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Radioautographic Incorporation of Tritiated Lysine Into Protein of Cells of the Spermatogenic Cycle

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RADIOAUTOGRAPHIC INCORPORATION OF TRITIATED LYSINE
INTO PROTEIN OF CELLS OF THE SPERMATOGENIC CYCLE

BY

Casimir F. Firlit, M.S., M.D.

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE
SCHOOL OF LOYOLA UNIVERSITY IN PARTIAL
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ABSTRACT

Surgically induced adult and prepubertal rat cryptorchidism was histologically studied at various times following abdominal retention. Similar histological observations were noted in both types of surgically created cryptorchidism, namely, the predominance of the Sertoli cell retinaculum with numerous Type "B" spermatogonia and occasional pachytene primary spermatocytes. The Sertoli cell retinaculum following 30 days of exposure to intra-abdominal temperature demonstrated cytoplasmic fibrosis and nuclear pleomorphism. All other germinal cells were spontaneously exfoliated during this period of surgically created cryptorchidism.

In comparing the incorporation of tritiated lysine into protein of cells of the cryptorchid versus the scrotal rat testis, it was found that a 40 fold increase in protein anabolism occurred in the "normal" appearing Sertoli cells of the cryptorchid testis and a 12 fold increase in the atrophic appearing Sertoli cells. In addition, cryptorchidism produced a 4 fold increase in protein labeling in both 'crust' spermatogonia and pachytene primary spermatocytes. The results of these studies demonstrated that the greatest degree of protein labeling in the cryptorchid testis occurs in the Sertoli cells.

Further studies concerned with the effect of glucose on the in vitro incorporation of tritiated lysine into protein of cells of the cycle of the seminiferous epithelium were performed. In the control system the greatest degree of protein labeling occurs in the resting primary spermatocytes with little or no labeling over the remaining cells of the spermatogenic cycle. The addition of glucose to incubating slices of rat seminiferous epithelium produced a profound increase in protein labeling in all the cells of the

spermatogenic cycle. However, the greatest degree of labeling occurred in the pachytene primary spermatocytes, whose chromosomes were crossing-over, and in the spermatids.

Finally, radioautographic studies of the effect of glucose on the incorporation of tritiated lysine into the successive cells of the cycle of the human seminiferous epithelium were performed. Following a 1 hour incubation period, slices of human testis in the presence and absence of glucose demonstrated a pattern of protein anabolism remarkably similar to that described for the rat. The principal cell types stimulated by the addition of glucose were the primary spermatocytes and spermatids, with the greatest degree of over-all stimulation occurring in the spermatids.

The fact that glucose exerts such a profound effect on the spermatids may offer a partial explanation for the atrophic appearance of the seminiferous epithelium associated with hypoglycemia. Any differences in protein biosynthesis of those cells of the spermatogenic cycle responsible for cell proliferation and renewal as compared to the more mature spermatids may have importance not only in investigations dealing with regulation of male fertility, but also in studies dealing with potential side-effects of drugs that may cause genetic damage with resulting congenital malformations.

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Dr. Casimir F. Firlit was born in Chicago, Illinois on December 7, 1939. He is married to the former Miss Sharon L. Chwierut, Chicago, Illinois and presently resides in Downers Grove, Illinois. Dr. Firlit received his undergraduate training at Loyola University, Chicago, Illinois and graduated with a B.S. degree in 1961. In 1961, he entered Loyola University Stritch School of Medicine and graduated in 1965 with the degrees of M.S. in Pharmacology and M.D. He entered internship (rotating) at Mercy Hospital Medical Center, Chicago, Illinois from 1965-1966.

Dr. Firlit is presently a resident physician (Urology) and a Research and Education Associate at the Veterans Administration Hospital, Hines, Illinois. His present training program began in September 1968 following a two year tour of active duty with the United States Navy (United States Marine Corps) in KheSanh, VietNam. In KheSanh, he organized an active Med Cap program for the Vietnamese and also a hospital infirmary for the indigent children. During the siege of KheSanh he served as Regimental Surgeon for the 26th Marine Regiment, coordinated and directed the medical activities of the 10 physicians and 250 corpsmen under his command. For his activities and duties during the siege, he was awarded by the Secretary of the United States Navy, the Purple Heart and the Bronze Star Award with the Combat "V".

While in undergraduate training (1957-1961), he began his research career with Dr. K. Kenneth Hisaoka and acquired experience in embryology and histological techniques. He mastered micro-technique and histochemical procedures for the preparation and interpretation of histological sections. He successfully utilized numerous histochemical procedures and became efficient in photographic microscopic techniques. During this period he held, jointly, authorship in six scientific publications in the areas of teleost embryology and ovarian histochemistry. He was awarded the Loyola University Award (Biology) for Undergraduate Research in 1960 and 1961.

From 1961-1965 he was actively enrolled in the combined M.S./M.D. program at the Stritch School of Medicine, Loyola University. His graduate research was under the guidance of Dr. Joseph R. Davis and was predominantly involved with testicular metabolism and the processes intimately associated with spermatogenesis. He actively utilized numerous biochemical, histological, histochemical, labeled-precursor and auto-radiographic techniques in his research. During this program of research he was jointly productive of eight published manuscripts.

In 1961 he was elected associate member to the Loyola University, Chapter of the Society of the Sigma Xi and presently holds full membership since 1965. In addition, he holds membership in the A.A.A.S., is a candidate member in the American College of Surgeons, and a regular member in the Society for the Study of Reproduction. In April, 1970 he was appointed Instructor in the Department of Pharmacology and Therapeutics, Loyola University. He currently holds medical licensure in the States of Illinois and California.

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CHAPTER I
INTRODUCTION

A number of investigators have studied the morphologic changes which occur in the cryptorchid testis of the rat (Campbell, 1942; Clegg, 1963a, 1963b; Gross and Jewett, 1957; Kiesewetter, 1959; Moore and Quick, 1924; and Niemi and Koromano, 1965), however, in all these instances, cryptorchidism was experimentally induced in adult animals by transplanting a previously descended testis from the scrotal sac to the abdominal cavity. The question arose in our minds as to the physiologic significance of such an experimental procedure for inducing cryptorchidism in animals inasmuch as cryptorchidism is known to occur in the human when an abdominal testis fails to descend normally into the scrotal sac.

The present experiments were designed to investigate the morphologic changes observed in cryptorchidism experimentally induced by fixation of an immature, abdominal testis to the dorsolateral abdominal wall as well as transposing a scrotal testis into the abdominal cavity of the adult rat. In producing cryptorchidism by these two different procedures it was possible to compare the histologic appearance of a cryptorchid testis induced by prevention of normal testicular descent with that induced by transplantation of a previously descended testis back into an abdominal environment.

Studies by Davis, Morris and Hollinger (1964 and 1965) have indicated that the incorporation of L-lysine-U-C¹⁴ into protein of slices of cryptorchid testes of the rat is markedly greater than the incorporation of L-lysine-U-C¹⁴ into protein of slices of scrotal testes obtained from the same animal. It is well known that the predominant histological feature of the cryptorchid testis is the absence of spermatids (Davis, et al., 1964). The question arose as to whether the increased protein labeling of the cryptorchid testis of the rat was, on the one hand, a reflection of this altered histological architecture with a resulting unmasking of the spermatogonia, primary spermatocytes and Serto-

cells, or, on the other hand, represented a true change in the protein-synthesizing capacity of the remaining testicular cell types of the germinal epithelium. The present experiments were designed to ascertain which of the cells of the seminiferous tubules of the rat testis remained 30 days after the experimental induction of cryptorchidism. The incorporation of tritiated lysine into protein of the remaining cells of the germinal epithelium of the abdominal testis was determined and a comparison made of the incorporation of tritiated lysine into protein of the corresponding cells found in the scrotal testis of the same animal.

Although numerous histological and biochemical studies have been performed on mature ejaculated spermatozoa, relatively few investigations have been performed on the metabolism of the spermatogenic cycle existing in the testis. It has been demonstrated that the effects of chronic, experimentally induced hypoglycemia in rats results in loss of germinal cellular maturation, suggesting the dependency of testicular cellular maturation upon glucose (Mancine, et al., 1960). Because of the highly complex nature of the process of cellular maturation precise metabolic functions of the various testicular cell types have been exceedingly difficult to ascertain.

In addition, although numerous cytological studies have been carried out on the meiotic behaviour of chromosomes, very little is known about protein synthesis occurring in cells undergoing meiotic division (Rhoades, 1961). Not only is meiosis the mechanism by which the diploid number of chromosomes is reduced to the haploid number of chromosomes found in the gametes, but also the recombination of chromosomal segments during the crossing-over phase of meiosis provides for constant changing of the cells genotype. The biochemical events associated with the transition from somatic mitosis to meiosis are almost entirely unknown. However, the definition by Leblond and Clermont of the cycle of the seminiferous epithelium of the rat in relation to the differentiation of the spermatids offers

an excellent opportunity to investigate this problem using radioautographic techniques. The present studies were designed to investigate the effect of exogenous glucose on protein labeling from L-lysine- H^3 in each of the successive cells of the cycle of the seminiferous epithelium of the rat as measured by radioautographic techniques.

Finally, the question as to whether the pattern of protein labeling in various successive cells of the seminiferous germinal epithelium of the rat would be similar in slices of human testis incubated with tritiated lysine will be presented. The problems of comparing experimental animal data versus human data are well known and the difficulties involved in performing dynamic biochemical studies on viable human testicular slices is no exception.

It is therefore the purpose of these studies to obtain information concerning metabolic aspects of the testicular germinal epithelium by specifically investigating protein anabolism utilizing radioautographic methods. It is hoped that the data obtained may be of value in treating clinical problems of male infertility.

TESTICULAR PROTEINS

Miescher (1897) reported the earliest studies on testicular proteins by observing changes in salmon sperm nucleohistones. Since then numerous histochemical studies describing nucleohistones in other species as well as localization of testicular enzymes have appeared in the literature. As a result of testicular enzyme localization, the complex histological pattern of the seminiferous epithelium has been widely investigated. However, still largely unexplored is the metabolism of the cells of the spermatogenic cycle. Although numerous biochemical studies have been performed on mature ejaculated spermatozoa, relatively few studies have been performed on the developing germ cells of the seminiferous epithelium.

1. Nuclear Proteins

A. HISTONES

The major proteins present in the nuclei of cells are histones, forming an essential constituent of chromatin. According to Zubay and Doty (1959), these proteins are chemically attached to the surface of deoxyribonucleic acid molecules (DNA) and comprise the outer surface of the chromosomes. The intimate association of histones with DNA molecules containing the genetic information required for cell division and self-perpetuation of species has provided increasing evidence in recent years to suggest that histones may play a role in the regulation of genetic expression.

One important feature of histones is their high content of basic amino acids. With the development of better separation techniques, histones have been chemically divided into four principal heterogenous fractions each of which can be further resolved into numerous minor histone fractions (Rasmussen et al., 1962). In comparing the amino acid content of histones from different tissues, Hnilica et al. (1962) have shown the same spectrum of histones in the thymus, spleen and liver of the calf. Similar results were obtained by Neelin and Butler (1961) for histones isolated from the chicken spleen, liver, kidney and heart. These data indicate that, in general, all cells in an animal may contain similar histones. One exception to this generalization involves nuclear proteins of the maturing germ cells of the testis.

B. HISTONE TRANSITION

During the latter stages of spermatogenesis the typically somatic-type histone, high in lysine content compared to arginine, is replaced by a new type of histone with a greater arginine content and higher basicity. In many species, this arginine-rich histone is replaced in the sperm by a nucleoprotamine. Protamines differ from histones in that they are short-chain polyamines containing mainly arginine and relatively few other amino acids, while histones have generally

a full complement of amino acids. According to Alfert (1956), protamines represent the extreme in basicity and simplicity of composition and structure of all the basic nuclear proteins.

The earliest reports of the chemical differentiation of nuclear proteins during spermatogenesis originated from the studies of Miescher (1897) and later from those of Kossel (1928). Their data indicated the presence of nucleohistones in the immature salmon sperm. More recent studies on salmon sperm by Alfert (1956), using the alkaline fast green method of Alfert and Geschwind (1953), have indicated that the change to a protamine-type nuclear protein occurs abruptly at a late stage of spermiogenesis. By extracting DNA and protamine with hot trichloroacetic acid (TCA) followed by histone staining with alkaline fast green, the stage of spermiogenesis when the fast green stainable histone is replaced by protamine can be detected by the absence of fast green staining in the spermatid nuclei.

Numerous other studies in vertebrates and invertebrates have described a transition in the basic nuclear protein of germ cells from the somatic lysine-rich type of histone to an arginine-rich histone, and finally to protamine in the maturing spermatozoa. The cytochemical methods used in addition to alkaline fast green are the Naphtol yellow S (Deitch 1955), Bromphenol blue (Block and Hew 1960a) and Sakaguichi reaction (McLeish et al. 1957; Deitch 1961). In some species of fish such as the genus Poecilia the transition from somatic type histones to protamines is so abrupt that the conversion may not involve a transition through an intermediate arginine-rich histone (Rasch et al., 1966). In other species, including the bull (Gledhill et al., 1966a), the grasshopper, Chortophaga viridifasciata (Bloch and Brack, 1964), and the fruit fly, Drosophila melanogaster (Das et al., 1964) the transition of nucleohistones is terminated at an arginine-rich histone stage. In the testis of the frog, Rana pipiens, however, Vendrely (1957) and Bloch (1962) have described no nucleohistone

transition either during spermatogenesis or in the mature sperm.

C. HISTONE SYNTHESIS

The shift to an arginine-rich histone in the nuclei of developing germ cells has been reinvestigated using radioautographic techniques and the results of such studies have suggested that the transition involves synthesis of a new histone rather than conversion of a protein initially present. Bloch and Hew (1960a) have shown in the maturing sperm of the pulmonate snail, Helix aspersa that the histone change is accompanied by incorporation of ^3H -arginine into spermatid nuclei. They also demonstrated, by comparing the timing of ^3H -arginine and ^3H -thymidine incorporation with independent DNA measurements, that histone synthesis was not accompanied by DNA synthesis--which is the normal case in premitotic chromosome duplication. In the mouse, Monesi (1964) has also demonstrated nuclear incorporation of ^3H -arginine at approximately the same time as the histone transition. In the nuclei of late spermatids, the incorporation of the label occurs as early as 15 minutes after administration beginning at stage 11 of spermiogenesis, increasing in stage 12, and reaching a peak in early stage 13 of spermiogenesis. Nuclear labeling then decreases through stage 14 and early stage 15, and is absent in late stages 15 and 16 of spermiogenesis. Injections of tritiated lysine, leucine, histidine, tryptophan, tyrosine and phenylalanine produced no corresponding spermatid nuclear labeling which supported the concept of a nuclear synthesis of an arginine-rich histone. In addition, the ratio of cytoplasmic to nuclear labeling was low indicating that the label was incorporated directly in the nucleus rather than in the cytoplasm and transferred later to the nucleus.

D. FINE STRUCTURAL CHANGES

Specific changes in the fine structure of the spermatid nucleus also parallel the replacement of the somatic-type histone. In the snail, fine filaments between 120 and 150A° in diameter are formed in the spade-stage spermatid nucleus, which

later aggregate into folded convoluted lamellar structures parallel to the long axis of the enlarging spermatid head (Bloch and Hew, 1960a). Later stages of spermatid development and maturing spermatophore sperm are characterized by a loss of these structures and appear relatively homogenous. This formation and aggregation of filaments into lamellae occurs at the same time as the histone transition to protamine. Dass and Ris (1958) have also reported the appearance of thin fibers in the nucleus of the grasshopper during mid-spermiogenesis. The authors have suggested that the thin fibers are derived from chromatin fibers by assuming that the fibers are comprised of DNA-histone molecules.

In the shell stage of developing cricket spermatids, a different phenomenon has been described by Kaye and McMaster-Kaye (1966). Thin nonhistone protein fibers, free of DNA and histone were formed in addition to the thicker chromatin fibers. The appearance of these nonhistone protein fibers occurred well before the transition to an arginine-rich histone and protamine. Their formation appeared to be by de novo synthesis and not derived from chromatin fibers since the loss of most of the granular nonhistone protein, eliminated from the nucleus earlier in spermatogenesis, did not affect the structure or diameter of the remaining chromatin fibers.

E. SIGNIFICANCE OF HISTONE CHANGE

Although a histone transition is a relatively general occurrence during spermatogenesis, the implications of the event are obscure. The timing of the transition occurs during the later stages of spermatid development and is constant in any one species, variable among different species, and terminates at either a protamine stage or an arginine-rich stage. The unique characteristic of a sperm in producing a DNA-protamine complex must be advantageous and necessary to the sperm for the successful fertilization and development of the zygote. Allfrey et al. (1963) have demonstrated that arginine-rich histones are capable of inhibiting the DNA-primed synthesis of messenger RNA in isolated calf thymus

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nuclei. Evidence is also accumulating to indicate that during early development up to the blastula stage, genetic activity is largely suppressed (Brachet and Denis, 1963; Gross and Cousineau, 1964; Gross et al., 1964). A histone transition could therefore be considered as providing a mechanism for the suppression of the genetic activity of cells. Any alteration or inhibition of this histone change could lead to infertility. Several reports of reduced fertility in men (Leuchtenberger et al., 1953) and in bulls (Leuchtenberger et al., 1956; Perez et al., Gledhill et al., 1966b) have suggested that the reduction in fertility may be due to a defective or immature sperm deoxyribonucleoprotein complex.

2. Cytoplasmic Proteins

A. RESIDUAL BODIES

A regular feature of the testis is the release of a large proportion of cytoplasm by the developing spermatids during the terminal events of spermiogenesis. Some of this cytoplasm is released in the seminiferous tubular lumen while the remainder is phagocytosed by the Sertoli cells. Regaud (1901) first described these detached cytoplasmic masses as residual bodies containing a large safranophilic mass, the sphere chromatophile. The content of this sphere chromatophile consists of ribonucleoprotein which becomes progressively concentrated into large basophilic globules (Daoust and Clermont, 1955) and glycogen (Firlit and Davis, 1965). The body arises from a fusion of numerous RNA-containing spermatid granules termed von Ebner granules (Regaud, 1901; Daoust and Clermont, 1955; Sud, 1961).

The protein of the sphere chromatophile has recently been suggested by Vaughn (1965, 1966) to be a nuclear protein derived from the lysine-rich histones which are displaced during the histone transition of the maturing spermatid nuclei. According to Vaughn, the histone transition begins at stage 13 of spermiogenesis in the rat (alkaline fast green), occurring

simultaneously with the appearance of basic protein granules (picric acid-bromphenol blue). In stages 15 and 16 of spermiogenesis, similar staining protein granules appear in the cytoplasm of the spermatids which coalesce to partially form the sphere chromatophile. In stage 17 and 18 of spermiogenesis, the homogenously distributed cytoplasmic ribonucleic acid (RNA) granules (azure B) coalesce to form the larger von Ebner granules. The majority of these granules localize in the sphere chromatophile of the spermatids found during early stage 19 of spermiogenesis. The precise origin of the basic protein of the sphere chromatophile could not, however, be deduced from cytochemical observations alone. Additional evidence has come from radioautographic and microspectrophotometric studies (Vaughn, 1966). The radioautographic data have indicated that the sphere chromatophile does not synthesize protein or RNA, nor is there any evidence of cytoplasmic synthesis of the basic protein. Microspectrophotometric data have shown that the amount of protein-bound lysine relative to the amount of RNA increases with increasing sphere chromatophile diameter. This indicates that the lysine and the RNA forming the body have different sources, rather than originating from the cytoplasmic ribonucleoprotein (RNP) granules. According to Vaughn, if both von Ebner granules and sphere chromatophile bodies arise by coalescence of ribosomes, the ratio of basic protein to RNA would be constant, but would not be constant if the basic protein component of the enlarging sphere chromatophile has a nonribosomal source. To demonstrate the hypothesis that the basic proteins of the chromatophile originate in the spermatid nucleus, two phases of nuclear lysine decreases were demonstrated. The first decrease in nuclear lysine content, amounting to 70% occurs between stages 9 and 12 of spermiogenesis, while the second decrease in nuclear lysine content occurs during the somatic-type histone replacement by arginine-rich histone. The first decrease in nuclear lysine content has

been stated by Vaughn not to involve histones since the histone transition has been noted to occur later in stage 13 of spermiogenesis. The second decrease in nuclear lysine content can only be demonstrated indirectly since no net change of lysine has been found to occur. The loss of lysine-rich histone is probably compensated for by the appreciable quantity of lysine contained in the newly synthesized, arginine-rich histone.

3. Protein Anabolism

A. TIMING AND SITE OF SYNTHESIS

Although numerous cytological studies have been carried out on the meiotic behavior of chromosomes, extremely little is known concerning protein synthesis occurring in cells undergoing meiotic division (Rhoades, 1961). Moreover, the biochemical events associated with the transition from mitotic to meiotic cell division are almost entirely unknown. The continuous nature of the spermatogenic cycle, beginning with a stem cell spermatogonium and ending with the mature spermatid has therefore made the radioautographic approach a unique opportunity to investigate protein labeling during the three main phases of spermatogenesis, namely, mitosis, meiosis and morphogenic differentiation.

In vitro incubation studies employing the incorporation of tritiated lysine into protein of the various cells of the seminiferous epithelium of the rat testis have indicated that the cells which incorporate the largest amounts of lysine into protein are the young primary spermatocytes (Davis and Firlit, 1965). The spermatogonia incorporated moderate amounts of tritiated lysine, with the highest degree of spermatogonial protein labeling occurring in type A spermatogonia, resulting from the second peak of spermatogonial mitosis at stage XII of the spermatogenic cycle. Immediately after the division of type B spermatogonia into resting primary spermatocytes, a marked increase in the degree of labeling over the resting primary spermatocytes occurs, followed by

a gradual decline in grain counts over the area of the transition primary spermatocytes, with little or no label appearing over the remaining cells of the cycle of the seminiferous epithelium. The in vivo incorporation of radioactive amino acids into mouse testicular proteins, followed by squash preparations of the isolated labeled seminiferous tubules, has been investigated by Monesi (1965). In spermatogonia, both nuclear and cytoplasmic protein synthesis has been found to occur at all stages of the cell division cycle following the intraperitoneal or subcutaneous injections of tritiated arginine, leucine, lysine, tryosine, histidine, phenylalanine or tryptophane. During interphase and prophase, the rate of protein synthesis is almost equal, but declines during late prophase, metaphase, and anaphase; this differs from ribonucleic acid (RNA) synthesis which ceases during metaphase and anaphase (Monesi, 1964). In the different types of spermatogonia, the rate of synthesis varies from being much greater in type A and intermediate as compared to that found in type B spermatogonia, and lower in dormant type A than dividing type A spermatogonia. A similar difference in the rate of synthesis between the different types of spermatogonia occurs for RNA synthesis and has been suggested by Monesi (1964) to be possibly due to the greater degree of nuclear condensation found in type B spermatogonia. Although protein labeling occurs in both the chromosomes and nuclear sap, incorporation into the nucleoli has not been determined because of their limited size in type A spermatogonia and their apparent absence in type B spermatogonia.

In primary spermatocytes, labeled amino acids are incorporated into both nuclear and cytoplasmic proteins at all stages of the meiotic cycle, with the greatest degree of labeling occurring in pachytene spermatocytes in stages II to V of the spermatogenic cycle. Chromosomal studies indicate a much greater incorporation occurring in the autosomal chromosomes than the sex chromosomes.

The presence of protein labeling in nucleoli, however, has been impossible to detect because of their small size during prophase. Monesi (1964) has suggested that a significant part of the chromosomal protein synthesized during meiotic prophase may be preserved within the chromosomes through cell division.

Cytoplasmic incorporation of tritiated amino acids occurs in most stages of spermiogenesis, with the highest amount occurring during stages 1 to 11 of spermiogenesis, then decreasing to zero in the mature spermatid found during stage 16 of spermiogenesis. Residual bodies shed from mature spermatids become labeled only after 3 days following injection of the isotope. Nuclear labeling is either small or absent during all stages of spermiogenesis except 11 and 14. Spermatid nuclei at these stages of development incorporate large quantities of ^3H -arginine, but are insensitive to the other tritiated amino acids. Incorporation of large quantities of ^3H -arginine demonstrates the timing of the nuclear histone transition as described above. Tritiated amino acids are readily incorporated in the cytoplasm and nucleus of the Sertoli cells. Rapid labeling of the nucleolus also occurs at about an equal density of labeling as the nucleoplasm. The satellite karyosomes which flank the nucleus however, are very poorly labeled. In contrast, Carneio and Leblong (1959) failed to observe any nucleolar incorporation of labeled amino acids in the Sertoli cells.

B. EFFECT OF TEMPERATURE

1. Incubation Temperature

Since the scrotal temperature is known to be decidedly lower than normal body temperature (Moore and Quick, 1923), studies were initiated by Davis et al. (1963) to determine a proper incubation temperature for investigating the in vitro incorporation of radioactive lysine into protein of rat testicular slices. Simultaneous incubations of testicular slices ranging from 20 to 40°C

were performed utilizing several temperature-controlled water baths surrounding a main Warburg apparatus. The manometers were arranged so that each flask was allowed to shake at the same rate during the incubation period. The results of the effect of temperature on the incorporation of radioactive lysine into protein of slices of adult rat testis, liver, kidney and spleen demonstrated that in the testis, maximal incorporation of lysine into protein occurred at 32°C. In the liver, kidney and spleen, maximal incorporation of isotope into protein occurred at 37.5°C. No radioactivity was found in protein when these tissues were incubated at 44°C. These data indicate that the protein-synthesizing systems of the rat testis are more sensitive to heat as compared to the protein-synthesizing systems of the rat liver, kidney and spleen. It would therefore appear that this increased heat lability of the protein-synthesizing systems of the testis may offer a partial biochemical explanation for the injurious effects of an increased abdominal temperature on spermatogenesis which occurs in cases of cryptorchidism.

In order to determine the viability of the rat testis slices incubated for 1 hour at various temperatures, Davis, et al. (1963) carried out studies employing uniformly labeled glucose as a precursor for the biosynthesis of CO₂ and glutamic acid. The amount of isotope transferred from radioactive glucose to CO₂ and glutamic acid in slices of rat testis was determined at 26, 32 and 37.5°C. In both instances, a linear increase of labeling with increasing temperatures of incubation was observed. It would therefore appear that slices of rat testes are still viable after a 1 hour incubation period at 37.5°C and that the enzyme systems responsible for protein biosynthesis in the testis may be selectively inhibited by an increase in temperature above the normal scrotal temperature.

2. Species Variation

Similar temperature experiments were repeated using slices of rabbit testes. During these experiments, the in vitro incorporation of radioactive lysine into rabbit testicular protein was found to be maximal, not at 32°C, but at 37.5°C (Buyer and Davis, 1966; Hall, 1965). By further studying the peritoneal and scrotal temperatures of the rabbit versus the rat, it was found that the scrotal and peritoneal temperatures of the rat were statistically lower than the corresponding temperatures of the rabbit. This difference in the effect of temperature on testicular protein labeling of the rat and the rabbit suggested that optimal testicular protein synthesis may occur at a temperature which is closest to that found in the scrotum of each animal. In the rat, mouse, and hamster, maximal incorporation of labeled lysine into testicular protein occurred at 32°C. On the other hand, maximal incorporation of isotope into testicular protein was at 37.5°C for the rabbit, guinea pig and dog, each having a higher peritoneal and scrotal temperature. It would therefore appear that optimal activity for protein labeling for each species of animal occurs at a temperature closest to that found in the scrotal sac and that the deleterious effects of temperature on the incorporation of lysine into testicular protein occur at a temperature closest to that of the normal body temperature of each animal.

C. EFFECT OF CRYPTORCHIDISM

1. General Effects

Cryptorchidism can be experimentally induced in the rat by gently forcing the testis into the abdominal cavity by applying pressure to the bottom of the scrotal sac. A fine silk suture is passed under the capsule of the testis and anchored to the lateral abdominal body musculature. The contralateral testis of the same animal is allowed to remain in the scrotal sac to serve as a control. The predominant histological feature of the cryptorchid testis

appears to be the absence of all of the spermatids and mature spermatozoa as well as most of the spermatocytes. However, the spermatogonia, Sertoli cells and the interstitial cells of Leydig appear to resist degeneration (Glegg, 1961; Davis and Firlit, 1966; Nelson, 1951; Niemi and Kormano, 1965; Williams and Cunningham, 1940).

The changes in testicular weights during a period of 30 days following abdominal fixation of the undescended prepubertal testis on the left and the abdominal transplantation of the adult testis on the right were studied. In both instances, the weight of the cryptorchid testis was markedly lower than that of the scrotal testis. In addition, the in vitro incorporation of L-lysine-U-C¹⁴ into protein of slices of the two different experimentally induced cryptorchid testes were studied. Protein labeling of both types of cryptorchid testes were found to be about four times as great as that of the normal scrotal testis (Davis et al. 1964, 1965).

