	2	4	0
Mean	2,537	3,273	3,345	3,604	3,931	3,961	4,289	4,825

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.24	4.34	4.39	4.43
LSR ($S_{\bar{X}}$ = 355.95)	1,392	1,452	1,488	1,523	1,545	1,563	1,577

[1]Steel and Torrie (151)

TABLE XXII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO DIGLYCERIDES IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	120,475,238	---	---
Treatment	7	92,170,890	13,167,270	15.05**
Replicate	4	3,878,455	969,614	1.11
Error	28	24,425,983	872,353	---

**($p<0.01$)

TABLE XXIII

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO DIGLYCERIDES IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	64	16	32	8	0	4	2
Mean	4,168	4,804	6,254	6,743	6,829	7,731	8,093	8,982

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	1,633	1,704	1,746	1,788	1,813	1,834	1,850

($S_{\bar{X}}$ = 417.69)

[1]Steel and Torrie (151)

TABLE XXIV

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* INCORPORATION OF ACETATE-1-^{14}C INTO TRIGLYCERIDES IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	23,024,274	---	---
Treatment	7	15,116,450	2,159,493	9.54**
Replicate	4	1,572,002	393,001	1.74
Error	28	6,335,822	226,280	---

** ($p<0.01$)

TABLE XXV

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* INCORPORATION OF ACETATE-1-^{14}C INTO TRIGLYCERIDES IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	64	32	8	64	4	2	0
Mean	2,439	2,763	2,913	3,012	3,041	3.043	3,115	4,658

Value of p (d.f. - 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	831.8	867.9	889.2	910.5	923.2	933.9	942.4

($S_{\bar{X}}$ = 212.73)

[1] Steel and Torrie (151)

TABLE XXVI

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO TRIGLYCERIDES IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	17,976,205,248	---	---
Treatment	7	17,956,136,090	2,565,162,299	4053**
Replicate	4	2,347,027	586,757	0.93
Error	28	17,722,131	632,933	---

**($p<0.01$)

TABLE XXVII

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO TRIGLYCERIDES IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	64	32	16	4	8	2	0
Mean	59,424	62,684	68,949	73,026	74,935	80,719	82,778	131,649

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	1,391	1,452	1,487	1,523	1,544	1,562	1,576

($S_{\bar{X}}$ = 355.79)

[1]Steel and Torrie (151)

TABLE XXVIII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* INCORPORATION OF ACETATE-1-^{14}C INTO NON-VOLATILE FATTY ACIDS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	15,950,519	---	---
Treatment	7	3,952,947	564,707	1.33
Replicate	4	114,752	28,688	0.07
Error	28	11,882,820	424,386	---

TABLE XXIX

DUNCAN'A NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* INCORPORATION OF ACETATE-1-^{14}C INTO NON VOLATILE FATTY ACIDS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	2	32	64	4	8	16	0
Mean	2,547	2,806	2,833	3,016	2,037	3,221	3,223	3,659

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	2.90	3.04	3.13	3.20	3.26	3.30	3.33
LSR	844.9	885.6	911.9	932.3	949.7	961.4	970.1

($S_{\bar{X}}$ = 291.33)

[1] Steel and Torrie (151)

TABLE XXX

ANALYSIS IF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO NON VOLATILE FATTY ACIDS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Source Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	250,257,922	---	---
Treatment	7	229,478,274	32,782,611	52.89**
Replicate	4	3,425,004	856,251	1.38
Error	28	17,354,644	619,809	---

**($p<0.01$)

TABLE XXXI

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO NON-VOLATILE FATTY ACIDS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	64	32	16	4	8	2	0
Mean	14,180	14,775	15,344	15,544	16,182	16,860	19,849	21,443

Value of p (d.f. = 28)	2	3	4	5	6	7	8
			1%				
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	1,377	1,436	1,472	1,507	1,528	1,546	1,560

($S_{\bar{X}}$ = 352.08)

[1]Steel and Torrie (151)

TABLE XXXII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO PHOSPHOLIPIDS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	14,122,726	---	---
Treatment	7	10,446,749	1,492,393	13.93**
Replicate	4	675,215	168,804	1.58
Error	28	3,000,762	107,170	---

** ($p<0.01$)

TABLE XXXIII

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO PHOSPHOLIPIDS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	8	4	32	128	64	2	0	
Mean	1,140	1,178	1,206	1,236	1,242	1,399	1,407	2,799

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	572	597	612	627	635	643	649

($S_{\bar{X}}$ = 146.4)

[1] Steel and Torrie (151)

