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Effect of Glucose on the Radioautographic Uptake of L-Lysine-H-p3-s by Cells of the Seminiferous Epithelium

Casimir F. Firlit
Loyola University Chicago

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EFFECT OF GLUCOSE ON THE RADIOAUTOGRAPHIC
UPTAKE OF L-LYSINE- H^3 BY CELLS
OF THE SEMINIFEROUS EPITHELIUM



BY

Casimir F. Firlit, B.S.

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

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LIFE

Casimir F. Firlit was born in Chicago, Illinois on December 7, 1939. He attended St. Rita High School, Chicago, Illinois and graduated in June 1957. In September of 1957 he entered upon his premedical studies at Loyola University and completed his course of studies in June 1961 at which time he received the degree of Bachelor of Science, major in Biology. During the course of his premedical studies he was presented with the Loyola University award for Outstanding Undergraduate Research in 1960 and 1961; and in May, 1961, was elected an associate member to the Society of the Sigma Xi.

In September 1961, he began his studies in medicine at the Stritch School of Medicine of Loyola University; and in addition, began his graduate studies in the Department of Pharmacology. In the summer of 1962, 1963 and 1964 he was the recipient of the Lederle Summer Fellowship in research, and in September 1964 was awarded the Royal E. Cabell award for research.

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FORWORD

Although numerous histological and histochemical studies have been performed on the mammalian testis with regard to the precise rhythmic pattern or "wave" of spermatogenesis, the regulating mechanism or mechanisms responsible for this phenomenon have remained obscure. It has been postulated that if such a mechanism, in fact, does exist, it would most probably exercise its influence at the moment of release of the mature spermatozoa from the seminiferous epithelium.

Moreover, it has been demonstrated in experimentally-induced hypoglycemia in rats that the effect of prolonged hypoglycemia on the seminiferous epithelium results in loss of maturation of germinal cells. As a result of these studies the relationship of glucose to the maturation of testicular cells appears to be of importance. Therefore, it is the purpose of this investigation to present a possible mechanism responsible for the precisely regulated maturation pattern of the seminiferous epithelium; and, in addition, to further evaluate the effects of glucose on the cells of the seminiferous epithelium by attempting to demonstrate this dependency in terms of individual testicular cell types.

ABSTRACT

A histochemical study has been carried out on the detached cytoplasm of the maturing spermatid of the mouse. The detached cytoplasm has been designated as the cytoplasmic body (CB) and its morphological development divided into four distinct phases. Phase CB-1 begins with the formation of the cytoplasmic tag during stage 11 of spermiogenesis and was found to contain numerous small granules of RNA, glycogen, polysaccharides with 1:2-glycol-groups and lipid. During phase CB-2 and CB-3, coalescence and fusion of these granules occurs which results in a centrally-oriented plaque of RNA and glycogen, peripherally-oriented satellites of 1:2-glycol-containing polysaccharides, and large globules of lipid. During phase CB-4, the cytoplasmic body membrane becomes approximated to the plaque and migration begins toward the basement membrane of the seminiferous tubule where some of the residual bodies undergo phagocytosis by Sertoli cells. A loss of the RNA and lipid components of the residual body becomes evident without an accompanying loss of the glycogen content. The 1:2-glycol-containing polysaccharides within the satellites appear to spread over the surface of the glycogen plaque which ultimately becomes no longer demonstrable by the staining techniques employed in these studies.

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 the 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. The data obtained indicate that in the absence of added glucose the most heavily labeled cells of the seminiferous epithelium are the resting primary spermatocytes, with little or no label appearing in the remaining cells of the spermatogenic cycle. 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 pachytene primary spermatocytes and spermatids. It is suggested that the transition from somatic mitosis to the first meiotic prophase is associated with a marked increase in protein labeling while the incorporation of radioactive lysine into protein of the pachytene primary spermatocytes and spermatids is the most sensitive to the addition of exogenous glucose.

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CHAPTER I
INTRODUCTION

CHAPTER I

INTRODUCTION

A) THE MORPHOLOGY OF SPERMATOGENESIS

The mitotic phase of spermatogenesis occurs throughout the divisions of the spermatogonia. The significance of this phase is threefold. (1) Through multiplication of cells the foundation is laid for a large cell population to be discharged into the seminal fluid. (2) During the multiplication phase most of the spermatogonia progressively change in order to ultimately undergo meiosis. (3) 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 spermatis 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 (52).

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 (48) 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 crusty spermatogonia.

Allen (2) also describing the spermatogonia of the rat, divided 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 (50) came to the conclusion 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 (11) 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 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 afforded by Nicander(44) who found no glycogen in any of the spermatogonia of the mouse, very little glycogen in other rodents and in the dog, and a large amount of glycogen in spermatogonia of the bull.