The significance of a greater uptake of radioactive lysine into protein in the cryptorchid testis than that found in the normal scrotal testis appears to be at least partially associated with the complex histological structure of the testis. The concept that a tissue which was rapidly regressing in size should have an increase rate of protein synthesis seemed most unusual. Because of this unusual display of protein synthesis, it would seem extremely interesting to determine which of the several cell types of the seminiferous germinal epithelium of the testis were particularly susceptible to the effects of temperature on protein biosynthesis.

2. Temperature Sensitivity in Various Cell Types

Since it is not possible to physically separate and isolate all the cells of the germinal epithelium at the present time, the technique of studying various preparations of testes which consisted of only a few cell types offer one partial approach to this problem. Davis et al. (1964) made a comparison

of the incorporation of labeled lysine into protein of slices of four different testicular preparations. Davis demonstrated that the adult normal scrotal testis consists of spermatogonia, spermatocytes, spermatids and Sertoli cells as well as interstitial cells of Leydig. The cryptorchid testis consists of spermatogonia, Sertoli cells and Leydig cells, with only a few remaining spermatocytes and a total absence of spermatids. The immature testis consists largely of primary spermatocytes. An interstitial cell testicular tumor of the mouse consists of only Leydig cells. The effect of incubation temperature on the incorporation of L-lysine- $U-C^{14}$ into protein of slices of the adult scrotal testis the cryptorchid testis, the immature testis and the interstitial cell testicular tumor. With the exception of the adult scrotal testis which was the only testicular preparation containing spermatids, protein labeling in each testicular tissue studied increased with an elevated temperature of incubation (Davis et al. 1964). These results point to the possibility that the inhibitory effect of an increased temperature on testicular protein synthesis occurs mainly in the spermatids which are the more differentiated seminiferous germinal epithelial cells.

The question whether the increased protein labeling of the cryptorchid testis was a reflection of an unmasking of the spermatogonia, primary spermatocytes, and Sertoli cells resulting from the disappearance of the spermatids, or represented a true temperature-induced change in the protein-synthesizing capacity of these remaining testicular cell types. In an attempt to answer this question, the in vitro radioautographic incorporation of tritiated lysine into each of the remaining cells of the cryptorchid rat testis 30 days following abdominal transplantation of the testis was carried out at 37.5°C (Firlit and Davis, 1966).

Three types of seminiferous tubules are found in approximately equal numbers in the cryptorchid rat testis 30 days after abdominal transplantation and have been designated as type A, B, and C cryptorchid tubules. Type A tubules contain spermatogonia of the "crust" variety, primary spermatocytes in the pachytene stage of meiosis and Sertoli cells. Type B tubules contain only spermatogonia of the "crust" variety and Sertoli cells, with the cytoplasm of the Sertoli cell extending as an apparent syncytium from the basement membrane to the lumen of the seminiferous tubule. Type C tubules contain essentially Sertoli cells with pleomorphic-appearing nuclei and fibrotic, hyalinized cytoplasm. Only four cell types therefore were found to occur in the germinal epithelium of the abdominal testis 30 days following experimental induction of cryptorchidism. These cell types were "crust" spermatogonia, pachytene primary spermatocytes, normal-appearing Sertoli cells and atrophic-appearing Sertoli cells, accounting for 31, 3, 28 and 38% respectively of the total cells remaining in the germinal epithelium of the seminiferous tubules of the cryptorchid testis. Grain counts over these respective cell types demonstrated that the induction of experimental cryptorchidism produced a marked increase in protein labeling in each of the remaining cells of the germinal epithelium. This data would indicate that the increase in protein labeling of the cryptorchid rat testis is due not only to an unmasking of the remaining cell types with a higher capacity for protein synthesis but is also due to a temperature induced stimulation of protein synthesis occurring in these same testicular cell types.

D. EFFECT OF GLUCOSE

1. Stimulation

Although glucose has been shown to maintain the oxygen uptake of adult testicular tissue (Ewing et al., 1966a; Paul et al., 1953; Schuler, 1944; Serfaty and Boyer, 1956; Tepperman et al., 1949), few studies have been carried out on the effect of this substrate on testicular protein biosynthesis. Davis

and Morris (1963) studied the effect of glucose on the incorporation of L-lysine- $U-C^{14}$ into protein of rat tissue slices at $37.5^{\circ}C$ and one hour incubation. In the case of the testis, the addition of $0.009M$ glucose to alternate flasks increased the incorporation of radioactive lysine into protein by over 600%. Protein labeling in slices of the head of epididymis was enhanced by approximately 150% and values for the remaining tissues studied ranged from essentially no change to only 50% stimulation of protein labeling including jejunal mucosa, the Walker 256 carcinosarcoma, spleen, thymus, submaxillary gland, pancreas, tail of the epididymis, kidney, brain, diaphragm, heart, regenerating liver, normal liver, lung, and seminal vesicle. It therefore appears that with respect to 17 different tissues of the adult rat, glucose exerts an apparently unique and characteristic enhancement of testicular protein biosynthesis. These data indicate that the enzymic systems involved in the incorporation of labeled lysine into protein of the testis are markedly more sensitive to the addition of exogenous glucose than those of other tissues of the rat.

The importance of glucose in maintaining the normal morphological appearance of rat testes has been emphasized by reports of histological damage of the seminiferous epithelium occurring in the presence of either experimentally induced hypoglycemia (Mancine et al., 1960) or alloxan diabetes (Deb and Chatterjee, 1963; Hunt and Bailey, 1961). In addition, male rats, rendered diabetic by pancreatectomy have been found to exhibit a decrease in fertility (Foglia et al., 1963). Reproductive function in the human male has also been shown to be markedly influenced by the diabetic state with its associated presence of hyperglycemia. The diabetic human male has been reported to have an increased incidence of impotence (Babbott et al., 1958; Rubin, 1958), a decreased sperm count (Schoffling et al., 1963), a deficiency of spermatozoa motility (Klebanow and Macleod, 1960), and atrophic changes in the seminiferous

germinal epithelium of the testis (Schoffling et al., 1963).

2. Site of Stimulation

The affect of exogenous glucose on the incorporation of radioactive lysine into protein of slices of four different testicular preparations was studied by Davis and Morris (1963). The absolute level of protein labeling of the immature testis, the cryptorchid testis and the interstitial cell testicular tumor were all greater than in the case of the adult scrotal testis, these tissues, which in no instance contained any spermatids, displayed essentially no effect of glucose on protein labeling as compared to the scrotal testis. These data suggest the possibility that the spermatids are the most sensitive to glucose with regard to regulation of protein synthesis.

In order to further investigate this possibility, as well as to compare protein labeling in mitotic versus meiotic cell division on the effect of exogenous glucose on protein labeling from tritiated lysine in each of the successive cells of the cycle of the seminiferous epithelium of the rat was studied in vitro by means of radioautographic techniques (Davis and Firlit, 1965).

Slices of normal adult rat scrotal testes were incubated in Kreba-Ringer bicarbonate buffer with 100 μ Ci of tritiated lysine in the presence and absence of exogenous glucose. At the end of the incubation period, the flask contents were poured through a 90-mesh stainless-steel sieve to collect the testicular slices which were then processed for radioautography.

In the glucose-supplemented system a marked increase in the overall degree of protein labeling from tritiated lysine was observed in all of the cells of the spermatogenic cycle. However, in contrast to the results obtained in the control system early pachytene primary spermatocytes were found to contain the most dense grain distribution in the presence of added glucose. During the two meiotic cell divisions a sharp decrease in the grain density was observed.

Whereas few grains were seen over the spermatids in the control system, the addition of exogenous glucose was found to produce a marked increase in the number of grains appearing over the spermatids.

The data indicate that of the various cell types found in the seminiferous epithelium of the rat, the primary spermatocytes demonstrate the highest degree of protein labeling from radioactive lysine. On the other hand, protein labeling of the spermatids appears to have the greatest sensitivity to the addition of glucose.

Further evidence which serves to support the finding that a major stimulatory effect of glucose on testicular protein labeling involves the spermatids is supported by Means and Hall (1968a) who found that marked stimulation of testicular protein labeling by glucose did not occur in rats before the age of 28 days. It is of interest to note that these biochemical results correlate with the previous histological studies of Clermont and Perey (1957) which demonstrated that spermatids first appear in the germinal epithelium of rats at approximately 20 to 30 days of age. Means and Hall also reported a gradual decrease in the stimulation of protein biosynthesis in hypophysectomized rats such that at 15 days after the operation, the response to glucose was only 50% of the control level and by 25 days there was no stimulation of protein biosynthesis by glucose. Spermatids have been reported to decrease in number following hypophysectomy to about 50% at 15 days post-operatively (Coombs and Marshall, 1956) and to disappear by 25 days after the operation (Steinberger and Nelson, 1955).

The data also suggest that glucose exerts a marked stimulation of protein labeling in the primary spermatocytes whose nuclei are at pachynema and whose chromosomes are undergoing crossing over. The possibility therefore exists that glucose may be involved in the mechanisms of recombination of chromosomal

segments during meiosis, thereby playing an important role in the constantly changing patterns of the genome (Davis, 1968). In addition, the data also suggest that the transition from somatic mitosis to the first meiotic prophase is associated with a marked increase in protein labeling.

3. The Protective Effects of Glucose

It is well known that the effect of temperature on biochemical systems is a complex phenomenon which involves not only changes in enzyme reaction rates, but also involves enzymic denaturation. In order to more accurately establish the temperatures above which testicular protein labeling is heat labile, preincubation experiments were carried out in which the effects of temperature on slices of rat testes were exerted prior to the period of incubation with the radioactive amino acid (Davis and Morris, 1967). In the absence of glucose during the preincubation period, the incorporation of lysine into protein remained constant following preincubation temperatures ranging from 30 to 32°C for the testis and 30 to 38°C for both the spleen and liver. A progressive decrease in protein labeling then occurred at a preincubation temperature of 34°C for the testis and 40°C for both the spleen and liver. When added glucose was present during the preincubation period, the incorporation of labeled lysine into testicular protein now remained constant following preincubation temperatures which ranged from 30 to 36°C. These data indicate a partial reversal of temperature-induced inhibition during the preincubation period. On the other hand, the presence of glucose during the preincubation period produced essentially no change in protein labeling in slices of spleen and liver at any of the preincubation temperatures studied. When glucose was added after the preincubation period simultaneously with the radioactive lysine a marked progressive decline in testicular protein labeling was noted following preincubation at each of the seven temperatures studied. It would appear that glucose is not capable of reversing the temperature induced inhibition of

of testicular protein labeling if added after exposure of the testis to these preincubation temperatures. In the case of the spleen and liver, the addition of glucose after the preincubation period resulted in a marked decrease in protein labeling only after preincubation at a temperature of 42°C.

The data presented suggest that the in vitro inhibition of rat testicular protein labeling caused by temperatures higher than the normal scrotal temperature of 32°C can be protected against by the addition of glucose. Glucose protection against elevated temperatures has been observed in various species of microorganisms (Allen, 1950). Escherichia coli exposed to a temperature of 54°C for several minutes in the presence of glucose have been found to undergo less thermal destruction than in the absence of glucose (Baumgartner, 1938). The tolerance of chicken hearts to elevated temperatures in tissue culture has also been shown to be increased by the addition of glucose to the culture medium (Hetherington and Craig, 1939). The possibility therefore exists that temperatures exceeding 32°C may lead to the rapid utilization of glucose by the maturing spermatids which have been shown to be most dependent upon glucose. This increased utilization of glucose by the maturing spermatids will in turn lead to a rapid depletion of available glucose levels in the testis. The resulting inadequate supply of glucose will be incapable of protecting certain enzymes associated with protein synthesis against an elevated temperature, leading to an irreversible damage to the testicular protein-synthesizing systems of the rat (Davis and Morris, 1967).

Means and Hall (1968b) have recently suggested that the mechanism by which glucose stimulates testicular protein biosynthesis may be by maintaining the levels of testicular adenosine triphosphate (ATP). These authors demonstrated that glucose prevented the decline of ATP during aerobic in vitro incubations, but under anaerobic conditions, glucose was unable to

stimulate increases in ATP concentration or protein biosynthesis. In addition, both the concentration of ATP and rate of protein biosynthesis increased when glucose was added to the incubation medium after incubating for 30 minutes without glucose. In immature rats, glucose was found to have no effect on either the level of ATP or the rate of protein biosynthesis.

E. EFFECT OF DRUGS

1. Nitrofurazone

Davis and Hollinger (1966) studied the effect of nitrofurazone on the in vitro incorporation of radioactive lysine into testicular protein. The nitro-furan group of chemotherapeutic agents has long been known to exert specific histological changes in the testicular germinal epithelium involving the arrest of spermatogenesis at the primary spermatocyte stage (Nissim, 1957; Prior and Ferguson, 1950; Rogers et al., 1956). The histological architecture of the nitrofurazone treated testis of the rat therefore closely resembles that of the cryptorchid rat testis in that it is characterized by a virtual absence of all the spermatids and mature spermatozoa. It would seem that the loss of spermatids and spermatozoa from a nitrofurazone treated testis offers a unique opportunity to make selective study of the metabolism of the remaining cells of the seminiferous epithelium as they exist in the natural environmental temperature of the scrotal sac rather than as they exist after exposure to the high environmental temperature of the abdominal cavity as is the case in experimental cryptorchidism.

The effect of 0.1% nitrofurazone in the diet for 30 days on the weight of the rat testis demonstrated a progressive decrease in testicular weight, the uptake of labeled lysine into testicular protein was found to progressively increase up to 30 days. At 30 days of nitrofurazone treatment, the uptake of isotope into testicular protein was approximately four times that observed for

testicular slices of nontreated control rats (Hollinger and Davis, 1966). It would therefore appear that the remaining cells of the seminiferous germinal epithelium, namely, the spermatogonia and early primary spermatocytes, found at the lower physiological environmental temperature of the scrotal sac after the administration of nitrofurazone are responsible for the observed increase of testicular protein labeling. The possibility may therefore exist that the clinical administration of nitrofurazone might produce subtle biochemical side effects on testicular protein biosynthesis which would not readily be detected employing ordinary histological testicular biopsy techniques.

2. Chlorpromazine

The effect of chlorpromazine on endocrine function has been studied extensively and reviewed by deWied (1967). Histologically, chlorpromazine appears to interfere with testicular function, causing a similar reaction to that seen after hypophysectomy (Chatterjee, 1965, 1967). Only recently, however has a direct effect of chlorpromazine on testicular protein synthesis been reported (Kirby et al., 1969). By comparing the in vitro effect of chlorpromazine on the incorporation of radioactive lysine into protein of slices of rat testis, kidney, brain and liver, it was possible to demonstrate that chlorpromazine causes a greater inhibition of testicular protein labeling than that of brain, kidney and liver. At a concentration of $10^{-4}M$, chlorpromazine resulted in a 54% inhibition of testicular protein labeling, while causing only a 24% inhibition of protein labeling of both brain and kidney, and essentially no effect in the liver. Protein labeling of the chlorpromazine-inhibited system of the testis can be more markedly stimulated by the addition of exogenous glucose than that of the other three tissues studied.

F. THE EMBRYOGENESIS OF THE MALE GENITAL SYSTEM (HUMAN)

The male genital tract is derived from the urogenital ridge. It begins to differentiate at about the sixth week of fetal life. The medial portion of the urogenital ridge proliferates to form a specialized genital ridge which parallels the mesonephric urinary ridge. During the second month of fetal development the genital ridge of mesenchyme differentiates into an outer, germinal epithelium and an inner, loosely arranged epithelial mass. At this stage in embryological development there is no sex differentiation and the primitive gonad is intersexual. By differential rates of growth and by more marked proliferation of the caudal end of this ridge, a recognizable intraceolomic testis is produced. It comes to lie just above the pelvis by approximately the third month of fetal development, a process termed the internal descent.

The further passage of the testis into its definitive adult position in the scrotum occurs in the 8th-9th months of fetal development and is designated as the external descent. The internal structures of the testis develop during the 3rd month of gestation. The external germinal epithelium differentiates into the tunica albuginea and vasculosa of the testis. The inner epithelial mass of included totipotential cells give rise to the tubular structures of the testis including the rete testis. At the time of birth, a well developed branching system of seminiferous tubules is present. These still lack a lumen and continue as solid cords until puberty. At puberty, the testis cords acquire lumens and undergo further maturation. Differentiation of secondary spermatocytes into spermatids and spermatozoa, which begins at this age, characterizes the adult form of spermatogenesis, which continues throughout life into extreme old age.

The duct system of the testis is derived from the mesonephric ducts, in reality the persistent pronephric ducts. Special ducts, therefore are not

formed along with the development of the testis. However, during the early stages of undifferentiated sexual development, a Mullerian female duct is also formed, which in the male almost completely regresses except for the extreme cephalad end that persists as the appendix of the testis and the extreme caudal end that gives rise to two vestigial structures in adult males known as the seminal colliculus and the prostatic utricle.

The external genitals begin to appear at about the sixth week of fetal life in the form of a conical protuberance in the midline of the body about midway between the umbilical cord and tail. This protuberance is designated as the genital tubercle. In the course of time it will develop a shallow ventral groove with lateral ridges that fuse to create a urethral canal. Progressive growth of this tubercle creates a cylindrical phallus that gives rise to either the penis in the male or the clitoris in the female. As with the primary gonad, the genital tubercle is a sexually undifferentiated structure that may give rise to either male or female external genitalia (Arey, 1965).

G. GROSS AND MICROSCOPIC MORPHOLOGY OF THE ADULT HUMAN TESTIS

In the adult the testis weighs approximately 12 gms. and measures approximately 5 x 3 x 3 cm. It is covered by a serosal membrane, the visceral layer of the tunica vaginalis, which is reflected off the hilus of the testis to form an inner lining of the scrotal sac, the parietal tunica vaginalis. Thus, in the normal adult, there is a completely closed serosal-lined potential space folded about the testis. The lining of this space is derived from the processus vaginalis of the peritoneum. Beneath the visceral tunica vaginalis there is a thick fibrous layer known as the tunica albuginea. Deep to this fibrous layer is the tunica vasculosa, which is in reality merely a condensation of loose areolar tissue and blood vessels on the inner surface of the

tunica albuginea. All the arteries, veins, lymphatics and nerves enter through the hilus. The epididymis is a concentric tubular organ lying along the posterolateral border of the testis. The rete testis (tubules) drain into the collecting tubules of the head of the epididymis, while the tail of the epididymis communicates with the vas deferens.

Histologically, the testis consists of branching tubular glands separated from adjacent glands by a scant, loose connective tissue stroma. These glands have distinct basement membranes that increase in thickness with age. The testis is divided into many lobules by condensed fibrous septums which all radiate from the hilus of the testis. Within the testicular tubules are found the sustentacular supporting cells and the maturing germinal epithelium that has already been mentioned in the preceding section on embryogenesis. Within the stroma there are scattered groups and nests of round to polygonal, epithelial-like Leydig cells having abundant acidophilic cytoplasm and large nuclei that contain coarse chromatin granules, and long, slender, crystalline structures, the crystalloids of Reinke. These interstitial cells are known to be a site of formation of androgens. The number of these Leydig cells bears an inverse ratio to the preservation and activity of the tubular germinal epithelium. In advanced age or acquired disease, with atrophy of the germinal epithelium, there is a reported proliferation of the Leydig cells.

H. THE MORPHOLOGY OF SPERMATOGENESIS

The mitotic phase of spermatogenesis occurs throughout the divisions of the spermatogonia. The significance of this phase is considered to be threefold. First, through multiplication of cells the foundation is laid for a large cell population to be discharged into the seminal fluid. Secondly, during the multiplication phase most of the spermatogonia progressively change in order to ultimately undergo meiosis. Thirdly, it is in the process of

multiplication of spermatogonia that the pattern of the spermatogenic cycle emerges. The number of spermatogonia which develop synchronously into mature spermatids and the number of cells which are set aside at definite times as stem cells determine to a large extent the morphological pattern of spermatogenesis (Roosen-Runge, 1962).

1. Spermatogonia

In the early days of research on spermatogenesis, spermatogonia were rarely identified with precision. Even after von La Valette St. George (1876) had named them, they were often confused with Sertoli cells because of their identification being primarily one of position in the periphery of the tubule.

The first elaborate description of spermatogonia in the rat led to a most significant conclusion, namely, that the term spermatogonium may be applied to several distinct generations of cells. Regaud (1901) described a first generation of cells as "dusty" because of the fine distribution of chromatin in their nuclei, and a second generation resulting from mitoses of the first as "crusty" because of their coarse chromatin granules. The dusty cells were found to be larger and Regaud demonstrated that their nuclei stained with safranin if fixed in acetic bichromate. The crusty cells were smaller, and their nuclei stained with hematoxylin if fixed in acetic bichromate.

Regaud stated that the best way to account for the numerical relationship between dusty and crusty spermatogonia and spermatocytes would be to assume, as a dominant mode of division, true mitosis for the dusty cells but amitosis for the crusty spermatogonia.

Allen (1918) also described the spermatogonia of the rat, dividing them into Type A spermatogonia and Type B spermatogonia. The latter he interpreted as very early spermatocytes in which the reorganization of the nucleus from mitotic telophase to meiotic prophase occurred.

Roosen-Runge (1952 and 1962) and Roosen-Runge and Giesel (1950) deduced that Type A spermatogonia divide at several different stages and finally after 2-3 divisions change into cells of Type B, which still undergo mitotic division.

Clermont and Leblond (1953, 1964 and 1968) were able to distinguish three kinds of spermatogonia: Type A; intermediate; and Type B. They reported five peaks of mitotic activity. A correlation of spermatogonial counts with an exact determination of the stages of spermatogenesis enabled these authors to describe the development of spermatogonia in the following manner: In the beginning of each spermatogenic cycle a spermatogonium of Type A gives rise to two spermatogonia, and these in turn give rise to four Type A spermatogonia. One of the four Type A spermatogonia becomes a "dominant" cell which will not divide until the next cycle. The other three Type A spermatogonia divide again in a synchronous fashion giving rise to six intermediate spermatogonia; these divide once more to form 12 cells known as Type B spermatogonia. When these divide, they give rise to 24 cells which Clermont and Leblond (1952) have designated as "resting spermatocytes" because they are post mitotic as well as being premeiotic. Morphologically, they are indistinguishable from Type A spermatogonia, but their position in relation to germ cells in other stages has permitted their correct identification.

a. Glycogen

An opportunity for comparing the glycogen content of the spermatogonia of many mammals has been demonstrated (Nicander, 1957). Nicander found no glycogen in any of the spermatogonia of the mouse, very little glycogen in other rodents and in the dog, and a large quantity of glycogen in the spermatogonia of the bull and human testis.

Kramer (1960) has described the spermatogonia of the bull in considerable detail. He recognized the Type A cells which in contrast to those found in

rodents and many other mammals contained granules which stained conspicuously with periodic acid-Schiff's (PAS) reagent.

Montagna and Hamilton (1952a and 1952b) utilizing histochemical methods on biopsy specimens of adult human testis demonstrated the presence of glycogen. They localized glycogen within the germinal cells of the seminiferous epithelium, namely the spermatogonia, primary spermatocytes and the Sertoli cells. The quantity of glycogen was considered to be of higher concentration than that found in the bull.

2. Spermatocytes

Data on the development of the primary spermatocytes has been extremely sparse. Merkle (1957) measured the nuclei of germ cells in the rat throughout their development and found that the nuclear volume of the primary spermatocyte increased four-fold from the stage immediately following the last spermatogonial mitosis to that immediately preceding meiotic metaphase.

Watson (1952) attempted to measure the growth of the primary spermatocyte employing the electron microscope and arrived at the conclusion that in the rat these cells show little growth until the beginning of the zygotene stage, after which they grow steadily and rapidly until they divide.

Daled (1951) investigated the RNA content of spermatocytes as demonstrated by the pyronine staining and found that the cytoplasmic RNA increased gradually during the early pachytene stages and then decreased until cell division took place. He suggested that this finding implied that an increase of RNA is a determining factor for the first meiotic division.

Daoust and Clermont (1955) described various changes occurring in the nucleoli of the spermatocytes. An increase in number of nucleoli occurred during the leptotene and zygotene stages, as well as an increase in size during the early pachytene stage. The nucleoli of the spermatocytes were

found to stain deeply with pyronine-Y at this stage of meiosis. In the later pachytene stages the nucleoli appeared to be spherical to ovoid and to have a well-defined RNA-negative center which expanded until it occupied most of the area of the nucleolus in the diplotene stage. At this time only a very thin rim of RNA-positive material was left. The evidence appeared to be good that the amount of RNA increased markedly in the early primary spermatocyte almost completely disappeared from both the cytoplasm and nucleus.

Montagna and Hamilton (1952) found that the early spermatocytes in man contained considerable amounts of glycogen, but that the glycogen disappeared in later stages of the primary spermatocytes. No glycogen was found in the secondary spermatocytes. These findings seemed to point to a rapid utilization of carbohydrate during the period of volume growth of the primary spermatocyte.

3. Spermatids

Very few studies have been carried out on the loss of cytoplasm from the spermatid. After their formation they show no tendency to grow (Regaud, 1901). In the rat their cytoplasmic volume remains unchanged until the time when the next generation of spermatids undergoes formation. At that time the older generation of spermatids has reached the early acrosome phase, during which both nucleus and cell elongate. It appears certain, however, that the spermatozoon has less than 25% of the volume of the early spermatid (Leblond and Clermont, 1952).

The loss of cytoplasm by the spermatid is a conspicuous phenomenon because the degenerating cytoplasm stains intensely with hematoxylin, fat stains and pyronine. Regaud (1901) originally described the development of these cytoplasmic remains, and named them "the residual bodies". Daoust and Clermont (1955) have demonstrated that the RNA of the spermatids is collected into larger and larger granules, until it is set free in the residual bodies shortly before the release of the mature spermatozoa from the seminiferous

Lacy (1960) has studied the residual bodies with both light and electron microscopy and has found that in addition to containing lipid bodies and a mass which contains numerous RNA particles, the residual bodies also include some mitochondria which tend to fuse with each other to form membranous bodies.

4. Sertoli Cells

The structure of the nurse cell in the mammalian testis has presented morphologists with a considerable challenge. In general, the original observations on fresh and macerated testicular material made by Sertoli and his immediate followers have revealed more than have most of the subsequent studies which have been carried out using fixed preparations.

Sertoli's (1865) first paper on the cells which were named after him is frequently quoted in the literature without the full appreciation that this contribution contains the major part of all that is presently known about the structure of this cell in the testis. Sertoli's experimental technique involved a slow dissociation of the seminiferous tubules of the testis. His best results were obtained by leaving pieces of human testis in a solution of mercuric chloride (0.15%) for several days. Sertoli described the nurse cells of the testis as having a large nucleolus as well as containing many fat droplets in the cytoplasm, which varied in amount and distribution. He regarded the cells as individual units, not as a syncytium, and emphasized that they did not generate germ cells.

The first investigation of spermatogenesis which extensively used embedded sections only served to confuse the issue of the supporting cells of the testis (von Ebner, 1871). Von Ebner considered the cells interdispersed between the germ cells to be syncytial and derived from leukocytes. Von La Valette St. George (1878) defended Sertoli's original ideas and the controversy was carried on for many years. The old controversy whether there

is true 'copulation' between spermatids and supporting cells has been unequivocally settled by means of the electron microscope which has failed to demonstrate a true union between these two testicular cell types (Burgos and Fawcett, 1955).

Histochemical methods have confirmed the cyclic nature of the metabolism in the Sertoli cell. Von Ebner (1888) first demonstrated in the rat that fat occurs in large masses in the basal parts of the Sertoli cells immediately after the release of spermatozoa into the seminiferous tubular lumen. During the morphological transformation of the spermatids, fat appears in the more central parts of the Sertoli cells in dustlike particles. Toward the end of spermatogenesis the cytoplasm of the spermatid begins to show increasing amounts of fat. Von Ebner (1888) considered that these findings demonstrated a nutritional current supplying the spermatids. According to his view, not only were the Sertoli elements essential in producing this current, but they retrieved the fat used by the spermatids and transformed it for re-use. Kunze (1922) emphasized the heterogenous character of the substances which had until that time been largely described as 'fats of the testis'. He found extratubular and intratubular fatty substances to be essentially identical in many species of mammals and in each case, found mixtures of natural fats, cholesterol esters and phospholipids to be present. Montagna and Hamilton (1952) used a variety of tests for lipids and demonstrated large droplets in the basal part and dustlike fat in the central part of the Sertoli cells.

Leblond and Clermont (1952) first described in the Sertoli cell of the rat, mouse, hamster and guinea-pig PAS-positive material. In all these animals there were cyclic changes in the amount and distribution of PAS-positive granules. These were most clearly observed in the mouse, where it was shown that PAS-stained material in the Sertoli cells is at a minimum soon

after the appearance of a new generation of spermatids. During the formation of the acrosome many fine granules appear between the Sertoli nuclei and the heads of the spermatids. Leblond and Clermont suggested that these granules may indicate the passage of nutritional material from the Sertoli cells to the acrosomic system of the spermatids. Clermont (1954) gave a more detailed account of this PAS-positive material in the Sertoli cells of the hamster. Cyclic changes were quite prominent and the largest part of these granules were shown to be glycogen. The glycogen diminished abruptly at the time that the heads of the spermatids elongated and did not increase again until the beginning of the acrosome formation of the next generation of spermatids.