TABLE XXXIV

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO PHOSPHOLIPIDS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	3,180,425,673	---	---
Treatment	7	2,964,598,427	423,514,061	63.44**
Replicate	4	28,910,053	7,227,513	1.08
Error	28	186,917,193	6,675,614	---

** ($p<0.01$)

TABLE XXXV

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTROCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO PHOSPHOLIPIDS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	64	32	4	2	16	8	0
Mean	7,390	10,166	13,060	19,481	19,566	20,360	20,489	37,498

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	4,518	4,714	4,830	4,945	5,015	5,073	5,119

($S_{\bar{X}}$ = 1155.47)

[1] Steel and Torrie (151)

TABLE XXXVI

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* INCORPORATION OF ACETATE-1-^{14}C INTO STEROLS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	4,584,199	---	---
Treatment	7	2,212,537	316,077	4.56**
Replicate	4	428,784	107,196	1.54
Error	28	1,942,878	69,389	---

**($p<0.01$)

TABLE XXXVII

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* INCORPORATION OF ACETATE-1-^{14}C INTO STEROLS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Treatment(hours)	128	64	32	16	4	8	2	0
Mean	623	762	871	1,016	1,067	1,144	1,288	1,347

Value of p (d.f. = 28)	2	3	4	5	6	7	8
				1%			
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR ($S_{\bar{X}}$ = 117.8)	461	481	492	504	511	517	522

[1]Steel and Torrie (151)

TABLE XXXVIII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO STEROLS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	415,073,658	---	---
Treatment	7	357,427,738	51,061,105	26.83**
Replicate	4	4,354,671	1,088,668	0.57
Error	28	53,291,249	1,903,259	---

**($p<0.01$)

TABLE XXXIX

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO STEROLS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	64	32	16	4	8	2	0
Mean	4,121	4,321	5,494	6,197	6,760	7,324	7,332	14,269
Value of p (d.f. = 28)	2	3	4	1% 5	6	7	8	
SSR	3.91	4.08	4.08	4.28	4.34	4.39	4.43	
LSR	2,412	2,517	2,579	2,641	2,678	2,708	2,733	

($S_{\bar{X}}$ = 616.96)

[1]Steel and Torrie (151)

TABLE XL

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* INCORPORATION OF ACETATE-1-^{14}C INTO STEROL ESTERS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	646,130	---	---
Treatment	7	229,384	32,769	2.56*
Replicate	4	58,099	14,525	1.13
Error	28	358,647	12,809	---

*($p<0.05$)

TABLE XLI

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* INCORPORATION OF ACETATE-1-^{14}C INTO STEROL ESTERS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	32	8	4	128	16	2	64	0
Mean	594	639	715	717	720	732	761	867
Value of p (d.f. = 28)	2	3	4	1% 5	6	7	8	
SSR	2.90	3.04	3.13	3.20	3.26	3.30	3.33	
LSR ($S_{\bar{X}}$ = 50.61)	147	154	158	162	165	167	169	

[1]Steel and Torrie (151)

TABLE XLII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO STEROL ESTERS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	35,565,501	---	---
Treatment	7	26,679,337	3,811,334	14.19**
Replicate	4	1,364,058	341,014	1.27
Error	28	7,522,106	268,647	---

** ($p<0.01$)

TABLE XLIII

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO STEROL ESTERS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	32	64	2	4	16	8	0
Mean	1,751	2,343	2,576	3,096	3,360	3,396	3,417	4,655

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	906	946	970	992	1,006	1,018	1,027

($S_{\bar{X}}$ = 231.79)

[1] Steel and Torrie (151)

TABLE XLIV

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO TOTAL LIPIDS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	39	561,509,691	---	---
Treatment	7	298,749,759	42,678,537	5.49**
Replicate	4	45,214,406	11,303,602	1.45
Error	28	117,545,526	7,769,483	---

**($p<0.01$)

TABLE XLV

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO TOTAL LIPIDS IN THE ABSENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	64	4	8	16	32	2	0
Mean[2]	252.3	271.4	292.4	298.6	300.7	303.3	303.3	353.6

Value of p (d.f. = 28)	1% 2	3	4	5	6	7	8
SSR	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR	4,874	5,086	5,211	5,335	5,410	5,472	5,552

($S_{\bar{X}}$ = 1,246,55)

[1]Steel and Torrie (151)

[2]Mean x 10^2

TABLE XLVI

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO TOTAL LIPIDS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Source of Variance	Degrees of Freedom	Sum of Squares[1]	Mean Square[1]	F Ratio
Total	39	241,293	---	---
Treatment	7	234,735	33,534	170.53**
Replicate	4	1,152	288.0	1.46
Error	28	5,506	196.6	---