Nicander (1957) investigated the glycogen content in the testis of horses, bull, sheep, dog, cat, rabbit, guinea-pig, rat and mouse. He found cyclic changes in tubular glycogen in all animals except bulls and mice. Most tubular glycogen was present in the Sertoli cells except in bulls, cats, and rats. Generally, the highest concentration was seen in the early stages of the spermatogenic cycle, the lowest in late stages of the spermatogenic cycle, but in stallions and cats, these conditions were essentially reversed. Nicander stated that the glycogen of the Sertoli cells appeared to be utilized in the course of spermiogenesis.

I. CRYPTORCHIDISM

Cryptorchidism is synonymous with undescended testes. This abnormality involves complete or incomplete failure of the intra-abdominal testes to descend into the scrotal sac. From the preceding account of the embryogenesis of the male genital ridge, it is apparent that the testis arises within the coelomic cavity, and then by differential growth of the body, as well as more rapid proliferation of the caudal end of the urogenital ridge, the testis comes to lie within the lower abdomen or brim of the pelvis, a process referred to

as the internal descent. Following this, the testis descends through the inguinal canal into the scrotal sac, a process referred to as the external descent. On the basis of this descriptive account of testicular descent, malposition of the testis can occur at any point in this pathway of descent (Wells, 1943). The following anatomical factors either singly or in combination, may predispose to testicular maldescent (Eccles, 1902).

1. An unusually long mesorchium (testicular mesentery), which allows the gland undue intra-abdominal freedom and renders engagement in the internal inguinal ring less likely (Sohval, 1954).
2. Mesorchial peritoneal adhesions (Scorer, 1964).
3. An abnormal persistence of the plica vascularis, which may anchor the testicle high (Abel and Van Dellen, 1949).
4. Short spermatic vessels or vas deferens (Burdick and Coley, 1926).
5. The diameter of the testis and epididymis greater than that of the inguinal canal (Hunter, 1926).
6. Testicular fusion (McManus, 1948; Moore and Tapper, 1940; Reveno and Paluvinska, 1966; Von Lenhossek, 1845).
7. Absent, unusually long, or inactive gubernaculum (Wells, 1944; Wells, 1945).
8. Cremasteric hyperactivity interfering with descent (Robinson and Engle, 1954).
9. Maldevelopment of the inguinal canal (Kiesewetter, 1959).
10. Scrotal maldevelopment, absence of a testicular cavity (Kunstadter, 1952).

Adhesions resulting from fetal peritonitis have been reported as a cause of testicular retention. Whether the peritonitis is the basic factor remains unproved, but during the performance of surgery for imperfectly descended

testes that did not descend with normal therapy, it has been regularly noted that the testis is firmly bound in position by dense adhesions.

Elongation or absence of the gubernaculum testis has repeatedly been adducted to explain imperfect descent, but what part the gubernaculum plays in the process is unknown; the excellent studies of Wells and State (1947) suggest that the gubernaculum is probably of no importance except to guide the descending testis. In the newborn, the testis together with its fascial coverings may be lifted out of the scrotum without tearing anything but a little superficial connective tissue (Hunter, 1926); this observation casts much doubt on the conception of the gubernaculum acting as a traction band and drawing the testicle downward.

The concept that inguinal cryptorchidism results from faulty muscular development in the groin coupled with a failure of the gubernaculum to adjust itself to the muscular growth has also been advanced. When the transversalis and internal oblique muscles and the adjoining tendon are underdeveloped, the testis is retained high in the inguinal canal. However, when the external oblique muscle is anomalous, low testicular retention occurs. These observations have been observed in clinical cases of testicular maldescent (Browne, 1949; Coplan, et al, 1957; Counseller, et al, 1940).

A scarcity or even absence of cremasteric muscle fibers in the cryptorchid testis has been held by some to be the essential mechanism (Moore, et al, 1940; Thompson and Johnson, 1960). Hart (1966) noted and described that the cremasteric muscle in marsupials exerted a milking action on the testes, while the gubernaculum serves only as a guide (Hart, 1966). Lewis (1948) in experimenting with rats, noted that division of the crural nerve before eight days of age caused unilateral failure of testicular descent and concluded that the defect resulted from cremasteric muscle paralysis.

During the past three decades, experimental studies and clinical observations have indicated that endocrine factors play the most important role in descent of the testicle. The fact that the testes sometimes descend spontaneously at puberty lends weight to the probably relationship of endocrine (androgen) factors to migration of the gland. To date, the precise mechanism by which the androgenic steroids of the secreting fetal testes, under the control of maternal gonadotropins, bring about testicular descent is unclear. In addition, the fetal testis must be physiologically normal in respect to its androgen production and secretion; and without any intrinsic defect, or imperfect descent of the testes as well as diminished masculinity may result (Wells, 1943, 1944; Wells and State, 1947; and Hilscher, 1969).

Deming (1937, 1939, and 1951) showed experimentally in the immature rhesus monkey that, following the injection of chorionic gonadotropin, the testis increased 50% in size because of testicular and interstitial cell enlargement. In addition, tubular enlargement of the epididymis was also observed.

Before puberty chorionic gonadotropin is present in greatest amount in the fetus, becoming almost absent in boys from birth to about 10 years of age, then again becoming present in large amounts during adolescence and continuing so until about the 40th year when its quantity declines.

1. Symptoms Related to Cryptorchidism

In cryptorchidism symptoms are either local, endocrine and or psychologic. These are largely dependent upon the age of the patient and whether one or both testes are involved. Except for the absence of the testes from the scrotum or a complicating torsion of the spermatic cord, the undescended testicle generally causes no manifestation in the infant (Deming, 1941). However, in older boys there may be severe cramplike pains in the testicle,

even associated with episodes of vomiting. Often this reflects torsion of the spermatic cord. Inguinal testes may be painful when the legs are crossed, or local discomfort may reflect an accompanying hernia (Boggon, 1933). As testicular atrophy occurs and particularly in the ectopic organ, especially perineal, the testes becomes insensitive or may be the site of intermittent dull or even acute pain (Koop, 1957). Epididymitis of an undescended testicle may suggest torsion or, by pain reference, an acute intraperitoneal surgical disease. Three clinical cases of infants with fatal peritonitis secondary to suppurative orchitis in an abdominal testicle have been reported in the literature (Dean, et al., 1952). Chorea, eclampsia infantum or even hystero-epileptic attacks have ceased following operation for undescended testes. Psychic disturbances rarely appear until late childhood and are often absent. There is no clinical distinction in incidence of symptoms as related to either unilateral or bilateral involvement. It is believed that the high concentration of human chorionic gonadotropin in the fetus is directly responsible for the usual 97 to 99% normal testicular descent between the seventh and ninth month in utero. Experimental and clinical observations (Deming, 1951 and 1963) have fostered conservative non-surgical treatment of cryptorchidism in boys, but this treatment has not been successful in more than 5 to 8/100 cases (Scorer, 1964). McIntosh (1959) estimated that one or both testes are or appear to be undescended at birth in some 10% of male infants. At the end of the first year of life this number has decreased to 1%. Studies of the adult population indicates an incidence of approximately 0.3% (Nelson, 1953). Military medical inductee evaluation have indicated slightly higher figures (Baumracker, 1946).

The incidence of neoplasm in the undescended testes is alarmingly high, at least in many reported series. Linke and Kiefer (1959) found nine of 48 testicular tumors in undescended testes. In a series of 510 testicular tumors

at Walter Reed Army Hospital the incidence of malignant degeneration in cryptorchid testes was 5 times greater than in the scrotal testes (Linke and Kiefer, 1959).

Clinical studies involving sperm counts of human ejaculate have implied that increasing environmental temperature of the scrotal testes of normal men decreases spermatogenesis (Newman and Wilhlm, 1950; Hand, 1959). Studies on the effect of light in the Japanese Quail have demonstrated decreased fertility and even testicular morphological changes associated with variations in duration of light exposure (Kato, 1968). Finally, experimentally, vascular insufficiency of the testis, has been associated with morphological changes similar to that of artificially-induced cryptorchidism (Murkerjee and Amesur, 1965; Steinberger and Tjive, 1969).

2. Histological Examination of Cryptorchid Testes

Histological studies of testes in a non-scrotal position after the age of 5 years generally demonstrates gradations of gross atrophy accompanied by progressive hyaline thickening of the basement membranes of the tubules with progressive increase of the interstitial connective stroma (MacCollum, 1935; Sohval, 1954). Concomittantly, spermatogenic activity diminishes or simply fails to begin and finally ceases. The germinal epithelium then undergoes progressive atrophy until only a few primitive spermatogonia and Sertoli cells persist. During this period, the basement membranes show increasing hyalinization and thickening so that eventually the tubules become replaced by dense cords of hyaline connective tissue outlined by the prominent thickened basement membrane. There is also an increase of interstitial stroma and usually some hyperplasia of the interstitial cells of Leydig. Numerous studies have demonstrated that placement of the normal gonad in the abdomen can alone produce reversible and ultimately, irreversible degenerative changes

(Lewis, 1948; Moore and Quick, 1924; Nelson, 1953; Newman and Wilhelm, 1950). Finally, increasing the environmental temperature of scrotal testes of normal men decreases spermatogenesis (Kato, 1968; MacLeod and Hutchkiss, 1941).

J. THE CYCLE OF THE RAT SEMINIFEROUS EPITHELIUM

Because periodic acid-Schiff positive material can be identified in the Golgi apparatus of young spermatids Leblond and Clermont (1952) were able to divide spermiogenesis of the rat into 19 distinct stages. The first 8 of these stages, the germinal epithelium contains old spermatids, which are released into the seminiferous tubular lumen when a new generation reaches stage 8. Hence, the new generation of spermatids is alone until they reach stage 15 of the cycle when another generation of spermatids appear. Therefore, stage 1 and stage 15 spermatids appear together, and the succession of cells associated with this appearance marks one complete cycle of the seminiferous epithelium. The 19 stages of spermiogenesis are further subdivided into four phases.

The first phase is the Golgi phase, which includes three of the stages of the cycle. In stage 1, the idiosome is in the Golgi zone and two distinct centrioles are near the chromatoid body. The fine filament from one of the centrioles eventually becomes the tail of the sperm. In stage 2, one to four granules appear in the idiosome. In stage 3 the fusion of the pro-acrosomic granules occurs.

The second phase is the cap phase, which consists of four stages of the cycle. In stage 4, the acrosome granules flatten on the nucleus. In stage 6, a cap is formed over the nucleus by the acrosome and is referred to as the acrosomic cap. The idiosome separates from the acrosomic granule, and the two centrioles move closer to the nucleus. In stage 7 of the cycle of the seminiferous epithelium the cap reaches maximal size. The proximal centriole

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adheres to the nucleus, and the flagellum remains attached to the distal centriole. The chromatoid body is loose in the cytoplasm at this time.

The third phase is the acrosome phase, which includes seven stages of the cycle of the seminiferous epithelium. The caudal tube is present at this time and the head caps are oriented toward the tubular wall. In stage 8, the granule and cap move toward the basement of the seminiferous tubule, and the cytoplasm shifts to the opposite pole of the nucleus. The chromatoid body surrounds the flagellum near its insertion to the distal centriole. In stage 9 of the cycle, the acrosomic granule elongates. In stage 10, the head cap moves toward the caudal end of the nucleus, and the apical end is pointed. In stage 11, the nucleus and head cap elongate. In stage 12 the nucleus is at its maximal size. In stage 13, the nucleus is thinner, and the distal centriole divides into a dot and ring. In stage 14, the head cap is loose over the nucleus, the cytoplasm condenses, and the spermatid begins to look more like a mature spermatozoon.

The fourth phase is the maturation phase which consists of 5 stages. In stage 15, the head cap has a fine filamentous, finlike appearance; the ring centriole separates from the centriole and forms the middle piece. In stage 16, elongation of the finlike membrane occurs. In stage 17 of the cycle of the seminiferous epithelium the acrosome and head cap move forward. In stage 18 the perforatorium appears and finally in stage 19 the staining capacity of the sperm is sharply reduced.

K. HUMAN SPERMATOGENESIS

The histological appearance of the human seminiferous epithelium has generally suggested a haphazard arrangement of its germinal cells. However, in contrast to the arrangement of cell associations in the rat (Leblond and Clermont, 1962) only a limited number of cell associations can be identified in various seminiferous tubular cross sections (Clermont, 1968). This

characteristic arrangement of germ cells implies that in any one area of the human seminiferous epithelium, cell associations follow each other in time. This orderly sequence of cellular associations is referred to as the cycle of the seminiferous epithelium (Heller and Clermont, 1963).

Each cell association may be arbitrarily described as a stage of this cycle of the seminiferous epithelium and can be identified and classified by using a variety of histological and cytological criteria. In addition, histochemical techniques are of extreme value in determining the stage of spermatid acrosomal development necessary for proper stage classification.

In the initial studies on the human seminiferous epithelium (Johnsen, 1964) the periodic acid Schiff's-hematoxylin technique clearly delineated the acrosomic structures of the spermatid. It is the progressive maturation of the spermatid as characterized by alterations in the shape of the acrosome that acts as the keystone upon which the stages of the cycle of the seminiferous epithelium is based.

1. The Cycle of the Human Seminiferous Epithelium

Based on the description of cellular associations made from hematoxylin-eosin stained sections of human seminiferous epithelium, six stages of the cycle of the human seminiferous epithelium are identifiable (Clermont, 1968).

Stage I of the cycle of the human seminiferous epithelium is characterized by two generations of spermatids. The first generation is composed of newly formed spermatids with spherical nuclei which are often depressed by a small acrosomic structure. In between these early generation of spermatids, the older, second generation of spermatids are arranged in bundles which appear deeply inserted in the Sertoli cytoplasm. Associated with the spermatids are primary spermatocytes at pachynema. Along the periphery of the seminiferous tubule are scattered type A and B spermatogonia.

Stage II of the cycle of the human seminiferous epithelium discloses the younger generation of spermatids with their spherical nuclei within polyhedral cytoplasm forming regular and compact layers (Sa). Lining the tubular lumen and in direct contact with these spermatids are the more mature spermatids which are at the last stage of spermiogenesis. Before their release into the seminiferous tubular lumen, these mature spermatids detach their cytoplasm which contains heavily stained material.

Stage III of the cycle of the human seminiferous epithelium demonstrates one generation of spermatids. This remaining generation of spermatids contain spherical nuclei with dark homogenously stained chromatin. Two generations of spermatocytes are found. One generation of mid-pachynema spermatocytes and a second early generation of resting primary spermatocytes. The resting primary spermatocytes have ovoid or spherical nuclei and contain generally a fine granular chromatin network. Along the periphery of the seminiferous epithelium are a few scattered type A spermatogonia.

Stage IV of the cycle is identified by spermatids with nuclear elongation. These nuclei are irregular in shape and are in contact with their cytoplasmic membranes (Sb). More peripherally the older generation of primary spermatocytes are at pachynema, and the younger generation of primary spermatocytes are beginning meiotic prophase. The nuclei of these young spermatocytes contain a fine filamentous configuration characteristic of leptotema. Type A spermatogonia are scattered along the periphery of the epithelium.

Stage V of the human seminiferous epithelium is characterized by one generation of maturing spermatids (Sc). These spermatids have typically pointed and deeply stained nuclei and are aligned towards the basement membrane. The older generation of primary spermatocytes have entered late pachynema step of the meiotic prophase as indicated by the large nuclei and thick chromosomes. The young generation of spermatocytes are in transition from the leptotene

to zygotene step by meiosis. The zygotene nuclei are characterized by long chromosomal threads along the nuclear limiting membrane.

Stage VI of the cycle is characterized by the primary and secondary spermatocytes undergoing the first and second maturation division. The division figures are identified by the thicker and longer chromosomes of the primary spermatocytes against the fine, short chromosomes of the secondary spermatocytes. The maturing spermatids with their elongated heads are deeply inserted between the primary spermatocytes. The young generation of primary spermatocytes are at either zygotema or early pachynema. Type A spermatogonia are also identified along the basement membrane. The described six stages of the cycle of the human seminiferous epithelium are presented in the sequence that they occur in the cycle of the human seminiferous epithelium.

CHAPTER II
MATERIALS AND METHODS

A. TISSUES

1. Rat Testes

In the studies on the effect of glucose on the radioautographic incorporation of L-Lysine- H^3 into protein of cells of the seminiferous epithelium and in studying the radioautographic incorporation of L-Lysine- H^3 into protein of cells of the germinal epithelium in cryptorchidism, 60 day old Sprague-Dawley rats, weighing 185-220 gms were utilized.

In the studies on the germinal epithelium of cryptorchid testes experimentally induced in prepubertal and adult rats, two groups of rats were utilized. The first group (5 animals) were 20 day old prepubertal rats. The prepubertal rats are those in which the testes had not yet begun their descent and were consequently located within the abdominal cavity. The second group (5 animals) consisted of 60 day old rats in which the testes had undergone a normal descent into the scrotal sac.

All the animals were obtained from Abrams Small Stock Breeders, Chicago, Illinois, and were fed ad libitum Rockland Rat diet.

2. Human Testicular Specimens

Orchiectomy specimens were obtained from two patients aged 65 and 70 years, performed because of adenocarcinoma of the prostate. The surgical specimens were obtained from the Veterans Administration Hospital, Hines, Illinois under cognizance and approval of the Associate Chief of Staff (Chief of Research and Education), Assistant Chief of Surgery and the Department of Pathology. As the experiments were designed and followed, no specimens from or use of human subjects were needed specifically for these experiments. All tissue experiments were carried out on routine surgical specimens without experimental risk or involvement of the subject.

a. Transportation of Surgical Specimens

The surgical specimens were removed in the operating room, Veterans Administration Hospital, Hines, and placed immediately in preoxygenated Krebs-Ringer Bicarbonate Buffer packed in ice within an insulated container. The entire assembly was directly transported to the Department of Pathology (V.A. Hospital, Hines) where the surgical specimens were examined by the staff pathologist. Adequate tissue samples were obtained at this time for gross and microscopic analysis as required per state law. The remainder of the testicular parenchymal and capsular mass was, without hesitation, transported to Loyola University Stritch School of Medicine, Department of Pharmacology, Maywood, Illinois. Incubation of human testicular slices was performed within 30 minutes following orchiectomy.

B. ORCHIECTOMY (SURGICAL TECHNIQUE)

Removal of the testicles is generally performed as a palliative measure in the treatment of adenocarcinoma of the prostate. It is easily performed through a longitudinal scrotal skin incision. The incision is made in the anterolateral aspect of the scrotum. It is carried down through the skin and all the intermediate layers to the testicle, but without opening the tunica vaginalis (Visceral). The testicle is bluntly freed from the scrotum and stretched downward bringing the lower spermatic cord into view (Plate XI, Fig. A). Then with upward traction the gubernaculum is identified as attaching the inferior pole of the testis to the floor of the scrotal sac. A single O-chromic suture is passed around the base of the gubernaculum and tied. The gubernaculum is subsequently cut, freeing the testicle from the scrotal sac, (Plate XI, Fig. B). Up to this stage, the vascularity has been maintained. Attention is then redirected to the spermatic cord. This time two heavy Kelly clamps are used to double clamp the spermatic cord and the cord is then

transected between the two clamps (Plate XI, Fig. C). The cord stump is suture-ligated with #1-chromic gut. The isolated testicle is immediately placed in iced oxygenated Krebs-Ringer bicarbonate buffer for immediate transportation to the pathologist and subsequently to the laboratory for isolated tissue incubation procedures (Plate XI, Fig. D).

1. Isolation of Testicular Sample from the Surgical Specimen

Following bilateral orchiectomy, the surgically removed testes having been placed in pre-oxygenated, iced Krebs-Ringer bicarbonate buffer (Plate XII, Fig. D) were immediately transported to the pathology department. The awaiting pathologist rapidly removed the surgical specimens from the iced Krebs-Ringer bicarbonate buffer, measured and examined each specimen. Plate XII, Fig. A illustrates the surgical specimen in toto. Following measurement and gross examination, the entire testis was transected in a mid-sagittal plane, carefully avoiding the epididymis (Plate XII, Fig. B). Figure C illustrates 50% of the parenchymal mass and the entire epididymis as retained by the pathologist for further processing and microscopic examination. The remaining 50% of the parenchymal mass (Plate XII, Fig. D), was placed into iced Krebs-Ringer bicarbonate buffer and rapidly transported to the laboratory.

In general the entire time period from transection of the spermatic cord to incubation of human testicular slices was 30 minutes.

C. CRYPTORCHIDISM

Abdominal fixation of undescended and descended 60 day old rat testes was performed under ether anesthesia. Through a midline abdominal incision, adequate exposure of the lower abdomen and right dorsal lateral abdominal wall was achieved. By applying gentle pressure to the bottom of the scrotal sag, in the 60 day old rat, translocation of the scrotal testis to the abdominal cavity was accomplished. Subsequently, the translocated right testis was suture

utilizing 6-0 surgical silk, to the right dorsolateral abdominal wall. The suture was passed just beneath the tunica albuginea beyond the point where the testicular artery entered the parenchyma of the testis. This placement was necessary to avoid embarrassing the vascular supply of the sutured testis.

In the case of the prepubertal rat (20 day old) the identical anesthesia and exposure were performed. This time the intra-abdominal testes were identified at a point just proximal to the internal inguinal rings. The right testes was mobilized for a distance of approximately 1 cm and subsequently sutured, utilizing 6-0 surgical silk to the right dorsolateral abdominal wall.

The left testis of each animal was allowed to remain in or descend to the scrotal sac and serve as a control.

The abdominal cavity was closed utilizing stainless steel clips.

D. PREPARATION OF TESTICULAR SECTIONS

The animals were sacrificed by decapitation. The testes were immediately removed and placed in cold Carnoy's solution (6:3:1), 5°C for overnight fixation. Following fixation, the tissue was dehydrated in three changes of cold absolute ethanol, cleared in methyl salicylate and embedded in Tissuemat (M.P. 56.0-58.5°C). Tissues were sectioned at 5 μ and mounted on glass slides. In place of the conventional albumin fixation, paraffin ribbons containing the tissue were applied to a layer of distilled water placed on the slide and the specimen allowed to expand to its original size at 53°C (Hisaoka and Firlit, 1962). The excess water was then removed by touching the edge of the slide to a piece of filter paper. The tissue was then permanently fixed to the glass slide by rolling a rubber stopper over a piece of moist filter paper placed over the specimen. The preparation was then allowed to dry at room temperature for a minimum of 1 hour prior to staining.

E. HISTOLOGICAL STAINING METHOD

1. Hematoxylin and Eosin

Hematoxylin (Harris) and Eosin Y (0.5 gm + 100cc 95% ETOH) were used generally throughout these studies. In the case of tissue slated for radioautography, it was found necessary to prestain the tissue in Harris' Hematoxylin followed by adequate color differentiation (Davis and Firlit, 1965), prior to coating the slides with radio-sensitive emulsion. No counter-staining with Eosin Y was used for the radioautographic sections.

2. Periodic Acid-Schiff Reaction

In the studies employing radioautography, alternate sections in addition to those previously stained with hematoxylin (Harris') alone, were stained with either hematoxylin, hematoxylin-eosin or periodic acid-Schiff reagent (Leblond and Clermont, 1952) with toluidine blue (Davis and Firlit, 1965) counterstaining in order to properly classify the 14 stages of the cycle of the seminiferous epithelium in the rat according to the description of Leblond and Clermont (1962).

Classification of the 6 stages of the cycle of the seminiferous epithelium in man was made according to the description of Clermont (1968).

F. ISOLATION AND INCUBATION OF TISSUES

The animals were sacrificed by decapitation and the testes rapidly removed. The outer capsule was cut with a scissors and the parenchyma gently expressed by manual pressure.

Human testis were immediately sectioned into 2cm² portions in the laboratory cold room. The parenchymal mass was noted to be pale-salmon colored and relatively firm in consistency.

Slices of either rat or human testis averaged 0.5mm in thickness were obtained with the aid of a Stadie-Riggs microtome at 4°C. The slices from

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a single testis, consisting of many intact seminiferous tubules, were placed in alternate Warburg flasks in a random fashion and incubated in a Warburg apparatus at 37.5°C for the rat, and 37°C for the human. It was found that shaking of the slices at 140 oscillations per minute during the incubation period caused the seminiferous tubules to be teased apart. Slices of human testis and slices of cryptorchid rat testes remained essentially as a slice of 0.5mm in thickness. Along the periphery of these slices a few separated tubules occurred, but generally remained as a slice. Inasmuch as the depth of the seminiferous tubules (adult rat) from the basement membrane to the lumen was determined to be similar in both the control and the experimental systems, this technique allows for a uniform penetration of substrates from the medium through the depth of each seminiferous tubule in the two systems. The grain counts obtained in the presence and absence of exogenous glucose were therefore independent of the depths of the counted sections from the surface of the seminiferous tubule. The main chamber of the Warburg flask contained 200 mg wet weight of tissue in 3.0ml of Krebs-Ringer bicarbonate buffer at pH 7.4. The side-arm contained 100 μ c of tritiated Lysine in a volume of 0.2ml. L-Lysine-H³ was obtained from the Nuclear-Chicago Corporation, Chicago, Illinois and had a specific activity of 91mc/mmole. The final concentration of L-Lysine-H³ in the incubation flask was 3.4×10^{-4} M. When glucose was employed as an exogenous substrate, the final concentration of glucose in the incubation flask was 0.009M. The gas phase was 95% O₂ and 5% CO₂.

G. PREPARATION OF TISSUES FOR HISTOLOGICAL STUDY

At the end of 1 hour of incubation period, the flask was removed from the manometer and the flask contents poured over a 90-mesh stainless-steel sieve to collect the testicular slices. The tissue held back by the sieve was

washed three times with Krebs-Ringer bicarbonate buffer and gently placed in cold Carnoy's solution by means of forceps. In one experiment, of each study destined for radioautography, the tissue was washed with buffer and then left in buffer containing 0.1% non-radioactive lysine for 30 minutes at 4°C in order to dilute any trace amounts of L-Lysine-H³ not removed by the buffered washings alone. Following fixation in Carnoy's solution overnight (Kopriwa and Leblond 1962), the tissue was dehydrated in absolute ethanol, cleared in methyl salicylate, and embedded in Tissuemat. Tissues were sectioned at 5 μ and mounted on glass slides. The sections were deparaffinized, hydrated, stained in hematoxylin (Harris'), color differentiated and then allowed to air dry prior to coating with radio-sensitive emulsion.

H. PREPARATION OF SLIDES FOR RADIOAUTOGRAPHY

The slides, having been previously stained and dried, were dipped in molten Kodak nuclear track emulsion, type NTB3 under a Wrattan safelight number 2. The slides were allowed to dry and were then stored in black plastic boxes, sealed with black plastic adhesive tape. The box was placed in the refrigerator (4°C) for a 96 hour period of exposure. The exposed slides were developed for 5 minutes in Kodak Dektol developer and fixed for 10 minutes in Kodak Fixer. The slides were removed from a water rinse, the surface blotted with filter paper and allowed to dry for 30 minutes. Following drying, the slides were re-stained with hematoxylin (Harris') and mounted in Permount for examination. In addition, alternate sections which were not dipped with nuclear emulsion were stained with either hematoxylin, hematoxylin-eosin or the periodic acid-Schiff reagent with toluidine blue counter-staining.

I. COUNTING RADIOAUTOGRAPHS

Grain counting was carried out under oil-immersion (960x) using a Whipple eyepiece micrometer grid. Grains were counted within 4 squared 50 μ areas

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giving a total area counted as grains per $200\mu^2$. Background counts were made of areas of emulsion not directly over tissues incubated with tritiated lysine and compared with grain counts obtained from areas of emulsion directly over tissues which were not exposed to tritiated lysine. In both instances, the average background count was found to be 8 grains per $200\mu^2$. The background was therefore routinely determined by counting grains in tissue-free areas in the neighborhood of the cells to be examined. The average number of grains per $200\mu^2$ above background per slide was determined by counting 5 adjacent microscopic fields consisting of $200\mu^2$ over a given area of cells. At least two slides from each animal were used for counting each stage in the cycle of the seminiferous epithelium. A total of 4 individual animals were used in these studies. After the proper classification of the tubule as to its stage in the cycle of the seminiferous epithelium, the average number of grains per $200\mu^2$ above background was determined over the following areas: spermatogonia in stages I-XIV; first layer of primary spermatocytes in stages VI-XIV; secondary spermatocytes in stage XIV; first layer of spermatids in stages I-XIV; and second layer of spermatids in stages I-VIII. The area of $200\mu^2$ used for grain counting was found to contain four cells for each of the successive cells for each of the successive cells of the cycle of the seminiferous epithelium with the exception of the second layer of primary spermatocytes in which two cells were found to occupy an area of $200\mu^2$. Because of the continuous nature of the spermatogenic cycle, it is possible in this manner to observe the various capacities of the successive cells of the cycle of the seminiferous epithelium of the rat to incorporate L-Lysine- H^3 into protein beginning with a stem cell spermatogonium in stage VIII and ending with the maturation phase of the spermatids.

In a similar fashion as in the rat, radioautographic grain counting in the human seminiferous epithelium was conducted in the following manner.

After proper classification of the tubule as to its stage in the cycle of the seminiferous epithelium, the average grains per $200\mu^2$ above background was determined over the following areas: spermatogonia in stages I-VI; first layer of primary spermatocytes in stages III-VI; second layer of primary spermatocytes in stages I-V; secondary spermatocyte in stage VI; first layer of spermatids in stages I-VI and second layer of spermatids in stages I-II.

CHAPTER III

RESULTS

A. THE GERMINAL EPITHELIUM OF CRYPTORCHID TESTES EXPERIMENTALLY INDUCED IN PREPUBERTAL AND ADULT RATS

A number of investigators have studied the morphologic changes which occur in the cryptorchid testis of the rat (Clegg, 1963a and 1963b; Moore, 1924; Moore and Quick, 1924; Niemi and Koromano, 1965). However, in all these instances, cryptorchidism was experimentally induced in adult animals by transplanting a previously descended testis from the scrotal sac to the abdominal cavity. The question arose as to the physiologic significance of such an experimental procedure for inducing cryptorchidism in animals inasmuch as cryptorchidism is known to occur in the human when an abdominal testis fails to descend normally into the scrotal sac.

The present experiments were designed to investigate the morphologic changes observed in cryptorchidism experimentally induced by fixation of an immature, abdominal testis to the dorsolateral abdominal wall and by transposing a scrotal testis into the abdominal cavity of the rat. In producing cryptorchidism by these two different procedures, it was possible to compare the histologic appearance of a cryptorchid testis induced by prevention of normal testicular descent with that induced by transplantation of a previously descended testis back into an abdominal environment.

1. Weight of Testes

Graph I indicates the change in the weight of testes following both abdominal fixation of undescended prepubertal testes and abdominal transplantation of previously descended adult testes. After abdominal fixation of the prepubertal testis, only a small increase in weight was observed for the cryptorchid testis. The contralateral scrotal testis, however, showed a marked increase in weight up to day 30 after the surgical procedure. After abdominal transplantation of the adult testis, a progressive decrease in

weight was observed for the cryptorchid testis while the contralateral scrotal testis did not change in weight to any appreciable extent during this period. These data indicate that the difference between the weight of the scrotal testis and that of a cryptorchid testis induced in either a prepubertal rat or in an adult rat is very similar 30 days following the surgical procedure.

2. Abdominal Transplantation of Adult Testis

a. 2 Days Post Surgery

Plate I, Figures 1-6 show the histologic appearance of the seminiferous epithelium of the adult rat testis following 2 days of abdominal implantation. Figures 1, 3 and 5 represent Stages I, VII, and XIII respectively, in the cycle of the seminiferous epithelium of the rat, according to the description of Leblond and Clermont (1962). The seminiferous tubules during Stages I-III, VII-VIII and XII-XIII consist of normally appearing spermatogonia, primary spermatocytes, spermatids and Sertoli cells. The cellular arrangement of these stages is similar to that of the normal seminiferous epithelium occurring in the scrotal testis.

However, Stages IV-VI, IX-XI and XIV of the cycle of the seminiferous epithelium demonstrate morphologic alterations of the tubular epithelium 2 days after abdominal implantation, as shown in Plate I, Fig. 2, 4 and 6 respectively. The initial abnormalities include migration of the early generation spermatids, which occur in these stages, away from their normal position within the seminiferous epithelium and toward the lumen of the seminiferous tubule. These spermatids in the cap phase of spermiogenesis appear to be highly sensitive to the increased intra-abdominal temperature and seem to be the earliest cell types to undergo such degenerative changes as cytoplasmic swelling and karyolysis. Exfoliation of the pachytene primary spermatocytes also begins to occur during these stages of the spermatogenic cycle. Stage XIV of the cycle of the seminiferous epithelium demonstrates a marked epithelial disarrangement 2 days after

the surgical induction of cryptorchidism by transplantation of mature testis from the scrotal sac to the abdominal cavity. At this time, the primary spermatocytes at diakinesis just prior to metaphase appear to have migrated toward the tubular lumen, resulting in numerous empty spaces within the seminiferous epithelium. In addition, many primary spermatocytes which were undergoing cellular division appeared to be halted at metaphase with their cytoplasm undergoing hyalinization. This hyalinization of the primary spermatocytes in metaphase at this stage of the spermatogenic cycle serves to demonstrate the marked sensitivity of the meiotic apparatus to heat. At the same time, the secondary spermatocytes and spermatids also undergo exfoliation toward the lumen of the seminiferous tubule.

b. 5 Days Post Surgery

Plate II, Figures 7-12 indicate the extent of dissociation of the seminiferous epithelium following 5 days of cryptorchidism induced by transplantation of an adult testis from the scrotal sac to the abdominal cavity. At this time only Stages III-IX could be identified according to their position in the cycle of the seminiferous epithelium. The remaining stages of the spermatogenic cycle showed such marked tubular changes that they were no longer identifiable according to the description of Leblond and Clermont (1962). These changes consist of the total loss of spermatids from the seminiferous epithelium and marked swelling of the primary spermatocytes in the tubular lumen. Although Stages III-IX could still be identified, there is a further loss of spermatids and primary spermatocytes into the seminiferous tubular lumen. The exfoliated spermatids of the cap and early acrosome phases of spermiogenesis were found in the lumen from multinucleated giant cells (Plate II, Fig. 9). With this further loss of spermatids and primary spermatocytes, large cell-free areas appeared in the remaining seminiferous epithelium.

The majority of tubules in Stages VII-VIII of the cycle of the seminiferous epithelium still closely resemble the corresponding stage seen in the normal scrotal testis. However, a few tubules in Stages VII-VIII have begun to demonstrate initial losses of early acrosome and late maturation phases of spermatids. In addition, an increased vacuolization can be noted in the cytoplasm of the Sertoli cells.

c. 7 Days Post Surgery

Plate III, Figures 13 and 14 are representative photomicrographs of Stages III-IV of the spermatogenic cycle of the cryptorchid rat testis after 7 days in the abdominal cavity. There is a further loss of spermatids and primary spermatocytes toward the lumen of the seminiferous tubule, with resulting multinucleated giant-cell formation.

d. 10-30 Days Post Surgery

Further examination of the cryptorchid testis at 10, 15 and 30 days after abdominal transplantation of an adult testis (Plate III, Fig. 15-17) indicate progressive seminiferous epithelial atrophy with a corresponding decrease in tubular size. The extent of seminiferous epithelial disorientation was so severe as to make identification of stages of the spermatogenic cycle, unfeasible at 10 days post surgery. After 30 days in the abdominal cavity, the seminiferous tubules were completely lined by Sertoli-cell cytoplasm and type B spermatogonia with only occasional pachytene primary spermatocytes. With a further decrease in tubular size, the opposite free borders of the Sertoli cells appeared to fuse resulting in the complete obliteration of the seminiferous tubular lumen. Approximately half of the Sertoli cells present at 30 days post surgery appeared to be markedly atrophic, displaying both fibrosis and hyalinization of the cytoplasm as well as nuclear pleomorphism, (Plate III, Fig. 18).

3. Abdominal Fixation of the Prepubertal Rat Testis

The immature testis of the prepubertal rat at 20 days of age is normally located in an abdominal position prior to its descent into the scrotal sac. It consists of many small developing seminiferous tubular cords and early tubules containing several layers of spermatogonia and transitional to early pachytene primary spermatocytes (Plate IV, Fig. 19). The pachytene primary spermatocytes circumscribe the lumen of the seminiferous tubules and at times appear to lie free within the tubular lumen. The early pachytene primary spermatocytes are the most advanced cell type present in the developing spermatogenic series of cells of the seminiferous epithelium of the rat testis at 20 days of age.

a. 5 Days Post Surgery

At 5 days following the abdominal fixation of the prepubertal testis, early changes occur within the pachytene primary spermatocytes. These changes include central migration of the pachytene primary spermatocytes into the tubular lumen where they undergo hyalinization (Plate IV, Fig. 20). Plate IV, Figure 21 indicates the normal histologic appearance of the corresponding inguinal testis of this animal at 25 days of age. The seminiferous tubules are loosely lined with transitional and pachytene primary spermatocytes.

b. 10 Days Post Surgery

At 30 days of age, following its descent from the abdominal cavity to the scrotal sac, the normal rat testis begins to demonstrate the onset of spermiogenesis. Approximately half of the tubules contain spermatids of the cap and acrosome phases of spermiogenesis. The remaining tubules contain only spermatogonia and resting or pachytene primary spermatocytes. Further degenerative changes can be noted within the 10-day cryptorchid testis following abdominal fixation. These changes include degeneration of the pachytene

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primary spermatocytes as indicated by cytoplasmic swelling, karyolysis and sloughing into the tubular lumen. These exfoliated primary spermatocytes can be observed to form multinucleated giant-cell masses within the seminiferous tubular lumen. Spermatogenesis in the 10-day cryptorchid rat testis appears to be halted at the pachytene primary spermatocyte level of development. These arrested pachytene primary spermatocytes appear to be the most advanced cell type found in the seminiferous epithelium following 10 days of intra-abdominal fixation.

c. 15 Days Post Surgery

The seminiferous tubules of the normal, scrotal testis at 35 days of age have increased in diameter and contain increasing numbers of spermatids (Plate IV, Fig. 22). However, seminiferous tubules possessing maturing spermatids in the maturation phase of spermiogenesis cannot yet be demonstrated at this time of development.

After 15 days of abdominal retention the seminiferous epithelium of the cryptorchid rat testis consists of primary spermatocytes and Type B spermatogonia found within the Sertoli retinaculum (Plate IV, Fig. 23). With the progressive degeneration and central migration of the pachytene primary spermatocytes, the Sertoli-cell retinaculum becomes increasingly evident within the substance of the seminiferous tubule.

d. 30 Days Post Surgery

The seminiferous epithelium of the normal scrotal testis at 50 days of age now contains spermatids in the maturation phase of spermiogenesis (Plate IV, Fig. 24). However, after 30 days of abdominal retention the seminiferous tubules of the cryptorchid rat testis appeared to consist of a Sertoli-cell retinaculum containing peripherally located Type B spermatogonia and occasional pachytene primary spermatocytes (Plate V, Fig. 25-27). Plate V, Figures 28-30 show higher magnifications of seminiferous tubules of the cryptorchid rat

testis following 30 days of abdominal retention of a previously undescended testis, illustrating the predominance of the Sertoli-cell retinaculum containing numerous Type B spermatogonia. With the progressive degeneration and exfoliation of the pachytene primary spermatocytes into the lumen of the seminiferous tubule, the underlying Sertoli-cell cytoplasm and the Type B spermatogonia remain as the principal cellular components of the seminiferous tubular epithelium following 30 days of exposure to an intra-abdominal temperature. In addition, approximately half of the Sertoli cells present at this time show fibrosis, hyalinization, and increased vacuolization of the cytoplasm with associated nuclear pleomorphism.

B. RADIO-AUTOGRAPHIC INCORPORATION OF L-LYSINE-H³ INTO PROTEIN OF CELLS OF THE GERMINAL EPITHELIUM IN CRYPTORCHIDISM

According to the studies of Davis, et al. (1964 and 1965) incorporation of L-Lysine-U-C¹⁴ into protein of slices of cryptorchid testes of the rat is markedly greater than the incorporation of L-Lysine-U-C¹⁴ into protein of slices of scrotal testes obtained from the same animal. It is well known that the predominant histological feature of the cryptorchid testis is the absence of all the spermatids (Moore, 1924; Nelson, 1951; Clegg, 1936b; Niemi and Karomano, 1965). The question arose as to whether the increased protein labelling of the cryptorchid testis of the rat was, on the one hand, a reflection of this altered histological architecture with a resulting unmasking of the spermatogonia, primary spermatocytes and Sertoli cells or, on the other hand, represented a true change in the protein synthesizing capacity of the remaining testicular cell types of the germinal epithelium. The present experiments were designed to ascertain which of the cells of the seminiferous tubules of the rat testis remained 30 days after the experimental induction of cryptorchidism. The incorporation of tritiated lysine into protein of

the remaining cells of the germinal epithelium of the abdominal testis was determined and a comparison made of the incorporation of tritiated lysine into protein of the corresponding cells found in the scrotal testis of the same animal.

Plate VI demonstrates the three types of seminiferous tubules which are found in the cryptorchid rat testis 30 days after abdominal transplantation and they have been designated in this report as types A, B and C cryptorchid tubules. Type A tubule contain spermatogonia of the "crust" variety as described by Leblong and Clermont (1962), primary spermatocytes in the pachytene stage of meiosis and Sertoli cells. Type B tubules contain only spermatogonia of the 'crust' variety and Sertoli cells, with the cytoplasm of the Sertoli cell extending as an apparent syncytium from the basement membrane to the lumen of the seminiferous tubule. Type C tubules contain essentially only Sertoli cells with pleomorphic-appearing nuclei and fibrotic, hyalinized cytoplasm.

The percentage distribution of the three types of seminiferous tubules found in the cryptorchid rat testis 30 days after transference to the abdominal cavity is indicated in Table 1. Type A tubules constitute almost 31% of the total seminiferous tubules of the cryptorchid rat testis. Type B tubules account for 30% of the total seminiferous tubules of the cryptorchid rat testis while the remaining 39% of the tubules consist of Type C tubules. It would therefore appear that the cryptorchid rat testis 30 days after transference of an adult testis from the scrotal sac to the abdominal cavity is composed of approximately equal numbers of each of the three types of seminiferous tubules described in the present experiments.

Table 2 presents the percentage distribution of the cell population of these three types of cryptorchid seminiferous tubules. The epithelium of Type A tubules consists of 55% spermatogonia of the 'crust' variety, 8.6%

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pachytene primary spermatocytes and 36.4% normal-appearing Sertoli cells.

The epithelium of Type B tubules consists of 43% 'crust' spermatogonia, a total absence of primary spermatocytes while the remaining 57% of the epithelium of Type B tubules is made up of normal-appearing Sertoli cells. The epithelium of Type C tubules consists essentially of atrophic appearing Sertoli cells with only 3.6% spermatogonia and no primary spermatocytes or normal Sertoli cells present. These data indicate that the germinal epithelium of the cryptorchid rat testis 30 days after abdominal transplantation is composed of only four types of cells, namely, 'crust' spermatogonia, pachytene primary spermatocytes, normal-appearing Sertoli cells and atrophic appearing Sertoli cells.

Plate VII demonstrates representative radioautograms following incubation of slices of cryptorchid rat testes with tritiated lysine. A very dense labeling pattern was observed over both the spermatogonia and the Sertoli cell cytoplasm in Type A tubules. The labeling of the Sertoli cell cytoplasm in Type B tubules was found to be quite similar to the labeling found over the Sertoli cell cytoplasm present in Type A tubules. However, the atrophic-appearing Sertoli cells found in Type C tubules were characterized by a marked decrease in the uptake of tritiated lysine into cellular protein.

Table 3 presents the radioautographic incorporation of tritiated lysine into each of the remaining cells of the cryptorchid rat testis 30 days after transference of the testis to the abdominal cavity as compared to those cells found in the normal, scrotal testis. The data are presented as grains/200 μ^2 above background over each of the designated cell types. The induction of cryptorchidism was found to produce an approximate four-fold increase in protein labeling of both 'crust' spermatogonia and pachytene primary spermatocytes. However, the induction of cryptorchidism was also found to result in a forty-fold increase in protein labeling from tritiated lysine in the

the normal-appearing Sertoli cells of both Type A and Type B cryptorchid tubules. Protein labeling from tritiated lysine in the atrophic-appearing Sertoli cells found in Type C cryptorchid tubules was, however, observed to be almost one-third that which was seen over the normal appearing Sertoli cells of Type A and B cryptorchid seminiferous tubules.

C. EFFECT OF GLUCOSE ON RAT TESTICULAR PROTEIN SYNTHESIS

Although glucose has long been shown to maintain not only the oxygen uptake but also the morphological integrity of adult testicular tissue few studies have been carried out on the effect of this important substrate on testicular protein biosynthesis. Davis and Morris (1963a) have reported that the addition of 0.009M glucose is capable of increasing the aerobic incorporation of radioactive lysine into acid-precipitable protein of slices of adult rat testes by 600%. Graph II. In marked contrast, the addition of 0.0009M glucose was found to increase protein labeling of the head of the epididymis by 150% while causing no change to only a 50% enhancement of protein labeling in slices of jejunal mucosa, spleen, thymus, submaxillary gland, pancreas, tail of epididymis, kidney, brain, diaphragm, heart, lung, seminal vesicle, normal liver, regenerating liver, Walker 256 carcinosarcoma, Jensen sarcoma and Morris hepatoma 5123. It therefore appeared that with respect to a total of 15 different normal tissues of the adult male rat, as well as 3 varieties of transplantable rat tumors and a rapidly growing non-malignant tissue such as the regenerating liver, that glucose exerted a unique and characteristic stimulation of testicular protein biosynthesis.

Graph III indicates the effect of exogenous glucose on the in vitro incorporation of L-Lysine-U-C¹⁴ into acid-precipitable protein of slices of four different testicular preparations, each having its own individual histological structure. Whereas the absolute level of protein labeling of the immature testis, the cryptorchid testis and the interstitial-cell testicular

tumor were all higher than the adult scrotal testis, these tissues, which in no instance contained any spermatids displayed essentially no effect of glucose on protein labeling as compared to the adult scrotal testis. In view of the fact that only the adult scrotal testis contained spermatids, these data pointed to the possibility that the spermatids were the most sensitive cell type of the seminiferous germinal epithelium with regard to glucose regulation of testicular protein synthesis. As suggested by Hall (1965), and Means and Hall (1968b) this remarkable stimulation of spermatid protein biosynthesis is probably due to the maintenance of testicular ATP levels by glucose. Additional evidence that the spermatids are highly sensitive to glucose with regard to regulation of protein synthesis is presented in Graph III. A marked increase in the stimulatory effect of glucose on protein labeling of slices of the normal rat testis was observed with an increase in the age of the animal from 20 to 50 days, corresponding to the appearance of the spermatids in the developing seminiferous tubular epithelium.

In order to further investigate this possibility, as well as to compare protein labeling in the mitotic versus meiotic cell division, the following study was undertaken.

D. THE EFFECT OF GLUCOSE ON THE UPTAKE OF L-LYSINE- H^3 IN CELLS OF THE RAT SEMINIFEROUS EPITHELIUM

Plate VIII presents the results of a histological examination of the seminiferous epithelium of the rat following a 1 hour incubation period of slices of testes at 37.5°C (Graph V). The 14 stages of the cycle of the seminiferous epithelium in the rat have been classified according to the description of Leblond and Clermont (1962). Plate VIII indicates representative photomicrographs of these different stages of the cycle of the seminiferous epithelium of the rat testis stained with hematoxylin. As is well known,

each seminiferous tubule will contain at least two adjacent stages inasmuch as the cycle of the seminiferous epithelium is a continuous one. For example, stage I shown in Plate VIII also contains a small segment of a late stage XIV of the cycle of the seminiferous epithelium. It was found that the seminiferous epithelium of the rat testis retains its morphological integrity during the incubation period of the slice. On rare occasions, however, there was some indication of displacement of the cells located above the basal layer. Care was always taken to avoid counting grains in these areas. It was also found that a satisfactory classification of the various stages of spermatogenesis could be obtained by staining with hematoxylin alone. However, the absolute identification of each of the 14 stages of the cycle of the seminiferous epithelium of the rat testis was confirmed by staining alternate sections with the periodic acid-Schiff reagent with toluidine blue counterstaining. No counterstain was used on the radioautographs inasmuch as the grains could be seen more easily without it and the hematoxylin alone was found to give sufficient background stain.

Plate IX indicates the degree of labeling observed in radioautographs of sections taken from slices of rat testes incubated with L-Lysine- H^3 in the presence of 0.009M glucose (Graph IV). An exposure time of 4 days was found to give adequate grain densities for counting in all of the present experimental series. Stages VII and VIII of the cycle of the seminiferous epithelium seem to be characterized by a quite dense labeling pattern over the area of the primary spermatocytes with few grains appearing over the area of the spermatids. Stage XIII, on the other hand, shows a more homogenous grain distribution over both the primary spermatocytes and the spermatids.

Plate X presents representative radioautographs of sections taken from slices of rat testes incubated with L-Lysine- H^3 in the presence and absence

of glucose. A marked stimulation of protein labeling upon the addition of exogenous glucose was observed in the grain distribution of the pachytene primary spermatocytes present in stage VII. Glucose was also found to cause a great increase in the number of grains appearing over the area of the spermatids. In the absence of glucose, the spermatogonia and young spermatocytes were found to be the most heavily labeled cells with a gradual decline of the labeling being observed over the transition and pachytene primary spermatocytes. Few or no grains appeared over the area of the spermatids at any of the various stages of spermiogenesis in the absence of glucose in the incubation medium.

Graph VI is a plot of the average grain count per $200\mu^2$ above background over each of the successive cells of the cycle of the seminiferous epithelium of the rat testis 1 hour after incubation with L-Lysine- H^3 . In the control system, the only cells found to incorporate appreciable amounts of the isotope into protein were the spermatogonia and the young primary spermatocytes. Immediately after the division of type B spermatogonia into the resting primary spermatocytes, there was a marked increase in the degree of labeling over these cells. A gradual decline in grain counts then occurred over the area of the transition primary spermatocytes with little or no label appearing over the areas of the remaining cells of the spermatogenic cycle. A marked increase in the overall degree of protein labeling from L-Lysine- H^3 in the cells of the spermatogenic cycle was observed in the glucose-supplemented system. Again, the most heavily labeled cells were found to be the primary spermatocytes. However, in contrast to the results obtained in the control system, the early pachytene primary spermatocytes contained the most dense grain distribution. During the two meiotic cell divisions, a sharp decrease in the grain count was observed, with the greatest decrease in labeling being seen over the secondary

spermatocytes in metaphase. Whereas few if any grains were seen over the spermatids in the control system, the addition of exogenous glucose produced a marked increase in the number of grains appearing over these cells.

Table IV presents a statistical evaluation of the data obtained in terms of the number of grains per $200\mu^2$ above background observed for each main group of the successive cells of the cycle of the seminiferous epithelium. The mean data for the control and the glucose supplemented system obtained for cells from each of the four individual animals employed in the present studies have been compared using the t-test. In every instance, the difference in incorporation of tritiated lysine into protein of cells of the rat seminiferous epithelium observed for the control and the glucose supplemented system proved to be highly significant.

Graph VII summarizes the effect of exogenous glucose on the incorporation of L-Lysine- H^3 into protein of successive cells of the cycle of the seminiferous epithelium of the rat testis. The addition of glucose was found to cause essentially a threefold stimulation of protein labeling from L-Lysine- H^3 in the spermatogonia and the resting leptotene, zygotene and transitional primary spermatocytes. However, glucose enhanced protein synthesis (labeling) from L-Lysine- H^3 by a factor of 20 in the pachytene primary spermatocytes. Glucose was found to have relatively little effect on protein labeling of the cells involved in the later stages of meiotic division, namely, the primary and secondary spermatocytes in metaphase. However, during the process of spermiogenesis in which the spermatids undergo their morphogenic transition into free spermatozoa, glucose was found to exert its most pronounced stimulatory effect on protein labeling observed in the various cells of the cycle of the seminiferous epithelium of the rat testis.

E. EFFECT OF GLUCOSE ON UPTAKE OF L-LYSINE- H^3 IN CELLS OF THE HUMAN SEMINIFEROUS EPITHELIUM

The question whether the previously described pattern of protein labeling of various successive cells of the seminiferous germinal epithelium of the rat would be similar in slices of human testis incubated with tritiated lysine. The problems of comparing experimental animal data versus human data are well known and the difficulties involved in performing dynamic biochemical studies on viable human testicular slices is no exception. Because we were fortunate to obtain large segments of normal human testes of two males aged 65 and 70 years, respectively, which were removed as a therapeutic measure for adenocarcinoma of the prostate, the following study was performed (Plate XII).

Plate XIII presents the results of a histological examination of the human seminiferous epithelium following a 1 hour incubation period of slices of testes at $37.5^{\circ}C$. The 6 stages of the cycle of the seminiferous epithelium in the rat have been classified according to the description of Clermont (1968). It was found that the seminiferous epithelium of the human testis retains its morphological integrity during the incubation period of the slice; however, it was notably more friable than the rat seminiferous epithelium. It was found that a satisfactory classification of the various stages of spermatogenesis could be obtained by staining with hematoxylin alone. No counterstain was used on the radioautograms inasmuch as the grains could be seen more easily without it and the hematoxylin alone was found to give sufficient background stain.

This plate indicates the degree of labeling observed in radioautograms of sections taken from slices of human testes incubated with L-Lysine- H^3 in the presence of $0.009M$ glucose. An exposure time of 4 days was found to give adequate grain densities for counting in all of the present experimental series.

Graph VIII is a plot of the average grain count per $200\mu^2$ above background over each of the successive cells of the cycle of the human seminiferous epithelium, 1 hour after incubation with L-Lysine- H^3 . In the control system, the only cells found to incorporate appreciable amounts of the isotope into protein were the spermatogonia, pachytene spermatocytes and young spermatids (Sa). Immediately after division of type B spermatogonia into the resting primary spermatocytes, there was a marked increase in the degree of labeling over these cells, summing in late pachynema. An abrupt decline in grain counts then occurred over the area of the secondary spermatocytes and the secondary spermatocytes at metaphase. Subsequently, a rapid increase in protein labeling over the area of young spermatids (Sa) is noted, which then drops abruptly over the remaining cells of the spermatogenic cycle.

A marked increase in overall degree of protein labeling from tritiated Lysine in the cells of the spermatogenic cycle was observed in the glucose supplemented system. Not only do the primary spermatocytes actively incorporate tritiated Lysine into protein, but there is also a rapid increase in protein labeling following division of the spermatogonia into primary spermatocytes. A precipitous drop in protein labeling is repeated over the secondary spermatocytes and the secondary spermatocytes at metaphase. This decrease was observed to be more pronounced than that occurring in the seminiferous epithelium of the rat. Whereas the grain distribution over the young spermatids (Sa) occurred rapidly then decreased at Sb in the control system, grain distribution in the glucose supplemented system increased rapidly in the young spermatids (Sa) but was further stimulated into incorporation of L-Lysine- H^3 into protein over the remaining cells of the spermatogenic cycle. Grain density dropped abruptly following the most mature phase (Sd) of human spermiogenesis. This observation was also noted in the studies on the seminiferous epithelium of the rat testis.

Graph IX summarizes the effect of exogenous glucose on the incorporation of L-Lysine- H^3 into protein of the successive cells of the cycle of the seminiferous epithelium of the human testis. The addition of glucose was found to cause essentially a twofold stimulation of protein labeling from tritiated lysine in the spermatogonia, threefold stimulation in the resting primary spermatocytes, a two and one-half fold stimulation in the leptotene primary spermatocytes, a twofold stimulation in the zygotene primary spermatocytes and a one and one-half fold stimulation in the pachytene primary spermatocytes. Glucose was found to have little effect on cells in the later stages of meiotic division namely the secondary spermatocytes in metaphase. In contrast to the effect of glucose on the incorporation of tritiated lysine into protein of the spermatids of the rat, the early stages of human spermiogenesis (Sa) has the lowest degree of overall stimulation of labeled protein in the presence of glucose. This effect closely duplicates the effect of glucose on the incorporation of tritiated lysine in the seminiferous epithelium of the rat. The overall ratio of stimulation of glucose on the incorporation of L-Lysine- H^3 into protein of testicular slices is lower in the human. This lower ratio may be explained by noting that in the control system of the human seminiferous epithelium, the incorporation of tritiated lysine in terms of grains/ $200\mu^2$ is generally twice the level of that in the control system of the rat. In addition, these results may be reflective of a reciprocal effect in overall incorporation of tritiated lysine into testicular protein of a young seminiferous epithelium versus an old seminiferous epithelium.

Table V presents the statistical evaluation of the data obtained in terms of the number of grains per $200\mu^2$ above background above each of the main groups of cells of the cycle of the human seminiferous epithelium. The mean data for the control and the glucose supplemented systems were obtained for

cells from each of the two human testes employed in these experiments. In addition, these mean values have been compared using the t-test. The statistical comparison between the control and the glucose supplemented systems demonstrate that the results obtained for the spermatogonia, resting primary spermatocytes, leptotene primary spermatocytes, zygotene primary spermatocytes, pachytene primary spermatocytes, secondary spermatocytes, Sb and Sc spermatids to be highly significant; however, no significant increase in protein labeling in the glucose supplemented system occurred in the metaphase secondary spermatocytes, Sa and Sd spermatids.