**($p<0.01$)

[1]Value x 10^{-6}

TABLE XLVII

DUNCAN'S NEW MULTIPLE RANGE TEST[1] APPLIED TO THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO INCORPORATION OF ACETATE-1-^{14}C INTO TOTAL LIPIDS IN THE PRESENCE OF GLUCOSE: EXPERIMENT 2

Treatment (hours)	128	64	32	16	8	4	2	0
Mean[2]	140.3	154.0	198.4	226.8	248.2	253.3	265.1	404.1

Value of p (d.f. = 28)	2	3	4	5	6	7	8
1%							
SSR_2	3.91	4.08	4.18	4.28	4.34	4.39	4.43
LSR^2	24.5	25.6	26.2	26.8	27.2	27.5	27.8

($S_{\bar{X}}$ = 6,271.2)

[1]Steel and Torrie (151)

[2]Value x 10^{-3}

TABLE XLVIII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO TRANSPORT OF GLUCOSE AS MEASURED BY THE PHOSPHORYLATION OF 2-DEOXYGLUCOSE-1-^{14}C: EXPERIMENT 3

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	23	522.7	---	---
Treatment	2	1.0	.51	.06
Replicate	7	396.4	56.62	6.33**
Error	14	125.3	8.95	---

**($p<0.01$)

TABLE XLIX

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VITRO OXIDATION OF GLUCOSE-U-^{14}C TO $^{14}CO_2$: EXPERIMENT 4

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	47	474.4	---	---
Treatment	2	3.8	1.90	1.12
Replicate	15	419.9	27.98	16.49**
Error	30	50.9	1.70	---

**($p<0.01$)

TABLE L

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VITRO* OXIDATION OF PYRUVATE-2-^{14}C TO $^{14}CO_2$: EXPERIMENT 5

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	47	5,328.6	---	---
Treatment	2	80.5	40.25	1.63
Replicate	15	4,505.1	300.34	12.13**
Error	30	743.0	24.77	---

**($p<0.01$)

TABLE LI

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE *IN VIVO* CONCENTRATION OF FRUCTOSE-6-PHOSPHATE: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Variance	Mean Square	F Ratio
Total	15	2,293.5	---	---
Treatment	1	14.6	14.6	.14
Replicate	7	1,540.7	220.1	2.08
Error	7	738.2	105.4	---

TABLE LII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF FRUCTOSE-1, 6-DIPHOSPHATE: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	15	2,677.15	---	---
Treatment	1	2.55	2.55	.04
Replicate	7	2,171.15	310.16	4.31*
Error	7	503.45	71.92	---

*($p<0.05$)

TABLE LIII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF 2-PHOSPHOGLYCERIC ACID: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	9	50.60	---	---
Treatment	1	.96	.96	.96
Replicate	4	43.98	10.99	7.77*
Error	4	5.66	1.42	---

*($p<0.05$)

TABLE LIV

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF PYRUVATE: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	15	100,337.6	---	---
Treatment	1	2,213.5	2,213.5	2.47
Replicate	7	91,862.6	13.123.2	14.67**
Error	7	6, 261.5	894.5	---

**($p<0.01$)

TABLE LV

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF LACTATE: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	15	9.43	---	---
Treatment	1	.05	.05	.05
Replicate	7	2.78	.40	.42
Error	7	6.60	.94	---

TABLE LVI

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF α-KETOGLUTARATE: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	15	.131	---	---
Treatment	1	.000	.000	.000
Replicate	7	.032	.005	.310
Error	7	.100	.014	---

TABLE LVII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF MALATE: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	13	.0678	---	---
Treatment	1	.0078	.0078	3.71
Replicate	6	.0474	.0079	3.76
Error	6	.0126	.0021	---

TABLE LVIII

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF ATP: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	15	110.4	---	---
Treatment	1	1.03	1.03	0.44
Replicate	7	93.28	13.33	5.80*
Error	7	16.08	2.30	---

*($p<0.05$)

TABLE LIX

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF NADH: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	9	11,262.6	---	---
Treatment	1	422.5	422.5	.34
Replicate	4	5,936.6	1,484.1	1.21
Error	4	4,903.6	1,225.9	---

TABLE LX

ANALYSIS OF VARIANCE OF THE EARLY EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM ON THE IN VIVO CONCENTRATION OF NADPH: EXPERIMENT 6

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	11	9,891.5	---	---
Treatment	1	.1	.1	.00
Replicate	5	6,789.0	1,357.8	2.18
Error	5	3,102.4	620.5	---

VITA

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