CHAPTER IV

DISCUSSION

A. THE USE OF L-LYSINE- H^3 AS A PROTEIN PRECURSOR FOR THE STUDY OF TESTICULAR PROTEIN SYNTHESIS

The tritiated amino acid L-lysine- H^3 was selected as a protein precursor for the following reasons. First this amino acid is unique in that it is generally incapable of entering into transamination reactions with other amino acids (Cammarata and Cohen, 1950; Cohen, 1939). Consequently, the possibility of other amino acids labeled as a result of transamination with H^3 -Lysine being incorporated into testicular protein is markedly reduced, and as a result, any radioactivity identified in protein after incubation of slices of rat or human testis samples in the presence of this isotope can be deduced as being due to the presence of only labeled lysine in the testicular protein.

Secondly, the catabolic fate of lysine involves the conversion of this diamino-monocarboxylic-hexa-carbon acid to the corresponding alpha-keto form which undergoes cyclization to pipecolic acid (Rothstein and Miller, 1953). By a series of subsequent reactions, not thoroughly identified, ring cleavage occurs, and the monoamino-dicarboxylic-hexa-carbon alpha-amino adipic acid results. This amino acid is then deaminated and subsequently oxidized via alpha-keto adipic acid to the glutaric acid derivative (Rothstein and Miller, 1953; Rothstein and Greenberg, 1959; Shapley, 1920). The critical point of this catabolic schema is that none of the products resulting from lysine degradation are incorporated into protein. In addition, only a small percentage of lysine is catabolised in various tissues of the rat which leaves the major portion available for incorporation into protein.

Thirdly, Shettles (1942, 1960) has demonstrated that L-lysine is a required amino acid for normal testicular morphogenesis.

Therefore, L-Lysine- H^3 was selected because its catabolic fate does not result in degradative derivatives entering protein synthetic pathways, that

only a small fraction of the amino acid enters the catabolic pathway, majority enters into anabolic pathways. Finally, lysine appears to be a required testicular amino acid, necessary for cellular integrity and consequent normal fertility.

B. THE GERMINAL EPITHELIUM OF THE CRYPTORCHID TESTIS EXPERIMENTALLY INDUCED IN PREPUBERTAL AND ADULT RATS

The data of the present experiments indicate that the weight of the abdominal testis of the rat is markedly less than the weight of the scrotal testis of the same animal following the experimental induction of cryptorchidism. It was found that the magnitude of this difference in testicular weight was the same in two different types of experimental procedures capable of inducing cryptorchidism; abdominal fixation of a previously undescended immature testis of a prepubertal rat, and abdominal transplantation of a previously descended scrotal testis of an adult rat. Such would seem to indicate that the deleterious effects of an increased environmental temperature on testicular weight are quite similar when both degeneration of existing mature phase of spermatogenesis and cessation of initial spermatogenic development are involved (Davis, et al., 1963).

It has long been known that the germinal cells of the testis undergo rapid deterioration when subjected to increased temperature. The more mature cells of the seminiferous germinal epithelium appear to be the most susceptible to temperature (Chowdhury and Steinberger, 1964; Williams and Cunningham, 1940). With the method of inducing cryptorchidism by transposition of a scrotal testis of an adult rat into the abdominal cavity, the most consistent changes observed in the abdominal testis following approximately 30 days retention in the abdominal cavity have been the appearance of seminiferous tubules lined by Sertoli cells, spermatogonia, and occasional pachytene primary spermatocytes. The present

studies indicate that with this method (transplanting a mature testis from the scrotal sac into the abdominal cavity), the earliest histologic changes observed after 2 days of abdominal retention occur in Stages IV-VI, IX-XI, and XIV of the cycle of the seminiferous epithelium. These changes involve migration of the early generation spermatids toward the lumen of the seminiferous tubule with exfoliation of some of the pachytene primary spermatocytes in the tubular lumen. The spermatids in the cap phase of spermiogenesis are the first germinal cell type to undergo such degenerative changes as cytoplasmic swelling and karyolysis. Following 5 days of abdominal retention, the exfoliated spermatids and pachytene primary spermatocytes were found to form multinucleated giant cells in the tubular lumen, leaving large cell-free areas in the remaining seminiferous epithelium. After 10-30 days in the abdominal cavity, the cytoplasm of the Sertoli cells was found to increase in size so as to actually obliterate the seminiferous tubular lumen. Numerous Type B spermatogonia as well as a few occasional pachytene primary spermatocytes were found within the now prominent Sertoli-cell retinaculum. However, by 30 days following abdominal retention, approximately half of the Sertoli cells present demonstrated both fibrosis and hyalinization of their cytoplasm as well as nuclear pleomorphism.

The present data also indicate that following 30 days exposure to an abdominal environment, similar histologic changes occurred in experimental cryptorchidism induced by abdominal fixation of a previously undescended immature testis. The seminiferous tubules were found to consist of a prominent Sertoli-cell retinaculum containing numerous Type B spermatogonia and occasional pachytene primary spermatocytes. In a similar fashion, approximately half of the Sertoli cells present after 30 days of abdominal retention showed fibrosis and hyalinization of the cytoplasm with nuclear pleomorphism. It would therefore appear that spermatogenesis is halted in the cryptorchid testis induced

by this more physiologic method in the pachynema stage of meiosis and that no further progression of spermatogenesis occurs in the abdominally retained testis. In addition, the similarity seen in the histologic appearance of cryptorchid testes experimentally produced in the rat by abdominal fixation of an immature testis and abdominally transplanted previously descended mature testis suggest that cryptorchidism induced by the latter method closely approximates the physiologic condition of an undescended testis as it "normally" occurs in the human.

Inasmuch as the cessation of spermatogenesis at the pachynema stage of meiosis caused by an increased abdominal environmental temperature occurred when the spermatids had undergone degeneration as well as when they failed to form, the possibility is suggested that the spermatids may contain a regulator which is necessary in some feedback mechanism for the normal development of spermatogenesis. This regulator may be intimately involved in hypophyseal gonadotropin secretion, as postulated by Johnsen (1964), or may act directly on the testis by a "trigger" mechanism through the loss of cytoplasmic material from the maturing spermatid.

Previous data from this laboratory (Firlit and Davis, 1965) has implicated the spermatid cytoplasm as being intimately involved in the trigger mechanism. Early studies by Roosen-Runge (1952) had implicated that the release of older spermatids from the lumen of the seminiferous tubule is in some unknown manner associated with the regulation of the development of earlier generations of the germinal epithelium. Lacy (1960) demonstrated that the bulk of the lipid observed in the Sertoli cells was derived from the phagocytosis of the residual bodies, and that after the release of the spermatozoa, there was a gradual reduction in the lipid content of the Sertoli cell, indicating the release of some substance that might influence spermatogenesis. For these reasons, it

was postulated that the local regulating mechanism involved in the successive replacement of germ cells about a radial axis during the spermatogenic cycle may be due to a Sertoli cell hormone, the production of which is initiated or accelerated by the phagocytosis of the residual bodies of the spermatids.

The data from this laboratory indicate that glycogen is a significant constituent of the cytoplasmic body (Firlit and Davis, 1965). After the development of the cytoplasmic body into the residual body at CB-4, two possible fates of this structure have been demonstrated. The residual body can either be phagocytosed by a Sertoli cell as shown by Lacy (1960), or can independently migrate to a final position along the basement membrane of the seminiferous tubule. In either case the possibility exists that the residual body may contribute appreciable amounts of glycogen either to the Sertoli cell or to the germinal epithelium (Daled, 1951).

C. RADIO-AUTOGRAPHIC INCORPORATION OF L-LYSINE- H^3 INTO PROTEIN OF CELLS OF THE GERMINAL EPITHELIUM IN CRYPTORCHIDISM

Unilateral cryptorchidism has been induced in adult rats by transplanting a mature testis from the scrotal sac into the abdominal cavity. Following exposure to an abdominal environment for 30 days, three distinct types of seminiferous tubules were found to occur in approximately equal numbers in the cryptorchid testis based on the cellular composition of their germinal epithelium. Only four cell types were found to remain in the germinal epithelium of the abdominal testis 30 days after experimental induction of cryptorchidism. These cell types were 'crust' spermatogonia, pachytene primary spermatocytes, normal-appearing Sertoli cells and atrophic-appearing Sertoli cells, accounting for 31%, 3%, 28% and 38% respectively, of the total cells remaining in the germinal epithelium of the seminiferous tubules of the abdominal testis.

A comparison of the in vitro capacity to incorporate a radioactive amino acid into protein of the remaining cells of the germinal epithelium of the cryptorchid rat testis with the corresponding cells found in the scrotal testis has been carried out. The data of the present experiments indicates that the experimental induction of cryptorchidism results in a forty-fold increase in the in vitro incorporation of tritiated lysine into protein of the normal-appearing Sertoli cells of the abdominal testis and a twelve-fold increase in the in vitro incorporation of tritiated lysine into protein of the atrophic-appearing Sertoli cells of the abdominal testis as compared to protein labeling of the Sertoli cells found in the corresponding scrotal testis of the same animal. In addition, cryptorchidism was found to produce a four-fold increase in protein labeling of both 'crust' spermatogonia and pachytene primary spermatocytes as compared to the corresponding cells found in the scrotal testis.

Little is known concerning the function and metabolic activity of the Sertoli cell, especially in cryptorchidism, other than their involvement in the phagocytosis of dead germ cells (Clegg and Macmillan, 1961) and the residual bodies discarded by maturing spermatids (Kingsley, Smith and Lacy, 1959; Firlit and Davis, 1965). Tepperman, Tepperman and Dick (1949) have attempted to relate an increase in oxygen uptake by the cryptorchid testis of the rat in vitro to a possible persistence of the Sertoli cells in the germinal epithelium following destruction of some of the other testicular cell types. Clegg (1963a) has reported that the induction of artificial cryptorchidism in the rat results in a significant increase in the number of Sertoli cells with the cytoplasm of the Sertoli cells becoming more prominent as the maturing cells of the seminiferous epithelium disappear. Inasmuch as no mitoses were seen in the Sertoli cells, the possibility of amitotic division occurring in the Sertoli cells is offered as a partial explanation of the apparent increase in Sertoli cells in cryptorchidism. The fact that cryptorchidism also results

in an increased incorporation of radioactive lysine into the Sertoli cell cytoplasm as observed in the present studies further suggest that amitosis may occur.

The data of the present experiments indicate that the previously reported increase in labeling of perchloric acid-precipitable protein from C^{14} -labeled lysine in the cryptorchid rat testis in vitro (Davis, et al., 1964) has been confirmed on a cellular level, employing radio-autographic techniques and tritiated lysine. In addition, a comparison of the capacities of the remaining cells of the germinal epithelium of the cryptorchid rat testis to incorporate tritiated lysine into protein indicates that the greatest enhancement of protein labeling due to an increased abdominal temperature occurs in the Sertoli cells.

D. THE EFFECTS OF GLUCOSE ON THE UPTAKE OF L-LYSINE- H^3 IN CELLS OF THE SEMINIFEROUS EPITHELIUM OF THE RAT

The effects of exogenous glucose on protein labeling of the successive cells of the seminiferous epithelium of the rat has been investigated utilizing radioautographic techniques. Lysine would appear to be a particularly useful labeled precursor for studying protein labeling radioautographically inasmuch as lysine has been shown to be a relatively stable molecule (Schoenheimer, et al., 1939), other than being incorporated into protein. Lysine does not readily undergo transamination (Campbell, 1942) and has been found to yield only extremely small amounts of tricarboxylic acid cycle intermediates during the course of its catabolism (Davis and Morris, 1963b).

A typical seminiferous tubule of the rat will contain a few spermatogonia located along the basement membrane, one or several layers of spermatocytes further in, and clusters of spermatids located along the lumen of the tubule. Because of the ease of identifying the first 14 stages of the development of the spermatids according to the description of Leblond and Clermont (1962),

it has been possible to determine the pattern of grain distribution from L-Lysine- H^3 over each of the cell associations during the development of the spermatogonia into free spermatozoa, including the following main phases of spermatogenesis: (1) the mitotic divisions of the spermatogonia, (2) the first meiotic prophase of the primary spermatocytes and (3) the morphogenic development of spermatids, known as spermiogenesis.

Five mitotic peaks have been found to occur during the divisions of the spermatogonia in the spermatogenic cycle. The first three peaks of spermatogonial mitoses, occurring at Stages I, IX, and XII, give rise to type A spermatogonia. The fifth peak of spermatogonia mitoses occurring at Stage VI and results in the division of type B spermatogonia into young spermatocytes resulting from the division of type B spermatogonia at Stage VI. This large increase in protein labeling, reaching a peak at the resting primary spermatocyte stage, apparently occurs at the transition from somatic mitoses to the first meiotic prophase. It is interesting to note that the premeiotic synthesis of DNA also takes place in resting primary spermatocytes and that no further synthesis of DNA has been found to occur in later stages of spermatogenesis (Monesi, 1962). Indeed, the data of the present studies indicate that virtually no protein synthesis occurs in these later stages of spermatogenesis in the absence of exogenous glucose.

The predominant feature of meiosis involves paring, crossing over and reduction in chromosome number (Rhoades, 1961). It was found that the addition of glucose produced an overall stimulation of protein labeling in all of the successive cells of the seminiferous epithelium of the rat. However, glucose was found to exert a marked stimulation of protein labeling in the primary spermatocytes whose nuclei were at pachynema and whose chromosomes were undergoing crossing over. These data suggest the possibility that glucose may be

intimately involved in the mechanisms of recombination of chromosomal segments during meiosis, thereby playing an important role in the constantly changing pattern of genes. In contrast to these results, a marked decrease in protein labeling in the presence of glucose was observed in the secondary spermatocytes just prior to the second maturation division. In a similar fashion, the first and second meiotic divisions occurring during the development of *Trillium* anther has been reported to be associated with a fall in protein sulfhydryl groups (Stern, 1958). The fact that glucose was found to exert such a remarkable stimulation of protein labeling in the more mature testicular cells undergoing spermiogenesis may offer a partial explanation for the atrophic changes of the maturing spermatids resulting from experimentally induced hypoglycemia (Mancine, et al., 1960). Any decrease from a normal intracellular glucose content in the testis which may result from a lowered blood glucose level may be reflected in a decrease of testicular protein biosynthesis with a resulting atrophy of the seminiferous germinal epithelium and an impairment of the spermatogenic cycle.

E. THE EFFECT OF GLUCOSE ON THE INCORPORATION OF L-LYSINE- H^3 INTO PROTEIN OF CELLS OF THE SEMINIFEROUS EPITHELIUM OF THE HUMAN TESTIS

The effect of exogenous glucose on the protein labeling of the successive cells of the cycle of the human seminiferous epithelium has been investigated utilizing radioautographic techniques. Following a 1 hour incubation period, slices of human testis in the presence or absence of glucose plus tritiated lysine, the pattern of protein labeling over each of the cell associations of the human seminiferous epithelium is remarkably similar to that described for the rat. Not only do the primary spermatocytes also most actively incorporate lysine into testicular protein, but there is also a rapid increase in protein labeling following division of the spermatogonia into primary spermatocytes.

However, in the control system, the resting and leptotene primary spermatocytes in the rat were predominantly active in utilizing tritiated lysine. In contrast, the resting and leptotene primary spermatocytes of the human seminiferous epithelium represent the lowest level of protein labeling in the primary spermatocytes of the human testis. As maturation of the primary spermatocytes occurs, the level of protein labeling increases to summit at late pachynema. It is of interest to note that the duration of the first meiotic prophase of the primary spermatocytes in the cycle of the human seminiferous epithelium occurs at a more rapid rate than in the rat. Consequently, a comparison of the two meiotic prophases discloses the presence of one resting primary spermatocytes in the human seminiferous epithelium and three resting primary spermatocyte cell stages in the rat. In addition, the transitional period of the first meiotic prophase which is characterized by the active process of pairing of chromosomal material is of such a short duration in the cycle of the human seminiferous epithelium so as not to be identified as a specific cell stage. In a similar manner the period of diakinesis, shortening of chromosomal segments, is also of short duration.

The only difference in the pattern of protein labeling in the successive cells of the seminiferous epithelium of the human testis as compared to the rat testis appears to involve the degree of stimulation that glucose exerts over the pachytene primary spermatocytes. The greater enhancement of protein labeling by glucose in the pachytene primary spermatocytes occurring in the rat seems to be due to the presence of lower control values of these cells in the rat. One possible explanation may be that in the absence of adequate levels of glucose, the earlier resting and leptotene primary spermatocytes have a higher capacity for protein synthesis than the later pachytene primary spermatocytes in younger animals as opposed to older animals.

In view of the fact that the human testicular specimens were obtained from patients aged 65 and 70 years respectively, the phenomenon of age in its effect on the degree of spontaneous protein synthesis and also in its responsiveness to the addition of exogenous glucose still remains to be determined.

The present studies have demonstrated characteristic quantitative radioautographic patterns of protein labeling in the various successive cells of the seminiferous germinal epithelium of both the rat and human. Although similar quantitative patterns for nucleic acid labeling are presently lacking for these two species, Monesi (1962 and 1965) has investigated the radioautographic incorporation of nucleic acid precursors in the mouse testis. DNA was found to be synthesized during the resting stage of the primary spermatocytes with RNA synthesis reaching a peak in middle pachytene, declining during late pachytene and then ceasing completely during metaphase and anaphase I and II. These observations on nucleic acid labeling in the mouse testis seem to be consistent with our findings for protein labeling in the various successive cells of the seminiferous germinal epithelium of both the rat and human testis. Any differences in protein biosynthesis of those cells of the spermatogenic cycle responsible for cell proliferation and renewal as compared to the more mature spermatids may have importance not only in investigations dealing with regulation of male fertility but also in investigations dealing with potential side-effects of drugs that may cause genetic damage with resulting congenital malformations. It is our opinion that the presently described radioautographic patterns of protein labeling over each of the successive cells of the seminiferous germinal epithelium may provide an extremely practical initial screening procedure for the detection of any such possible side-effects of drugs on spermatogenesis. Drugs which are commonly administered to the male prior to the breeding performance may alter the radioautographic pattern of protein

labeling over the successive cells of the spermatogenic cycle in a more sensitive manner than a usual histological examination of the testis. Any drugs that are found to influence initially the radioautographic pattern of protein labeling of the successive cells of the seminiferous epithelium could then be studied further employing the more laborious techniques of mating experiments with subsequent examination of offspring.

CHAPTER V
CLINICAL CORRELATION .

A. BIOPSY OF THE TESTES

Biopsy of the human testis has become an accepted clinical procedure for evaluating the state of cellular activity within the seminiferous epithelium as well as the interstitial tissue (Montagna and Hamilton, 1952; Nicander, 1957). Nelson (1951, 1953) has commented that testicular biopsy serves a role in the evaluation and diagnosis of male gonadal defects and functional gonadal disturbances which is even superior to the endometrial biopsy of the female. The latter reflects the character of ovarian endocrine function without revealing defects of gametogenesis, whereas the testis biopsy furnishes an exact observation of the male sex cells. Testicular biopsy is now currently employed for three specific purposes: to differentiate between azoospermia due to obstruction or incomplete spermatogenesis (Johnson, 1964; Lezebnik and Kamhi, 1958); to diagnose and evaluate disturbances in the endocrine systems (Bongiovanni and Root, 1963); and for research purposes in which the normal and abnormal processes that characterize the testis in puberty, adolescence, maturation and senility are investigated (Dela Chapelle and Aortiling, 1962; Deming, et al., 1949).

A biopsy of the testes serve a most useful and precise purpose in establishing a diagnosis between obstructive azoospermia and absence of sperm in the ejaculate due to faulty spermatogenesis. The clue to the correct diagnosis may be obtained from the history or the physical examination but often there is no specific guide. A history of bilateral epididymitis strongly suggests that an obstruction has caused a block preventing the passage of spermatozoa from the epididymis to the seminal vesicles. Small, soft testes may be identified during a physical examination and little doubt remains to account for the azoospermia on the basis of arrested or absent spermatogenesis. These examples serve to identify the clinical situations in which the etiology of infertility can be ascertained. However, it is not uncommon to find a

well-developed and otherwise normal man having semen devoid of spermatozoa and without a report of an illness to indicate a probable cause for the azoospermia. This particular individual would greatly benefit from a testicular biopsy in disclosing the etiology of his azoospermia (Gross and Jewett, 1957; Gross and Peplogue, 1963).

Testicular biopsies are advocated by many (Goddum, 1968; Hamilton, 1961) when marked oligospermia is present. It is rare to find important discrepancies in the testicular biopsies of each testis of the same individual particularly if both are of equal size, are normal in scrotal position and neither has been subjected to trauma or surgical procedures or coexisting disorders such as a varicocele. Testicular biopsy may disclose a progressive testicular parenchyma disease such as peritubular fibrosis and established an unfavorable prognosis in men with oligospermia (Sohval, 1954).

Testicular biopsy has been a most important tool in research where it has been extensively employed to elucidate the variety of alterations in spermatogenesis and abnormalities in the interstitial cells. It has been an extremely valuable aid in the study of endocrinology and it is clinically employed to advantage in the evaluation of endocrine disorders in which fertility is not the prime or sole consideration (Shettles, 1942 and 1950; Wells, 1943).

B. HISTOLOGICAL EVALUATION OF TESTICULAR BIOPSY (HUMAN)

1. Normal Seminiferous Tubule

The peritubular membrane is represented by a thin line separating the interstitial from the spermatogenic cells. All the testicular blood vessels are found in the interstitial areas and it is the present presumption that the nutritive elements must pass through the membrane to reach the germinal cells. In a like manner the waste products of metabolism must pass out of the tubule by perfusion through the peritubular membrane. Spermatogonia are

are found in the single roll of cells that are arranged along the basement of the seminiferous tubule (Lockwood, 1887; O'Connor, 1960).

a. Sertoli Cells

The Sertoli cells are oval or fusiform in shape and are located just above the spermatogonia, and project radially into the lumen of the tubule. They have the only oval nuclei to be found in the germinal epithelium. Primary spermatocytes have the largest nuclei around a chromatin network which is clearly visible and changes in consistency during the meiotic prophase. The primary spermatocyte is clearly larger than the secondary spermatocytes. The spermatids are recognized by their small dark nuclei near the border of the seminiferous tubular lumen. The spermatid undergoes a series of morphological alterations during its maturation into a mature spermatozoa. Usually spermatozoa are identified as embedded in the cytoplasm of the Sertoli cell. Because of this morphological relationship a nutritive or trophic function has been attributed to the Sertoli cells (Deming, 1963).

2. Germinal Aplasia

Here the seminiferous tubules are usually uniform in size, however, the striking feature is a complete lack of germ cells present within the tubules. The sole remaining epithelial cells are the Sertoli cells. The cytoplasm of the Sertoli cells is rather vague in outline and faintly acidophilic. Occasionally a few tubules may show a sprinkling of germ cells, but mitosis does not to beyond the spermatogonia or primary spermatocyte stage. The Leydig cells are usually histologically normal (Shapley, 1920; Steinberger and Tjioe, 1969).

3. Spermatogenic Arrest

Spermatogenic arrest is characterized by a failure of spermatogenesis to go on to completion. The tubules are of normal size, the spermatogonia are normal in numbers, and the Sertoli cells are not unusual. Cellular arrest

may occur at the level of the primary spermatocytes the secondary spermatocytes, or at the spermatid stage. Clinically, the results are the same. Few mature spermatozoa, if any, are produced. If insufficient cells are present in the germinal layer, there will be an abundance of cellular debris in the lumen of the seminiferous tubules (Barr, 1957; Bunge and Bradbury, 1960; Dahl and Herrick, 1959).

4. Generalized Peritubular Fibrosis

The seminiferous tubule varies in size in relation to the degree of fibrosis. When a dense fibrotic layer is present in the periphery of the tubule, there appears a consequent shrinkage in size of the seminiferous tubule. In addition, spermatogenesis is often proportionate to the degree of peritubular fibrosis. The process is probably a progressive one and continues until all germ cells are eliminated by the thickening of the peritubular connective tissue. Eventually complete sclerosis of the tubule occurs (Dela Chapelle and Aortiling, 1962; von Ebner, 1871; Heller and Nelson, 1945).

5. Incomplete Maturation and Disturbed Spermatogenesis

The seminiferous tubules of such testis are found in men who have very few sperm or in whom the sperm count is below the average (20,000,000 - 80,000,000/mm³). Usually the diameters of the tubules are normal or somewhat smaller than usual. Peritubular fibrosis may be present in varying degrees so that the testis which produces a small number of sperm may have all the components of the normal and abnormal testis (Lockwood, 1887). The commonly recognized defects in this classification are a sloughing of the immature cells; atypical mitosis, particularly at the division; and abnormal nuclei, mature sperm and immature sperm present in the seminiferous tubular lumen. These cells appear sloughed into the tubular lumen rather than being confined to the sustentacular cells of Sertoli. A distortion of cell division of abnormal nuclear construction may be seen (Goddum, 1968; Johnsen, 1964).

Much speculation is given to the causes for these abnormalities since histologic evidence for inflammation is not present within the testis. Causes such as x-rays, toxins, genetic influences and nutritional disturbances either from deficiencies or faulty intermediary metabolism are suspected. It is evident that whatever the specific etiologies to date they still remain obscure (Shettles, 1942 and 1960).

C. CLINICAL SIGNIFICANCE

Metabolic, histological and radioautographic studies of the cells of the cycle of the seminiferous epithelium have been discussed. We have been able to demonstrate that surgically induced (artificial) cryptorchidism in the adult (mature) rat produced a seminiferous epithelium whose histological appearance was identical with that which occurred in the prepubertal surgically-induced cryptorchidism. In this early study, it was demonstrated that the spermatids, spermatozoa, secondary spermatocytes were highly heat labile. In clinical situations of human cryptorchidism, histological examination of biopsy specimens illustrate a morphologic picture essentially similar to that of experimental rat cryptorchidism. This demonstrated the applicability of this experimental model in studying clinical problems. In cryptorchid testes germinal maturation fails to advance to the spermatid stage. The tubules are filled with a Sertoli cell retinaculum with occasional peripherally scattered spermatogonia and rare primary spermatocytes. Clinically these tubules are described as demonstrating germinal arrest. Cryptorchidism is felt to be the result of an increased body, ambient temperature causing death of the mature germinal cells or frankly preventing the advanced maturation (spermiogenesis) of the primitive cells, namely the spermatogonia and primary spermatocytes.

Metabolic studies on slices of cryptorchid testes demonstrated the increased rate of incorporation of ^{14}C -labeled Lysine into testicular protein. To clarify whether this represented a true alteration (increase) in overall

testicular cellular maturation or simply an unmasking phenomena secondary to the exfoliation of germinal cells, radiographic studies utilizing ^3H -Lysine were performed. These studies confirmed the increased level of protein synthesis and further disclosed that this increased level of protein synthesis was as a result of an increased overall cellular metabolic rate. It appeared that the increased ambient temperature, although toxic to mature germinal cells, stimulated enzymatic protein anabolic pathways in the primitive cells of the spermatogenic cycle, namely the Sertoli cells, spermatogonia and primary spermatocytes. This study presented one explanation for the persistence of these primitive germinal cells in the cryptorchid testis. .

An investigation of the metabolic significance of glucose on protein metabolism was performed utilizing radioautographic techniques. The data indicated that in the control situation, protein synthesis occurred at a higher rate in the more primitive cells of the cycle of the seminiferous epithelium in the rat. However, with the addition of glucose into the incubation media a marked facilitation of protein anabolism occurred in the more mature cells of the cycle namely, pachytene primary spermatocytes and spermatids. The apparent dependency on glycolytic-energy producing pathways of these mature cells in synthesizing cellular protein was demonstrated. The need to further investigate other biochemical parameters is necessary to fully analyze the tenuous metabolic sensitivity of the maturing germinal cells. The clinical significance of these data prompted two human experiments utilizing orchiectomy specimens and ^3H -Lysine as the protein precursor. Radioautography demonstrated a phenomenally similar metabolic profile of human protein anabolism to the rat. Specifically, it demonstrated a high rate of protein synthesis in the primitive germinal cells and the pronounced glucose stimulating effect in the maturing germinal cells. Because of the demonstrated heat lability of mature germinal

cells and the acute sensitivity of these cells to glycolytic energy producing pathways in facilitating cellular protein synthesis, further clinical experimentation is needed. Testicular biopsy specimens of patients experiencing germinal failure, spermatogenic arrest, peritubular fibrosis and incomplete and disturbed spermatogenesis are necessary to further correlate these data in an attempt to alleviate these problems of male infertility.

As a result of these investigations, we have speculated that each clinical situation identified above may be the net reflection of a specific heat-labile enzymatic pathway, a specific metabolic precursor deficiency (complete or incomplete) a hormonal deficiency or finally a combination of any of these and in varying proportions.

CHAPTER VI

SUMMARY

1. The germinal epithelium of the cryptorchid testis of the rat was histologically studied at various time periods following abdominal retention of the testis.
2. Cryptorchidism was experimentally induced by two different procedures: abdominal fixation of a previously undescended immature testis of the prepubertal rat, and abdominal transplantation of a previously descended mature testis of the adult rat.
3. Similar morphologic changes in the seminiferous tubules were observed following both types of cryptorchidism, including the predominance of a Sertoli-cell retinaculum containing numerous Type B spermatogonia and occasional pachytene primary spermatocytes.
4. It was found that half of the Sertoli cells present following 30 days of exposure to an intra-abdominal temperature demonstrated cytoplasmic fibrosis and hyalinization as well as nuclear pleomorphism.
5. A comparison of the in vitro capacity to incorporate a radioactive amino acid into protein of the remaining cells of the germinal epithelium of the cryptorchid rat testis with the corresponding cells found in the scrotal testis has been carried out.
6. The data indicates that the experimental induction of cryptorchidism results in a forty-fold increase in the in vitro incorporation of tritiated lysine into protein of the normal-appearing Sertoli cells of the abdominal testis and a twelve-fold increase in the in vitro incorporation of tritiated lysine into protein of the atrophic-appearing Sertoli cells of the abdominal testis as compared to the protein labeling of the Sertoli cells found in the corresponding scrotal testis of the same animal.
7. Cryptorchidism was found to produce a four-fold increase in protein labeling of both 'crust' spermatogonia and pachytene primary spermatocytes as compared to the corresponding cells found in the scrotal testis.

8. No mitoses were seen in the Sertoli cells; the possibility of amitotic division occurring in the Sertoli cells is offered as a partial explanation for the apparent increase in the Sertoli cells in cryptorchidism.
9. The fact that cryptorchidism also results in an increased incorporation of radioactive lysine into the Sertoli cell cytoplasm as observed in these studies further suggest that amitosis may occur.
10. The data presented indicate that the previously reported increase in labeling of perchloric acid-precipitable protein from C^{14} labeled lysine in the cryptorchid rat testis in vitro has been confirmed on a cellular level employing radio-autographic techniques and tritiated lysine.
11. A comparison of the capacities of the remaining cells of the germinal epithelium of the cryptorchid rat testis to incorporate tritiated lysine into protein indicates that the greatest enhancement of protein labeling due to an increased abdominal temperature occurs in the Sertoli cells.
12. The radioautographic incorporation of L-Lysine- H^3 into protein of cells of the seminiferous epithelium of the rat has been studied following a 1 hour incubation of slices of testis with labeled precursor. In addition, the effect of exogenous glucose on protein labeling of the successive cells of the cycle of the seminiferous epithelium of the rat has been investigated.
13. In the absence of added glucose, the most heavily labeled cells of the seminiferous epithelium were the resting primary spermatocytes, with little or no label appearing in the remaining cells of the spermatogenic cycle.
14. The transition from somatic mitosis to the first meiotic prophase has been found to be associated with a marked increase in protein labeling from radioactive lysine.
15. The addition of glucose caused a significant increase in protein labeling from tritiated lysine in all the cells of the spermatogenic cycle with

the greatest degree of stimulation being noted in the primary spermatocytes and spermatids.

16. Glucose was found to exert a marked stimulation of protein labeling in the primary spermatocytes whose nuclei were at pachynema and whose chromosomes were undergoing crossing-over. These data suggest that glucose may be intimately involved in the mechanisms of recombination of chromosomal segments during meiosis, thereby playing an important role in the constantly changing pattern of genes.
17. The observation that glucose was found to exert such a remarkable stimulation of protein labeling in the more mature testicular cells undergoing spermiogenesis may offer a partial explanation for the atrophic changes of the maturing spermatids resulting from experimentally induced hypoglycemia. Any decrease from a normal intracellular glucose content in the testis which may result from a lowered blood glucose level may be reflected in a decrease of testicular protein biosynthesis with a resulting atrophy of the seminiferous germinal epithelium and an impairment of the spermatogenic cycle.
18. The effect of exogenous glucose on the incorporation of tritiated lysine into protein of the successive cells of the human seminiferous epithelium has been investigated utilizing radio-autographic techniques.
19. Following a 1 hour incubation period, slices of human testis in the presence or absence of glucose plus tritiated lysine illustrated a pattern of protein labeling over each of the successive cells of the cycle of the human seminiferous epithelium remarkably similar to that described for the rat.
20. The primary spermatocytes in the control system most actively incorporate lysine into protein.

21. The only difference in the pattern of protein labeling in the successive cells of the human seminiferous epithelium as compared to the rat testis appears to involve the degree of stimulation that glucose exerts over the pachytene primary spermatocytes.
22. The greater enhancement of protein labeling by glucose in the pachytene primary spermatocytes occurring in the rat as compared to the human appears to be due to the lower control values (specific activity) of these cells in the rat.
23. One possible explanation may be that in the absence of adequate levels of glucose, the earlier resting and leptotene primary spermatocytes have a higher capacity for protein synthesis than the later pachytene primary spermatocytes in younger animals as opposed to older animals (human).
24. Glucose exercises a marked stimulation of incorporation of tritiated lysine into protein of slices of human testis. The overall degree of stimulation is less than that occurring in the rat seminiferous epithelium; but this may be reflective of either a higher level of control protein synthesis or a particular insensitivity to glucose existing in an older seminiferous epithelium.
25. Differences in protein biosynthesis of those cells of the spermatogenic cycle responsible for cell proliferation and renewal as compared to the more mature spermatids may have importance in investigations dealing with regulation of male infertility.
26. Investigations dealing with potential side effects of drugs that may cause genetic damage with resulting congenital malformations may also reflect changes in protein synthesis.
27. The presently described radioautographic patterns of protein labeling over each of the successive cells of the seminiferous germinal epithelium may

provide an extremely practical initial screening procedure for the detection of any such possible side effects of drugs on spermatogenesis.

28. Drugs which are commonly administered to the male prior to coitus may alter the radioautographic pattern of protein labeling over the successive cells of the spermatogenic cycle in a manner more sensitive than that of histological examination.
29. Any drugs that are found to initially influence the radioautographic pattern of protein labeling of the successive cells of the seminiferous epithelium could then be studied further by employing more laborious techniques. Specific experiments investigating changes in mating patterns with subsequent examination of the offspring, would then be indicated.

CHAPTER VII

PLATES

PLATE I

Figures 1.- 6. Representative 5 μ sections of cryptorchid rat testis 2 days after abdominal transplantation of adult testis (H & E, X700). Fig. 1. Stage I: normal-appearing Sertoli cells, spermatogonia, primary spermatocytes, and spermatids within seminiferous epithelium. Fig. 2. Stage IV: arrow shows early migration of cap phase spermatids toward tubular lumen. Fig. 3. Normal Stage VII 2 days after abdominal transplantation. Fig. 4. Stage IX: arrow indicates exfoliation of pachytene primary spermatocytes into tubular lumen. Fig. 5. Normal Stage XIII 2 days after abdominal transplantation. Fig. 6. Stage XIV: pronounced epithelial disarrangement with central migration of primary spermatocytes toward tubular lumen, resulting in large empty areas within seminiferous epithelium. Arrows point to primary spermatocytes at metaphase with cytoplasmic hyalinization.

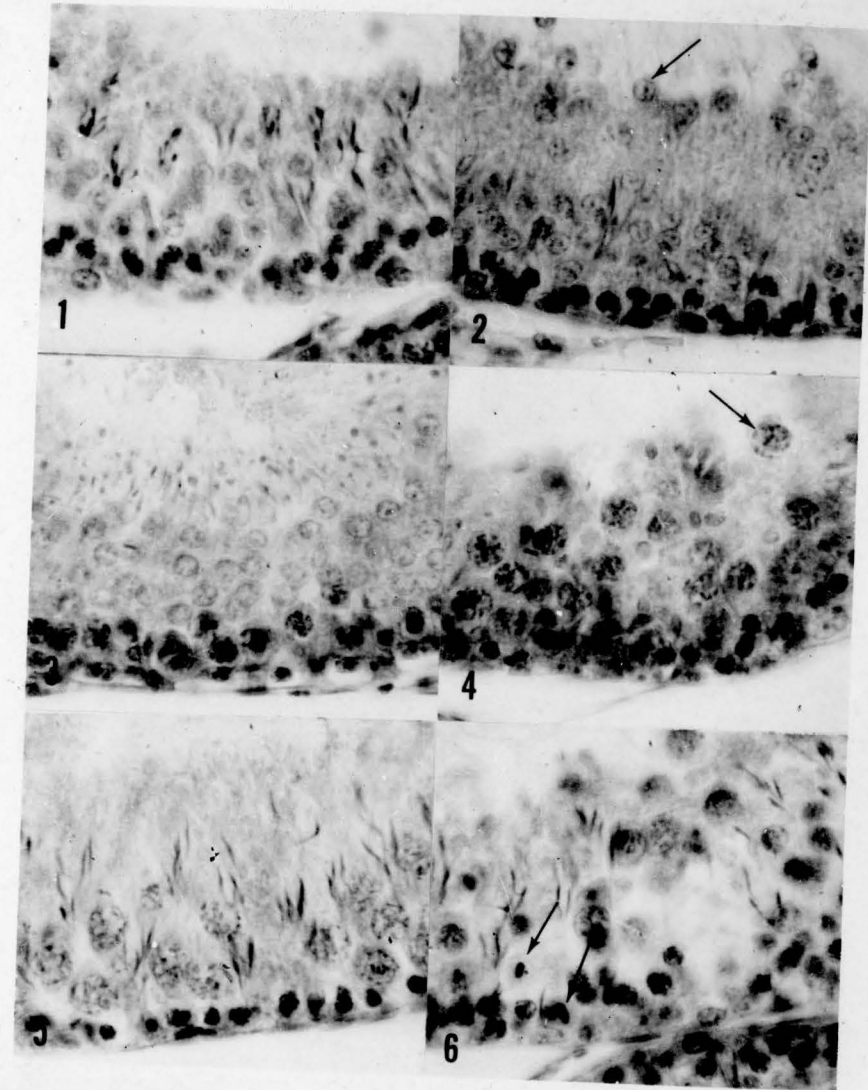


PLATE I

PLATE II

Figures 7 - 12. Representative 5 μ sections of cryptorchid rat testis 5 days after abdominal transplantation of adult testis. (H & E, X700). Fig. 7. Marked cellular sloughing of seminiferous epithelium, now only spermatogonia, primary spermatocytes, and Sertoli cells. Fig. 8. Loss of pachytene primary spermatocytes into seminiferous tubular lumen. Arrows point to primary spermatocytes with pyknotic nuclei and hyalinized cytoplasm. Fig. 9. Stage IV: arrows point to giant multinucleated masses formed from migrating cap phase spermatids. Fig. 10. Stage X: exfoliation of pachytene primary spermatocytes and spermatids. Fig. 11. Within seminiferous epithelium, many of the remaining spermatids and peripherally located primary spermatocytes appear pyknotic. Fig. 12. Several tubules in Stage VII still closely resemble corresponding stage of spermatogenesis found in normal scrotal testis.

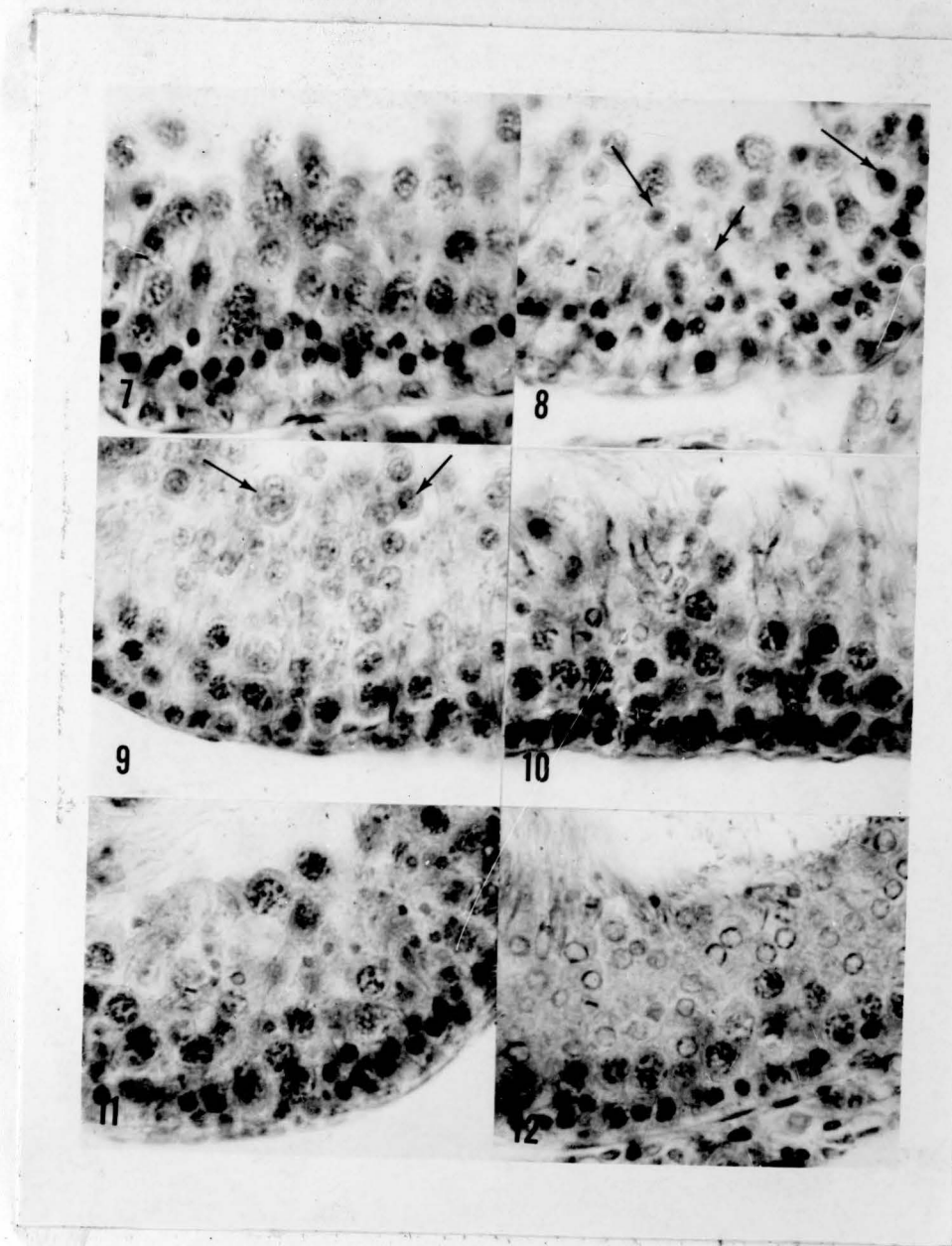


PLATE II

PLATE III

Figures 13 - 18. Representative 5 μ sections of cryptorchid rat testis 7 - 30 days after abdominal transplantation of adult testis. Fig. 13 and 14. Stages III-IV: loss of cap phase spermatids toward lumen of seminiferous tubule, where they form giant multinucleated cell masses indicated by arrows. (H & E, X700). Fig. 15 - 17. Appearance of seminiferous tubules after 10, 15 and 30 days, respectively, of exposure to intra-abdominal temperature. Note persistence of Sertoli cell retinaculum with peripherally located Type B spermatogonia. (H & E, X700). Fig. 18. Sertoli cell retinaculum after 15 days. Arrow points to Sertoli cell with nuclear pleomorphism. (H & E, X1400).

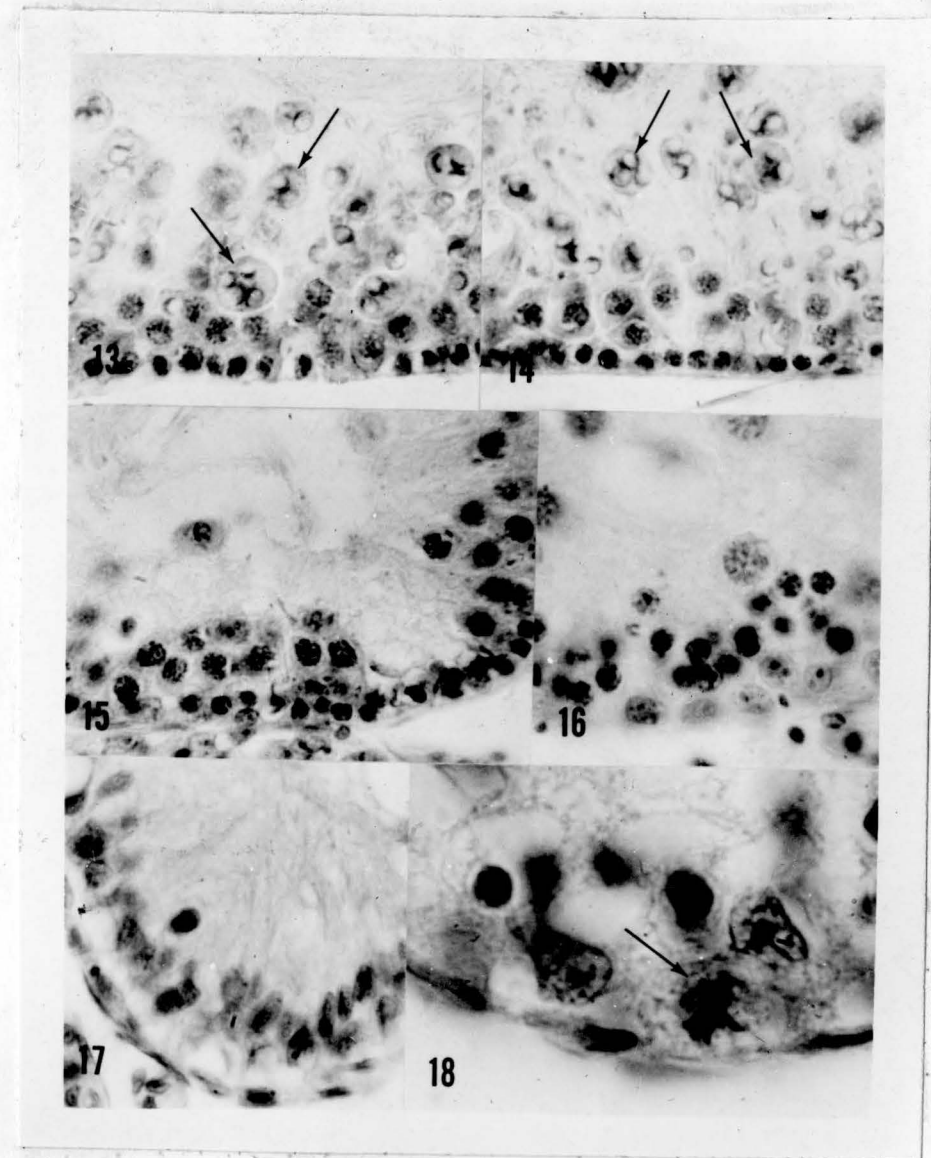


PLATE III

PLATE IV

Figures 19 - 24. Representative 5 μ sections of cryptorchid rat testis after abdominal fixation of prepubertal, undescended testis, and corresponding normal testis of same animal (H & E, X150). Fig. 19. Normal appearance of 20 day-old prepubertal testis in abdominal cavity. Fig. 20. Effect of 5 days of intra-abdominal temperature on development of retained prepubertal testis: sloughing of pachytene primary spermatocytes into tubular lumen. Fig. 21. Inguinal testis of 25 day-old rat prior to its normal descent, showing early tubular formation with resting and pachytene primary spermatocytes loosely surrounding lumen of the seminiferous tubules. Fig. 22. Arrow indicates onset of spermiogenesis in normal rat testis at 35 days of age, after descent into the scrotal sac. Fig. 23. Effects of 15 days of exposure to intra-abdominal temperature on seminiferous epithelium of retained prepubertal testis: tubules consist predominantly of peripherally located pachytene primary spermatocytes and Type B spermatogonia found within the Sertoli retinaculum. Fig. 24. Appearance of maturation phase spermatids in 50 day-old normal scrotal testis.

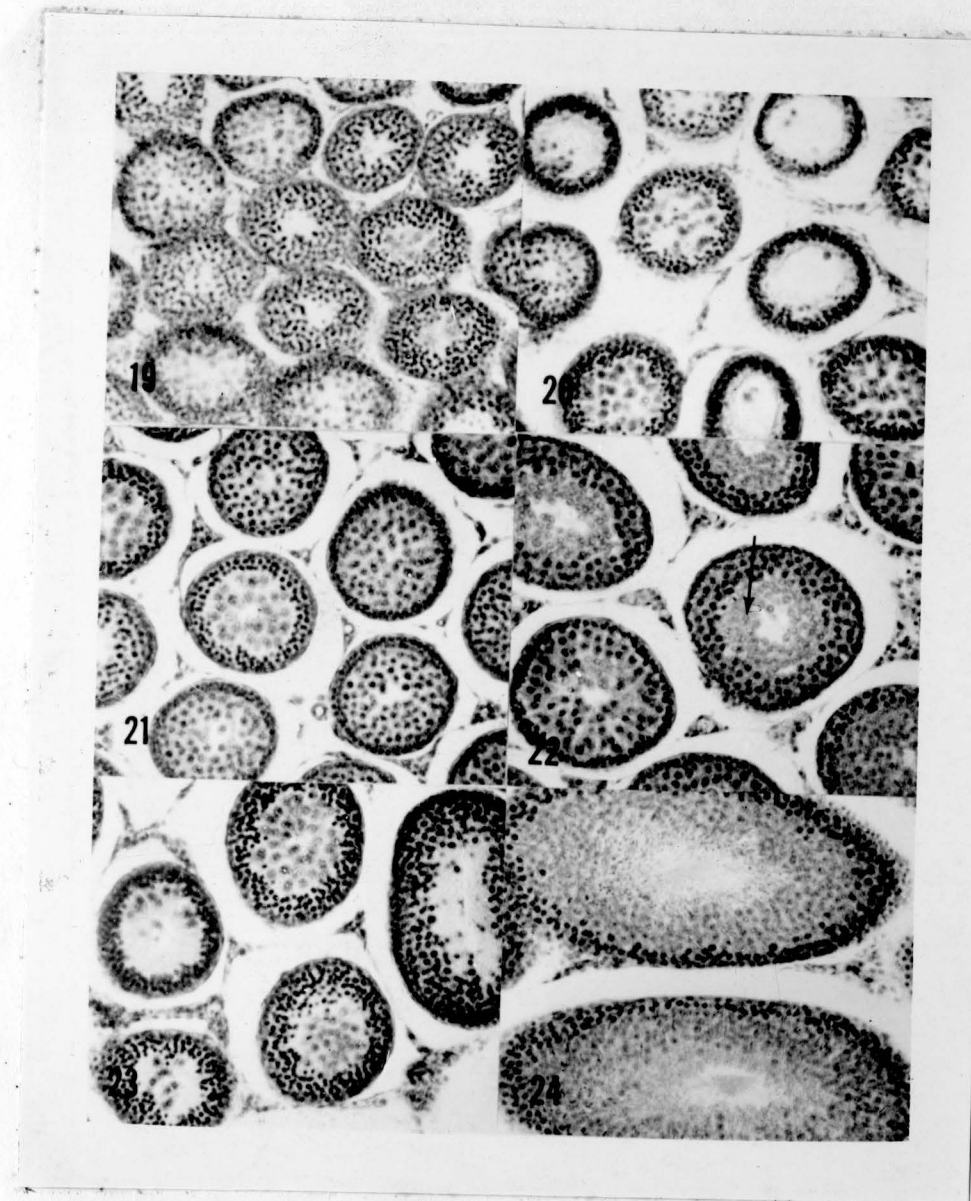


PLATE IV

PLATE V

Fig. 25 - 30. Representative 5 μ sections of cryptorchid rat testis 30 days after abdominal fixation of undescended prepubertal testis. Fig. 25 - 27. Effects of intra-abdominal temperature on seminiferous epithelium of cryptorchid rat testis 30 days after abdominal fixation. Seminiferous tubules are composed essentially of Sertoli cell retinaculum with peripherally located Type B spermatogonia and occasional pachytene primary spermatocytes. (H & E, X150). Fig. 28 - 30. Higher magnifications of seminiferous tubules of the prepubertal testis after 30 day exposure to intra-abdominal temperature. (X700) The predominant component of seminiferous germinal epithelium at this time are Sertoli cells and Type B spermatogonia (arrows), with varying numbers of degenerating pachytene primary spermatocytes in lumen of seminiferous tubule.



PLATE V

PLATE VI

Representative photomicrographs of the three types of seminiferous tubules found in the cryptorchid rat testis 30 days after abdominal transplantation of an adult testis. The tissues were sectioned at 5μ and stained with hematoxylin and eosin (magnification X700). A. Type A tubule containing 'crust' spermatogonia, pachytene primary spermatocytes and normal appearing Sertoli cells. B. Type B tubule containing 'crust' spermatogonia and normal appearing Sertoli cells. C. Type C tubule containing essentially only atrophic appearing Sertoli cells with pleomorphic nuclei and fibrotic, hyalinized cytoplasm.

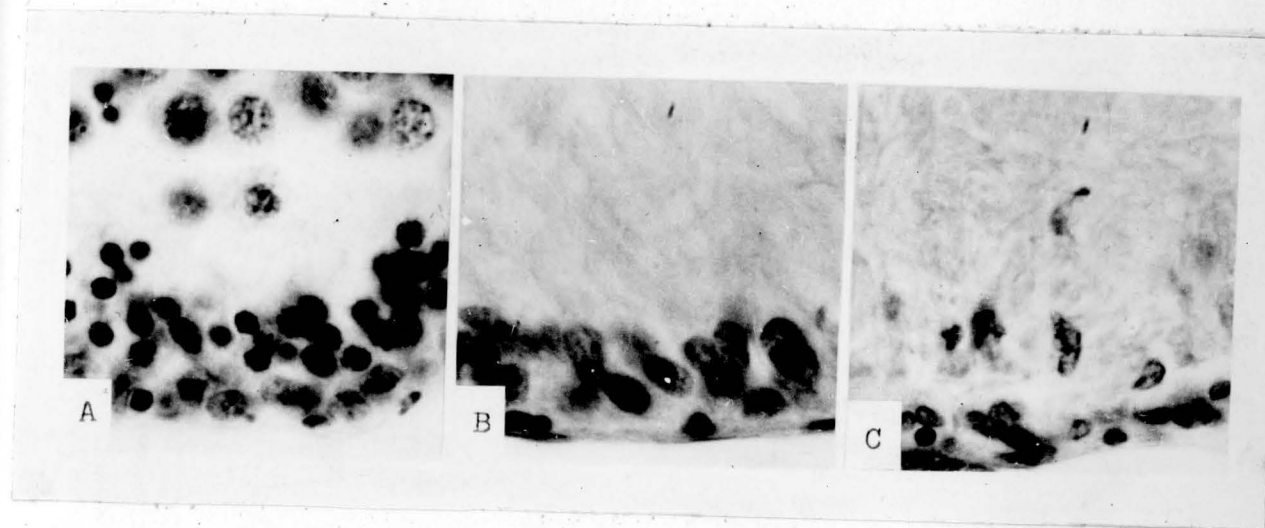


PLATE VI

PLATE VII

Representative radio-autograms following incubation with tritiated lysine of the three types of seminiferous tubules found in the cryptorchid rat testis 30 days after abdominal transplantation of an adult testis (magnification X700).

A. Type A tubule demonstrating a dense labeling pattern over 'crust' spermatogonia and the Sertoli cell cytoplasm. B. Type B tubule demonstrating a labeling pattern over the Sertoli cell cytoplasm which is similar to that seen in Type A tubules. C. Type C tubule demonstrating a marked decrease in labeling over the cytoplasm of atrophic Sertoli cells.

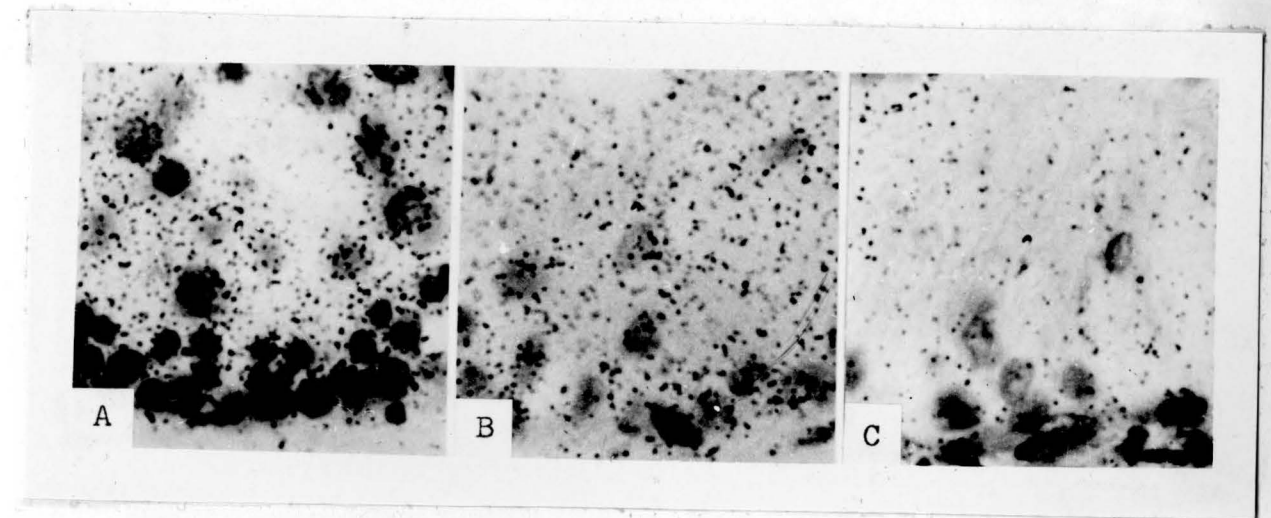


PLATE VII

PLATE VIII

Histological examination of the seminiferous epithelium following a 1 hour incubation period of slices of rat testis at 37.5°C. Figures 1 - 14 illustrate the 14 stages of the cycle of the seminiferous epithelium in the rat according to the description of Leblond and Clermont (1962). Fixation in Carnoy's solution; 5 μ sections stained with hematoxylin. Magnifications approximately X880.

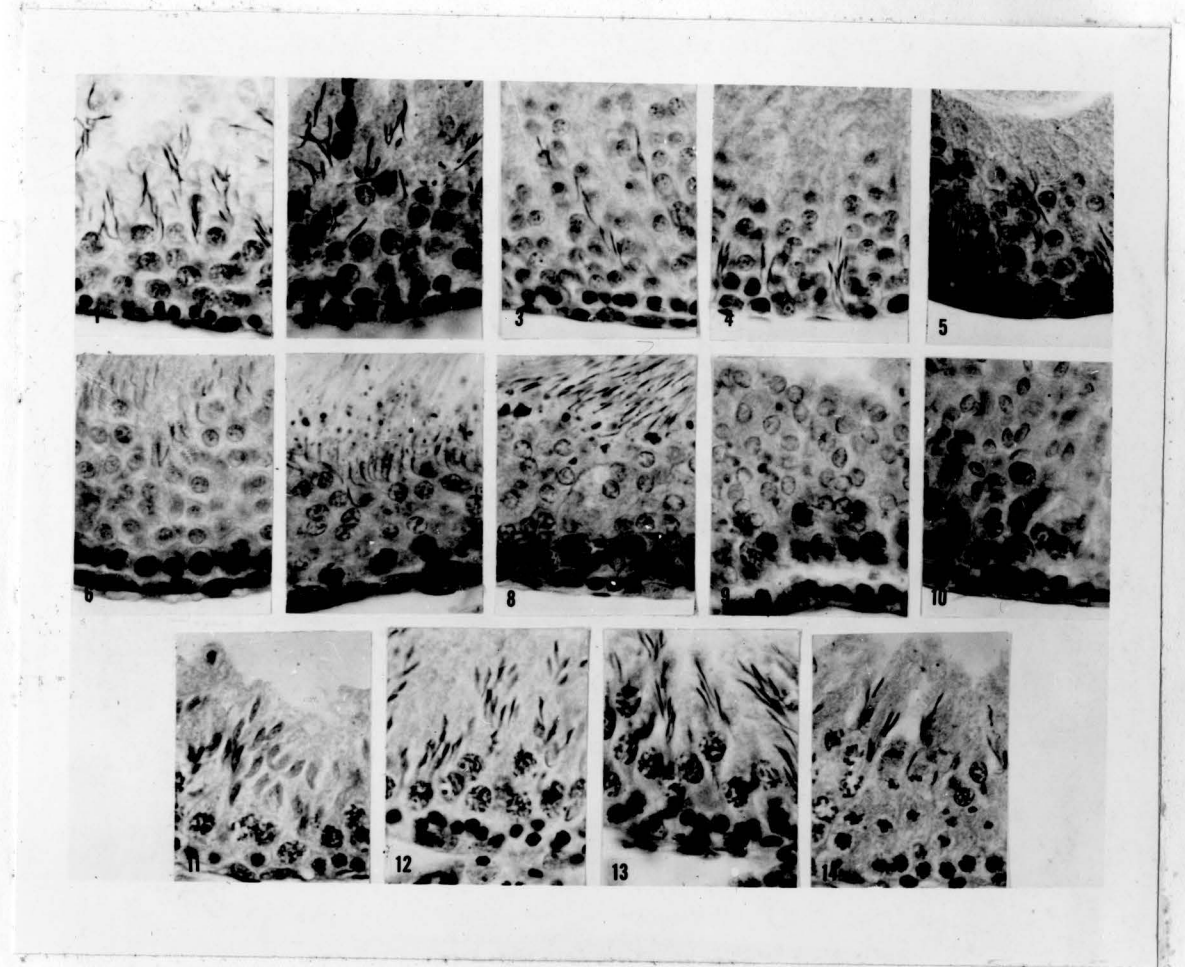


PLATE VIII

PLATE IX

Radioautographs of sections taken from slices of rat testes incubated for 1 hour with L-Lysine- H^3 in the presence of 0.009M glucose. Figures 2, 4 and 6 represent stages VII, VIII and XIII respectively, while Figures 1, 3 and 5 represent the corresponding alternate serial section stained without being dipped in nuclear emulsion. All sections were cut at 5μ and stained with hematoxylin. Exposure of the radioautograph was for 4 days. Magnification approximately X700.

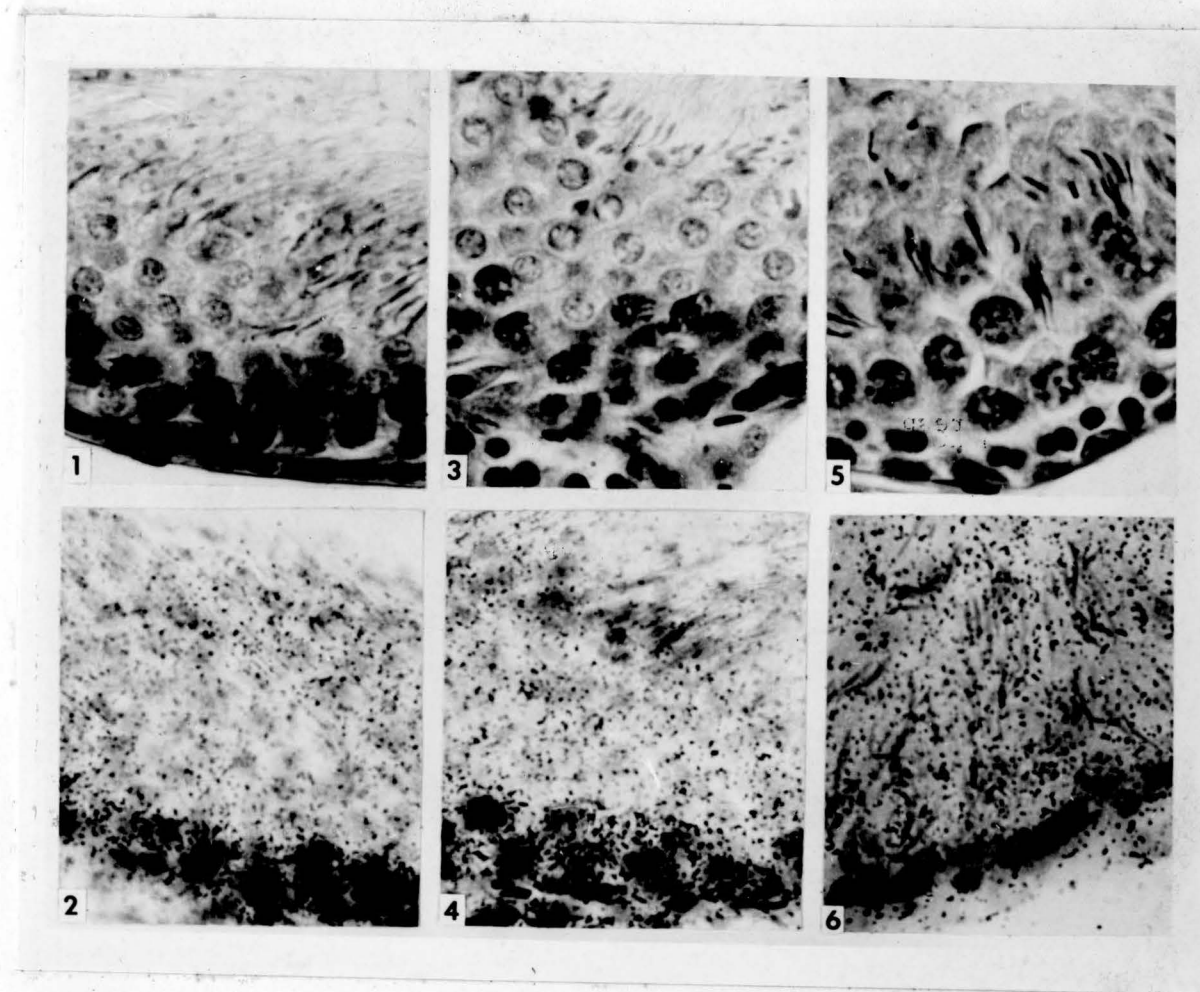


PLATE IX

PLATE X

Radioautographs of sections taken from slices of rat testes incubated for 1 hour with L-lysine- H^3 in the presence and absence of 0.009M glucose. Upper row of radioautographs represents labeling in the absence of glucose while the lower row represents labeling in the presence of glucose. Figures 1 and 2 represents stage I; 3 and 4, Stage V; 5 and 6, stage VII; 7 and 8, stage IX; 9 and 10, stage XIII. All sections were cut at 5μ and stained with hematoxylin. Exposure of the radioautograph was for 4 days. Magnification approximately X745.

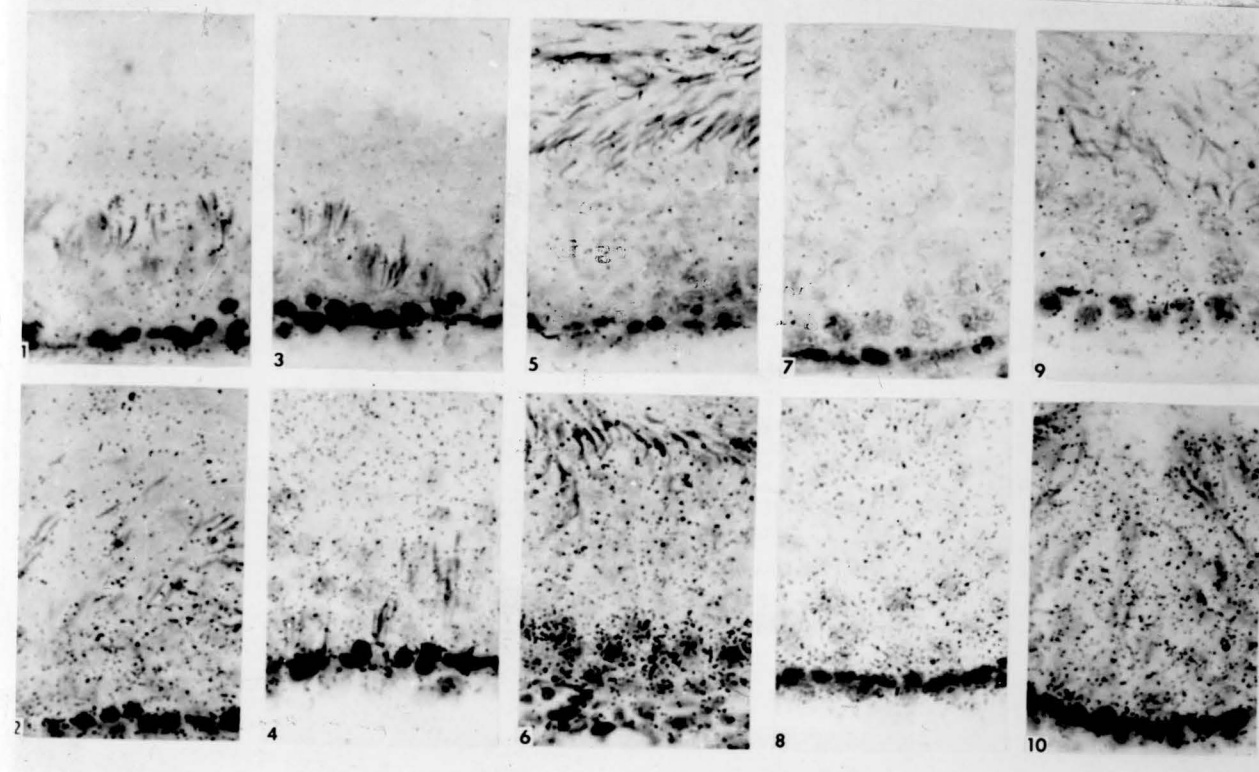


PLATE X

PLATE XI

Human orchiectomy procedure as performed for the treatment of adenocarcinoma of the prostate. Figure A identifies the longitudinal, anterolateral incision with the spermatic cord stretched outward and upward by umbilical cord tape passed around the cord. Figure B, ligation and transection of the gubernaculum. Note continuity of the testicular vascular supply. Figure C illustrates the ligated and transected spermatic cord and the removal of the testis. Figure D, placement of surgical specimen into pre-oxygenated Krebs-Ringer bicarbonate buffer within a 250cc glass jar packed in ice within an insulated container.

ORCHIECTOMY

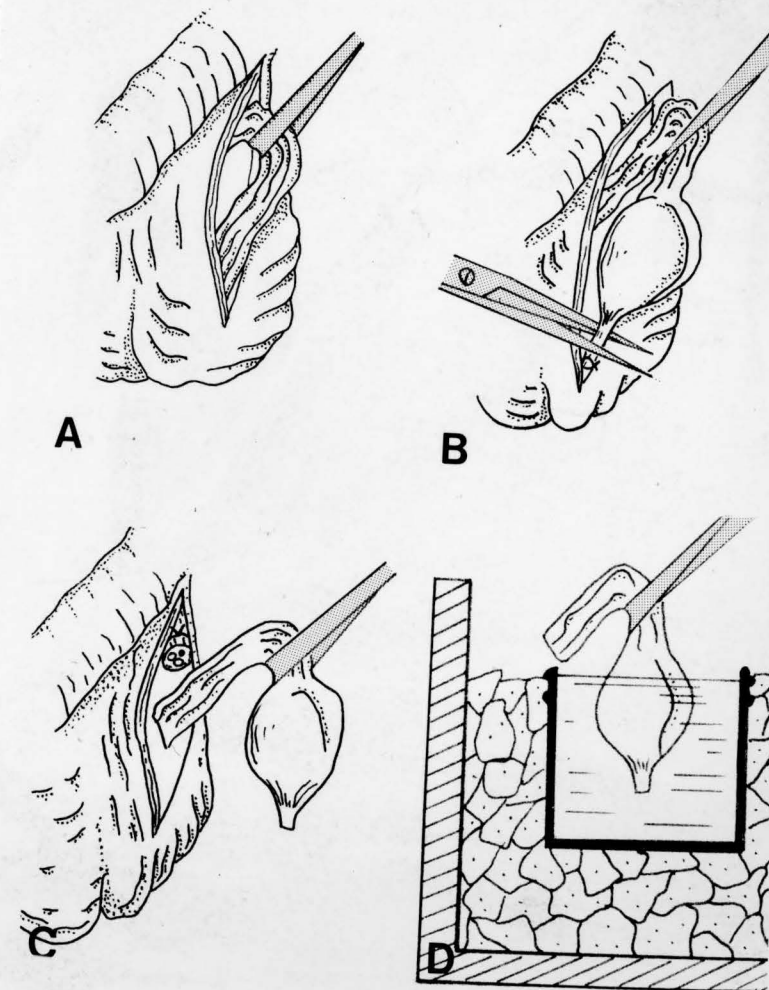


PLATE XII

Isolation of experimental testicular sample from surgical specimen. Figure A illustrates surgical specimen in toto. Figure B, mid-sagittal transection of the testicular parenchymal mass. Note head and tail of epididymis being reflected laterally and preservation of entire epididymis. Figure C, 50% of parenchymal mass and entire epididymis representing the pathologist specimen. Figure D, 50% of parenchymal mass, experimental testicular sample. Figure E, identifies experimental specimen in pre-oxygenated Krebs-Ringer bicarbonate buffer packed in ice within an insulated container.

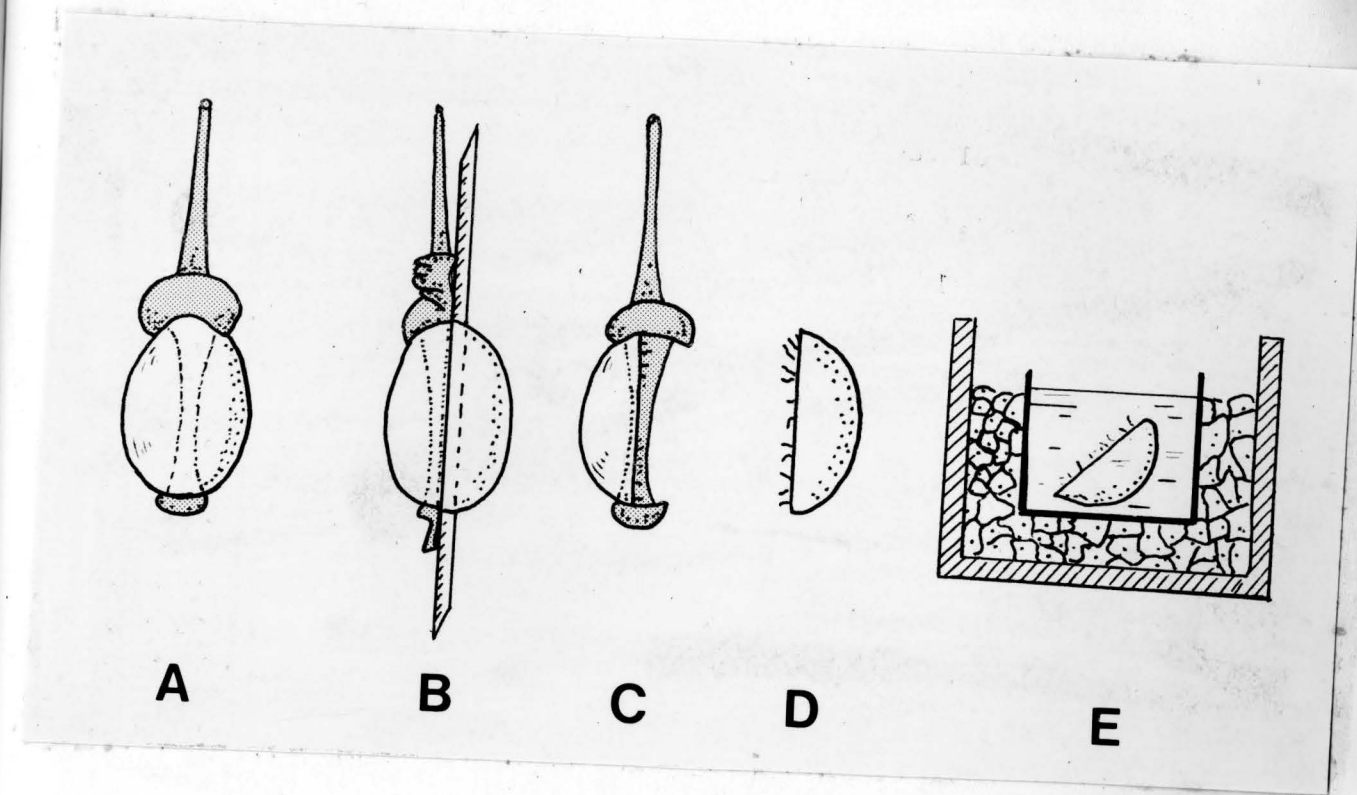
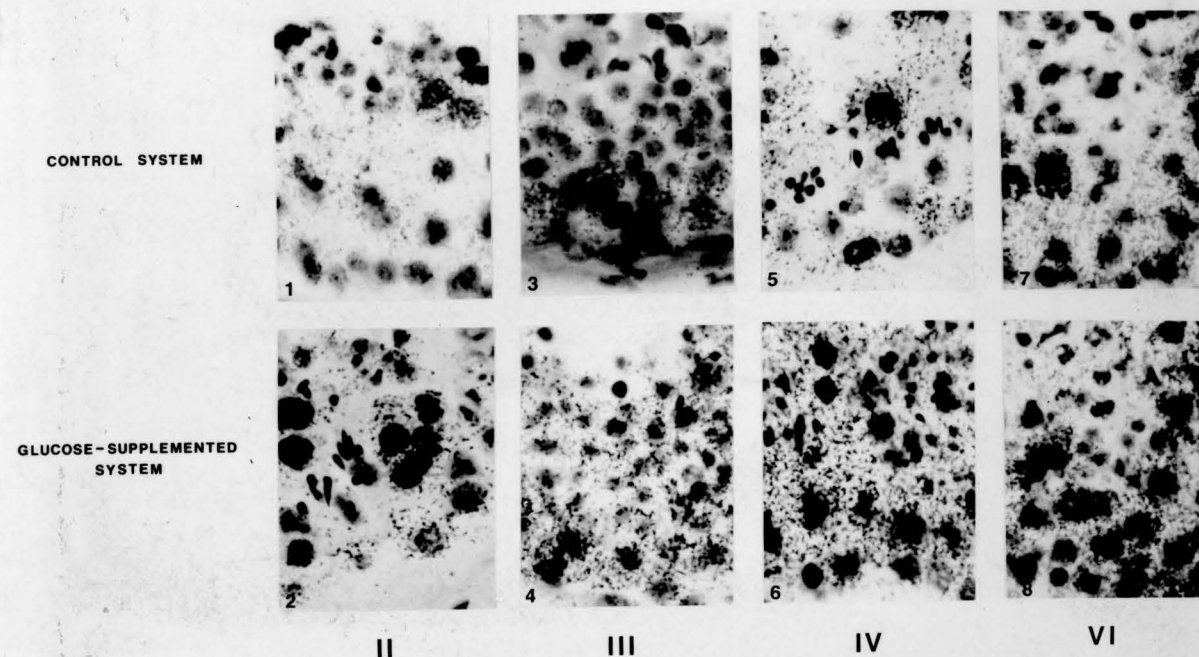


PLATE XII

PLATE XIII

Radioautographs of sections taken from slices of human testes incubated for 1 hour with tritiated lysine in the presence and absence of 0.009M glucose. Figures 1 and 2 represent stage II; 3 and 4, stage III; 5 and 6, stage IV; 7 and 8, stage VI. Classification of stages of the cycle of the human seminiferous epithelium were based on the description of Clermont (1968). Magnification approximately X1,000.

EFFECT OF GLUCOSE ON THE INCORPORATION OF L-LYSINE- H^3 INTO
PROTEIN OF CELLS OF THE SEMINIFEROUS EPITHELIUM OF
HUMAN TESTIS SLICES

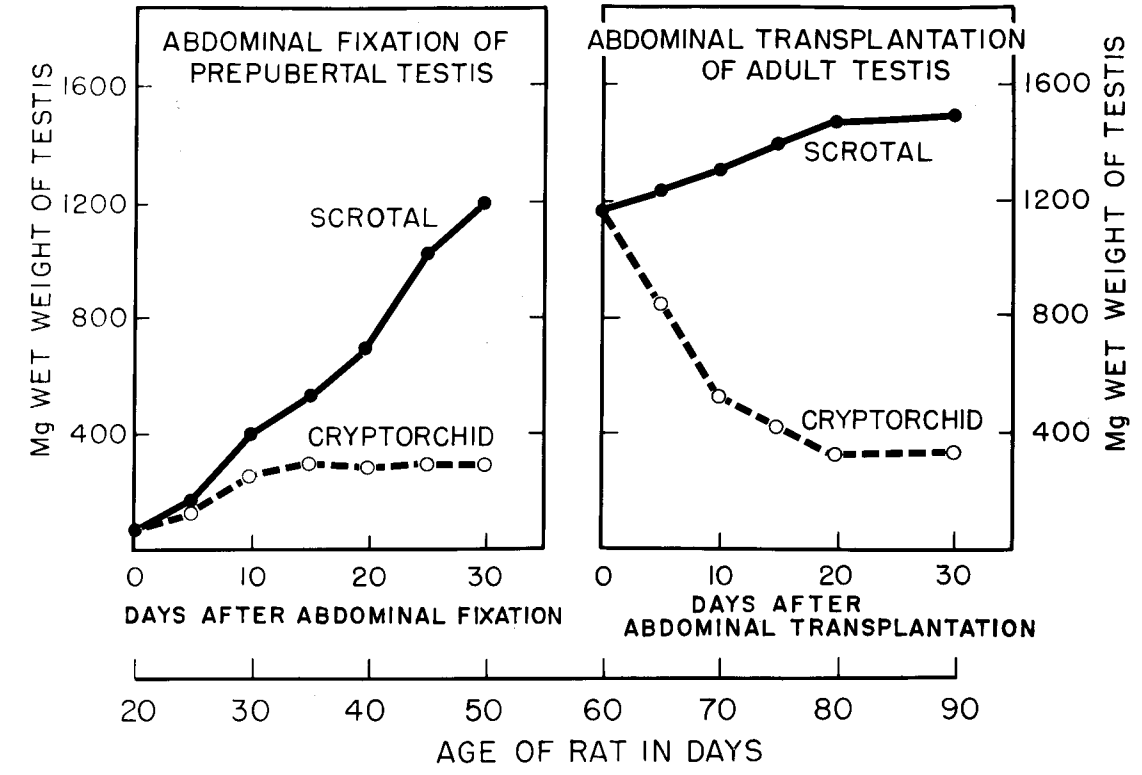


CHAPTER VIII

GRAPHS

GRAPH I

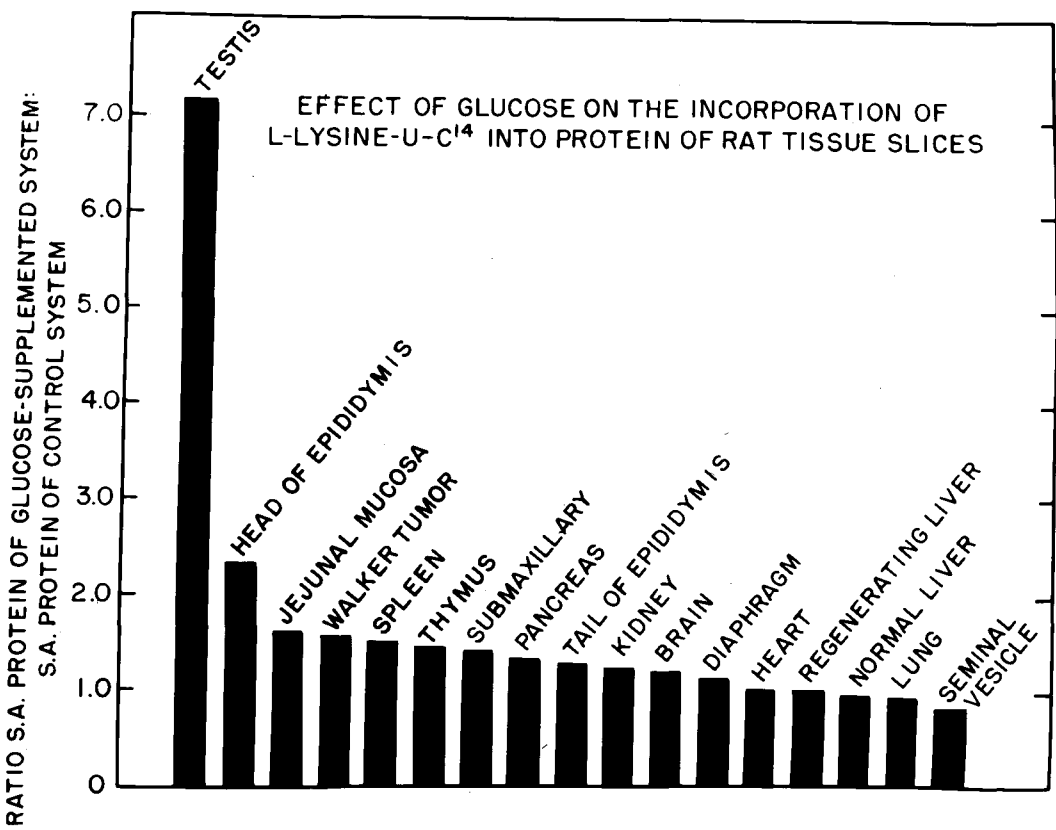
Graph I demonstrates the changes in testicular weights following abdominal fixation and abdominal transplantation of testes in prepubertal and adult rats respectively. Each point on the graph represents an average of 5 animals.



GRAPH I

GRAPH II

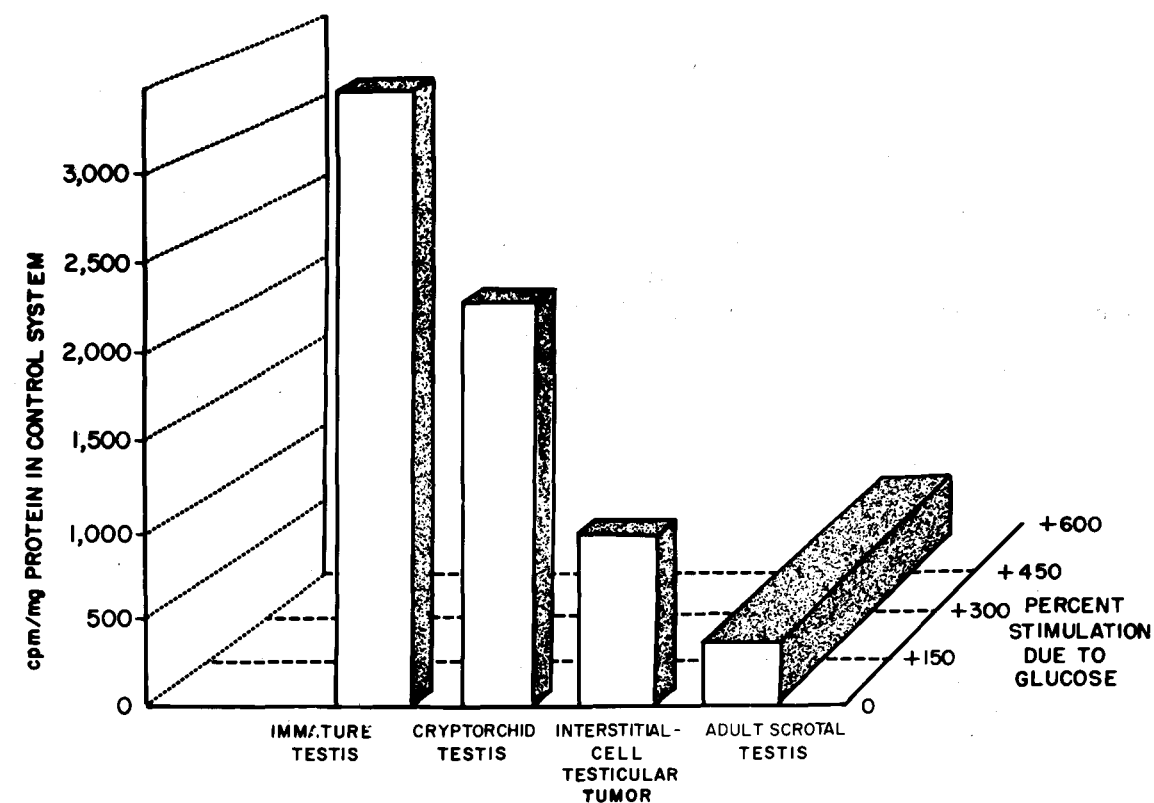
Effect of glucose on the incorporation of L-Lysine-U-C¹⁴ into protein of rat tissue slices: Slices of various rat tissues were incubated for one hour in the presence of 250,000cpm of L-Lysine-U-C¹⁴. Control flasks contained no glucose while experimental flask contained 0.009M concentration of this substrate. Incubation temperature was 37.5°C. The ordinate represents the per cent difference between control and experimental in each tissue (Davis, 1969).



GRAPH II

GRAPH III

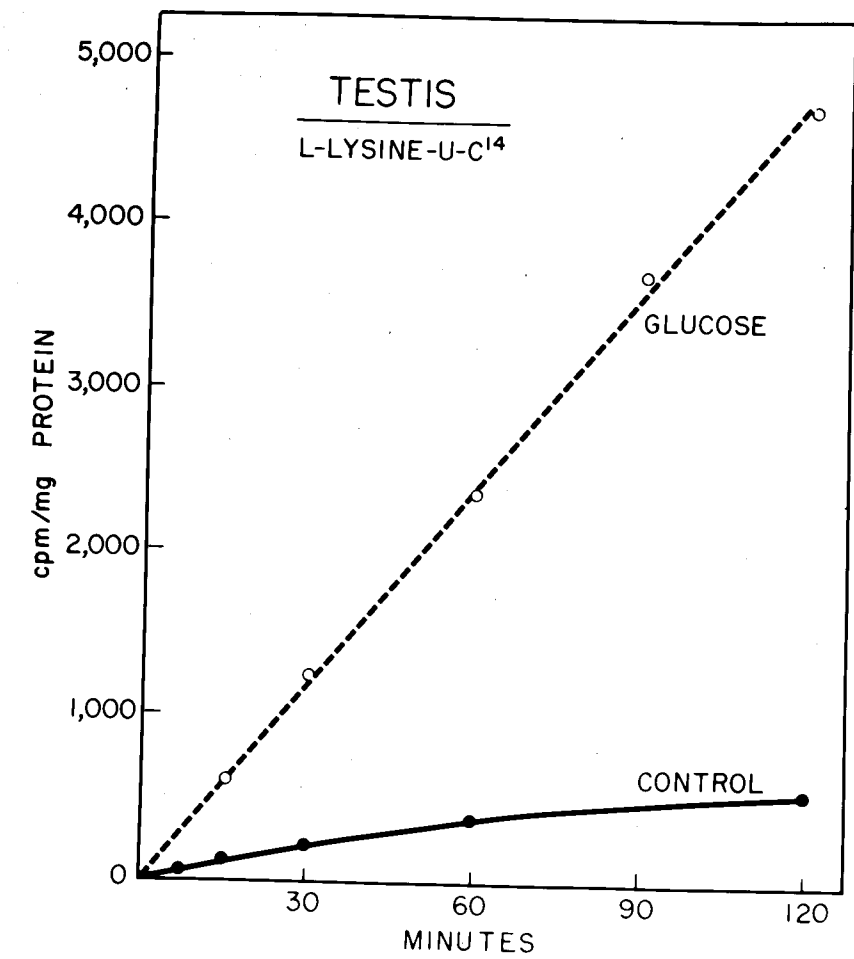
Effect of glucose in the incorporation of L-Lysine-U- C^{14} into protein of testis slices. Alternate flasks containing 0.009M glucose were incubated for 1 hour at 37.5°C. The height of each bar represents the labeling of protein in the absence of glucose while the depth of each bar represents the percentage stimulation of protein labeling by glucose (Davis, 1969).



GRAPH III

GRAPH IV

This graph demonstrates the rate of aerobic incorporation of radioactive lysine into protein of slices of adult rat testis in the presence and absence of 0.009M glucose. The incubation temperature was 37.5°C (Davis, 1969).

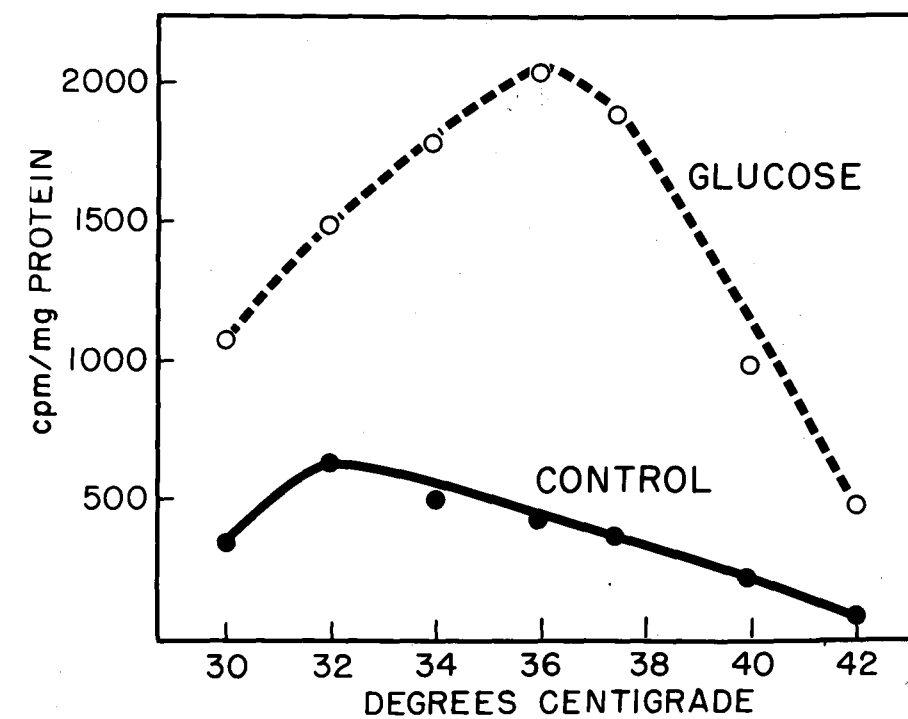


GRAPH IV

GRAPH V

This graph illustrates the effect of glucose on the aerobic incorporation of L-Lysine-U-C¹⁴ into protein of adult rat testis slices incubated for 1 hour at various temperatures (Davis, 1969).

AEROBIC INCORPORATION OF L-LYSINE-U-C¹⁴
INTO PROTEIN OF ADULT RAT TESTIS SLICES

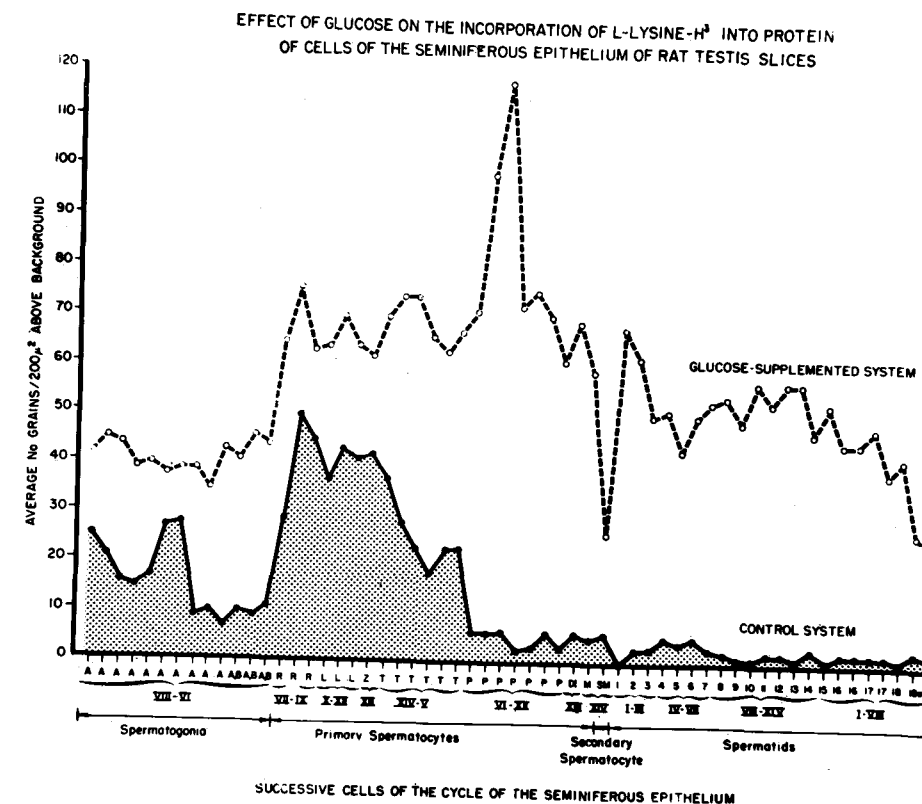


GRAPH V

GRAPH VI

Incorporation of L-Lysine- H^3 into protein of cells of the seminiferous epithelium of rat testis. Flask contents: L-Lysine- H^3 ($3.4 \times 10^{-4}M$), $100\mu c$ in 0.2 ml; 0.009M glucose in alternate flasks; Krebs-Ringer bicarbonate buffer, pH 7.4, to a total volume of 3.2 ml. The gas phase was 95% O_2 and 5% CO_2 . Flasks were incubated for 1 hour at $37.5^\circ C$. Each point on the curve represents the average of 4 individual experiments, comprising 20 microscopic fields from each animal or a total of 80 determinations for each of the designated cells of the cycle of the seminiferous epithelium. Representative standard errors for a total of 80 such determinations for cells in the control system approached up to ± 3 grains/ $200\mu^2$ above background; that for cells in the glucose-supplemented system up to ± 7 grains/ $200\mu^2$ above background.

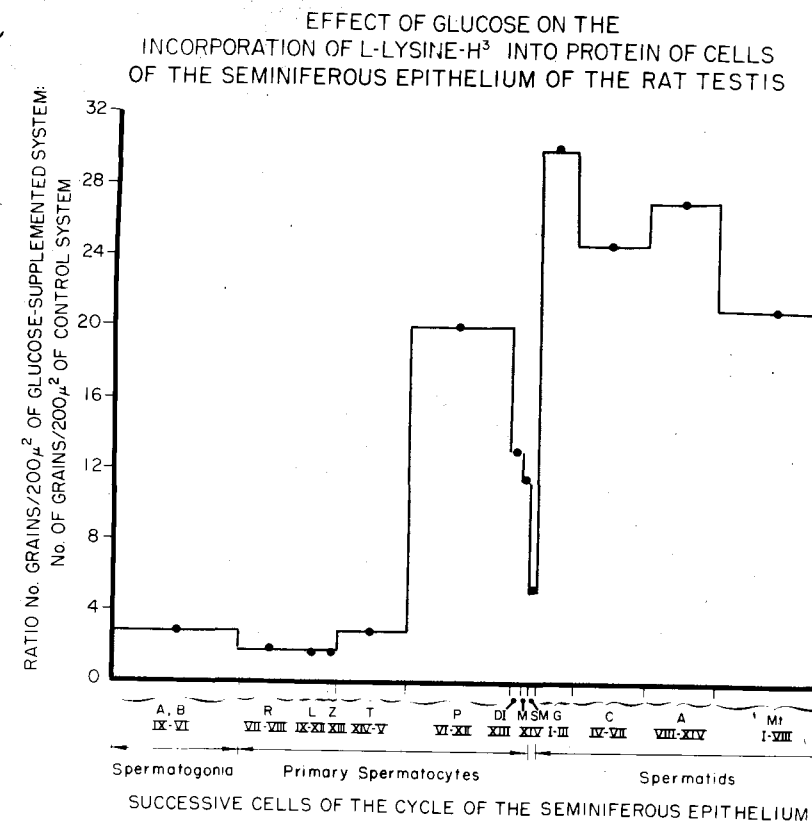
I-XIV refer to stages of the cycle of the seminiferous epithelium of the rat according to the description of Leblond and Clermont (1962). Numbers 1 - 19 refer to spermatids at various stages of spermiogenesis. The following letters refer to: A, type A spermatogonia; B, type B spermatogonia; R, resting primary spermatocytes; L, leptotene primary spermatocytes; Z, zygotene primary spermatocytes; T, transition primary spermatocytes; P, pachytene primary spermatocytes; DI, diplotene primary spermatocytes; M, primary spermatocytes in metaphase; SM, secondary spermatocytes in metaphase.



GRAPH VI

GRAPH VII

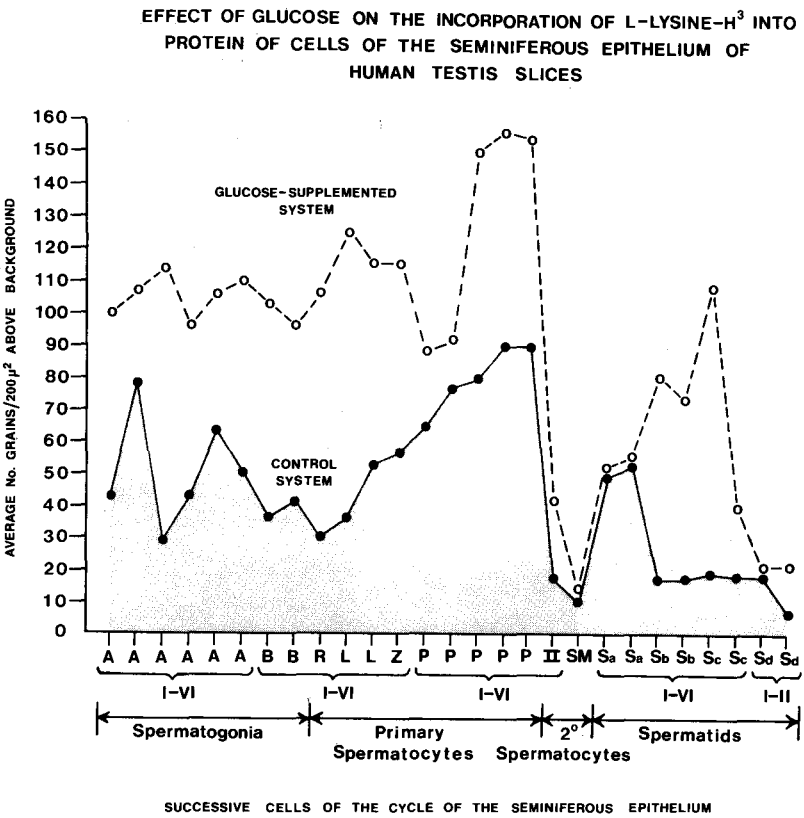
Effect of glucose on incorporation of L-Lysine- H^3 into protein of successive cells of the cycle of the seminiferous epithelium of the rat testis. For experimental details see Graph VI. Each point on graph represents average of designated cells of the cycle of the seminiferous epithelium as obtained in Table IV for groups of four rats. The space allotted to each of the successive cells of the spermatogenic cycle shown on the abscissa is proportional to its relative duration (Leblond and Clermont, 1962). The following letters refer to; G, spermatids in the Golgi phase; C, Spermatids in the cap phase; A, spermatids in the acrosome phase; Mt, spermatids in the maturation phase.



GRAPH VII

GRAPH VIII

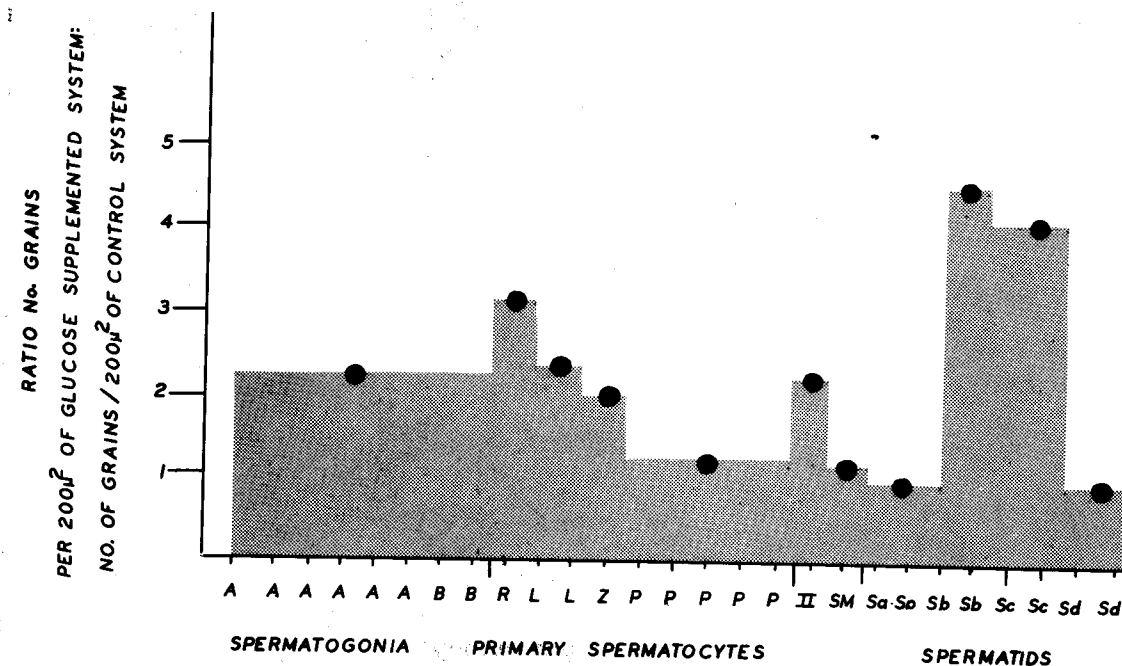
Effect of glucose on the incorporation of tritiated lysine into protein of the successive cells of the cycle of the seminiferous epithelium of the human testis. Each point on the curves represents the average from 2 individual experiments, comprising 10 microscopic fields from each human testis or a total of 40 determinations for each of the designated cells of the cycle of the seminiferous epithelium. I - VI refer to stages of the cycle of the seminiferous epithelium of the human according to the description of Clermont (1968). Sa-Sd refer to spermatids at various steps of spermiogenesis, with Sa being the immature form and Sd being the most mature form.



GRAPH VIII

GRAPH IX

The effect of glucose on the incorporation of L-Lysine- H^3 into protein of the successive cells of the cycle of the human seminiferous epithelium. Each point on the graph represents an average of designated cells of the cycle of the seminiferous epithelium as obtained in Table V for the groups of two human testes. The space allotted to each of the successive cells of the spermatogenic cycle shown on the abscissa is proportional to its relative duration. The following letters refer to: A and B spermatogonia, R, L, Z, P, primary spermatocytes, II secondary spermatocyte, SM secondary spermatocyte in metaphase, Sa, Sb, Sc, and Sd spermatids.



SUCCESSIVE CELLS OF THE CYCLE
OF THE SEMINIFEROUS EPITHELIUM
(HUMAN)

GRAPH IX

CHAPTER IX

TABLES

TABLE I

Percentage distribution of seminiferous tubules in the cryptorchid rat testis 30 days after abdominal transplantation.

A total of 130, 195, 206 and 164 seminiferous tubules were observed in each of the four animals used.

All figures are percentages of total seminiferous tubules \pm standard error.

COMPARISON OF ABNORMAL SEMINIFEROUS TUBULES FOUND IN THE CRYPTORCHID RAT TESTIS 30 DAYS AFTER ABDOMINAL TRANSPLANTATION

All figures are percentages of total seminiferous tubules found in the cryptorchid testis

Type A tubules.....	30.9 \pm 2.1
Type B tubules.....	30.1 \pm 0.74
Type C tubules.....	39.0 \pm 1.6

TABLE I

TABLE II

Percentage distribution of cell population in seminiferous tubules of the cryptorchid rat testis 30 days after abdominal transplantation. The number of cells were determined in approximately twenty-five seminiferous tubules of each type for each of the four animals used. All figures are percentages of total tubule cells \pm standard error.

COMPARISON OF ABNORMAL SEMINIFEROUS TUBULES FOUND IN THE CRYPTORCHID RAT TESTIS 30 DAYS AFTER ABDOMINAL TRANSPLANTATION

All figures are percentages of total tubule cells

	Type A tubule	Type B tubule	Type C tubule
Type B spermatogonia	55.0 \pm 0.81	43.6 \pm 2.67	3.6 \pm 0.31
Pachytene 1° spermatocytes	8.6 \pm 1.09	(0)	(0)
Normal Sertoli cells	37.1 \pm 1.40	57.4 \pm 3.02	(0)
Atrophic Sertoli cells	(0)	(0)	96.4 \pm 0.30

TABLE II

TABLE III

Radio-autographic incorporation of L-Lysine-H³ into cells of the germinal epithelium of the cryptorchid rat testis 30 days after abdominal transplantation. Grains/200 μ^2 were determined over each of the designated cell types in approximately 100 microscopic fields for each of the four animals used. The data are present as grains/200 μ^2 above background \pm standard error.

RADIOAUTOGRAPHIC INCORPORATION OF L-LYSINE-H³ INTO CELLS OF THE CRYPTORCHID RAT TESTIS 30 DAYS AFTER ABDOMINAL TRANSPLANTATION

The data are presented as grains/200 μ^2 above background

	Normal scrotal testis	Type A tubules	Type B tubules	Type C tubules
Type B spermatogonia	19.3 \pm 0.69	67.1 \pm 1.03	66.2 \pm 1.8	-
Pachytene 1 $^\circ$ spermatocytes	7.03 \pm 0.72	27.5 \pm 0.70	-	-
Spermatids	2.4 \pm 0.41	-	-	-
Normal Sertoli cell	1.6 \pm 1.6	67.0 \pm 0.63	61.5 \pm 1.12	-
Atrophic Sertoli cell	-	-	-	19.1 \pm 2.4

TABLE III

TABLE IV

The individual values presented for each animal represents the average of the following successive cells of the cycle of the seminiferous epithelium: type A and B spermatogonia in stages IX-VI; resting primary spermatocytes in stages VII-VIII; leptotene primary spermatocytes in stages IX-XII; zygotene primary spermatocytes in stage XIII; transition primary spermatocytes in stages XIV-V; pachytene primary spermatocytes in stages VI-XII; diplotene primary spermatocytes in stage XIII; metaphase primary spermatocytes in stage XIV; metaphase secondary spermatocytes in stage XIV; Golgi phase spermatids in stages I-III; cap phase spermatids in stages IV-VII; acrosome phase spermatids in stages VIII-XIV; and maturation phase spermatids in stages I-VIII. Means are expressed in grains per $200\mu^2$ above background.

Animal No.	Spermatogonia		Resting 1° Spermatocytes		Leptotene 1° Spermatocytes		Zygotene 1° Spermatocytes		Transition 1° Spermatocytes	
	Control	Glucose	Control	Glucose	Control	Glucose	Control	Glucose	Control	Glucose
1	11	40	39	70	37	68	35	58	18	74
2	18	38	41	68	50	52	34	68	32	60
3	11	43	44	71	37	67	40	58	13	66
4	17	44	35	72	41	73	39	57	31	74
Mean	14	41	40	70	41	65	37	60	24	68
SD	2.8	2.0	3.0	1.2	4.4	6.4	2.0	3.8	6.8	4.8
t	11.6		13.2		13.7		7.7		23.7	
P	<0.001		<0.001		<0.001		<0.001		<0.001	
Animal No.	Pachytene 1° Spermatocytes		Diplotene 1° Spermatocytes		Metaphase 1° Spermatocytes		Metaphase 2° Spermatocytes		Golgi Phase Spermatids	
	Control	Glucose	Control	Glucose	Control	Glucose	Control	Glucose	Control	Glucose
1	4	84	8	65	1	56	2	25	2	57
2	3	74	7	67	4	54	3	21	4	66
3	3	77	7	66	5	58	6	22	0	56
4	7	89	7	77	8	60	13	33	3	61
Mean	4	81	7	69	4	57	6	25	2	60
SD	1.4	4.8	0.4	4.0	2.0	1.8	3.6	3.8	1.0	3.2
t	69.0		22.3		27.0		16.4		24.0	
P	<0.001		<0.001		<0.001		<0.001		<0.001	
Animal No.	Cap Phase Spermatids		Acrosome Phase Spermatids		Maturation Phase Spermatids					
	Control	Glucose	Control	Glucose	Control	Glucose				
1	0	38	1	49	1	36				
2	5	58	3	55	4	52				
3	2	46	2	53	2	39				
4	11	53	2	59	1	39				
Mean	4	49	2	54	2	41				
SD	3.2	6.2	0.6	3.4	1.0	5.0				
t	9.2		24.5		34.8					
P	<0.001		<0.001		<0.001					

TABLE IV

TABLE V

The individual values presented for each human testis represents the average of the following cells of the cycle of the seminiferous epithelium: type A and B spermatogonia in stages I - VI; resting primary spermatocytes in stage III; leptotene primary spermatocytes in stages IV-V; zygotene primary spermatocytes in stage VI, pachytene primary spermatocytes in stages I - V; secondary spermatocytes in stage VI; secondary spermatocyte at metaphase in stage VI; Sa spermatids in stages I-II; Sb spermatids in stages III-IV; Sc spermatids in stages V-VI; and Sd spermatids in stages I-II. The mean are expressed in grains per 200 μ^2 above background.

HUMAN SEMINIFEROUS EPITHELIUM

HUMAN TESTIS NO.	SPERMATOGONIA		RESTING 1° SPERMATOCYTES		LEPTOTENE 1° SPERMATOCYTES		ZYGOTENE 1° SPERMATOCYTES	
	CONTROL	GLUCOSE	CONTROL	GLUCOSE	CONTROL	GLUCOSE	CONTROL	GLUCOSE
	1	44	104	30	109	45	121	55
2	45	103	34	102	43	119	57	116
MEAN	44.5	103.5	32.0	105.5	44.0	120.0	56.0	115.0
S.D.	0.22	0.22	1.41	4.95	1.41	1.41	1.41	1.41
t	186.24		14.27		38.00		29.50	
P	<0.001		<0.01		<0.001		<0.01	
HUMAN TESTIS NO.	PACHYTENE 1° SPERMATOCYTES		2° SPERMATOCYTES		METAPHASE 2° SPERMATOCYTES		S a SPERMATIDS	
	CONTROL	GLUCOSE	CONTROL	GLUCOSE	CONTROL	GLUCOSE	CONTROL	GLUCOSE
	1	80	128	15	40	9	13	49
2	80	107	16	41	11	13	52	44
MEAN	80.0	117.5	15.5	40.5	10.0	13.0	50.5	46.5
S.D.	1.41	10.0	0.22	0.22	1.41	1.41	2.12	2.00
t	10.24		78.91		1.50		1.37	
P	<0.01		<0.001		<0.5		<0.5	
HUMAN TESTIS NO.	S b SPERMATIDS		S c SPERMATIDS		S d SPERMATIDS			
	CONTROL	GLUCOSE	CONTROL	GLUCOSE	CONTROL	GLUCOSE		
	1	22	78	18	77	26	21	
2	12	75	19	80	24	20		
MEAN	17.0	76.5	18.5	78.5	25.0	20.5		
S.D.	7.07	2.12	0.22	2.12	1.41	0.22		
t	8.06		28.14		3.10			
P	<0.05		<0.01		<0.1			

TABLE V

CHAPTER X
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APPROVAL SHEET

The dissertation submitted by Casimir F. Firlit, M.S., M.D. has been read and approved by five members of the faculty of Loyola University.

The final copies have been examined by the Dissertation Director and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

This dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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DATE

Joseph R. Davis, M.D., Ph.D.
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