Kramer (25) has described the spermatogonia of the bull in considerable detail. He recognized Type A cells which in contrast to those found in rodents and many other mammals contained granules which stained conspicuously with the periodic acid-Schiff (PAS) reagent.

Montagna (41) utilizing histochemical methods on biopsy specimens of adult human testis demonstrated glycogen as being predominantly localized in the germinal cells of the seminiferous epithelium. Here the spermatogonia, primary spermatocytes and Sertoli cells contained appreciable amounts of glycogen.

2. Spermatocytes:

Data on the development of the primary spermatocyte has been extremely sparse. Merkle (38) measured the nuclei of germ cells in the rat throughout their development and found that the nuclear volume of the primary spermatocyte increased fourfold from the stage immediately following the last spermatogonial mitosis to that immediately preceding meiotic metaphase.

Watson (63) 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 (14) investigated the RNA content of spermatocytes as

demonstrated by 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 (15) 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 pyronin Y at this stage of meiosis. In the later pachytene stages the nucleoli appeared to be spherical to ovoid and 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 but that during the subsequent period of vigorous growth of the spermatocyte almost completely disappeared from both the cytoplasm and nucleus.

Montagna and Hamilton (41) 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 (48). 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 aerosome 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 (33).

The loss of cytoplasm by the spermatid is a conspicuous phenomenon because the degenerating cytoplasm stains intensely with hematoxylin, fat stains, and osmium tetroxide. Regaud (48) originally described the development of these cytoplasmic remains, and named them "the residual bodies". Dacoust and Clermont (15) 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 epithelium.

Lacy (29) 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 first paper (55) 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 (20). von Ebner considered the cells interdispersed between the germ cells to be syncytial and derived from leukocytes. Von La Valette St. George (31) 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 any true union between these two testicular cell types (8).

Histochemical methods have confirmed the cyclic nature of the metabolism in the Sertoli cell. von Ebner (21) 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 spermiogenesis the cytoplasm of the spermatid begins to show increasing amounts of fat. von Ebner 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 (28) emphasized the heterogeneous 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 (40) used a variety of tests for lipids and demonstrated large lipid droplets in the basal part and dustlike fat in the central part of the Sertoli cells.

PAS-positive material was first described in the Sertoli cell of the rat, mouse, hamster and guinea-pig by Leblond and Clermont (33). 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 nutritive material from the Sertoli cells to the acrosomic system of the spermatids. Clermont (13) 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 was 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 (44) 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.

B) FACTORS INFLUENCING MITOTIC ACTIVITY

1. Nutrition:

It is now generally agreed that a cell which is about to enter into division must have completed the production of spindle protein and the duplication of the essential cellular components (?). The preliminary nutritional requirements of the cell must therefore have been very diverse. These requirements may perhaps vary slightly from tissue to tissue or from species to species and in the case of any particular cell type they can only be defined adequately by in vitro studies (59).

Although it is well known that certain abnormal diets may

strongly influence the mitotic rate, it is most improbable that well-fed and healthy animals ever suffer from any shortage of raw materials for the construction of new cells. In particular it is most improbable that the control of mitotic activity in any tissue can normally be exercised in terms of limitations in the supplies of basic raw materials, except in the sense of a specific inhibition, perhaps by hormones, of some particular metabolic pathway (?).

2. Respiration:

It is now evident that the ability of a cell to enter mitosis is closely dependant on the establishment of an energy store which is sufficiently great to satisfy the needs of the cells throughout the whole mitotic process (?). Mouse epidermis, as an example of an adult tissue, when kept in vitro in a saline medium develops more mitoses when such substances as glucose, fructose, pyruvate or lactate are added and when oxygen is introduced in the gaseous phase (4).

Epidermal mitotic activity ceases in anaerobic conditions, although the cells can survive without oxygen for approximately 1 week (36). Adult epidermis is evidently unable to develop normal mitotic activity under anaerobic conditions in spite of the claim that it is a tissue with a high rate of glycolysis and a low respiratory quotient (4). Rothman (47) has doubted the existence of the Krebs cycle in the epidermis, however, it is now known

that enzymes associated with the Krebs cycle are present in the mitotically active basal cell layers (43) and that the mitochondria are especially large and abundant in these layers (42).

Both for mammalian epidermis (4) and for cleaving eggs (59) the conclusion has been reached that the energy needed by a cell during division must be mobilized and stored before that division can begin and that this store is most probably in the form of some energy-rich and presumably phosphorylated molecule. Swann (59) also considers that this energy reservoir may prove to be a special one which is reserved exclusively for use during cellular division, and that the final energy transfer may be through ATP.

The demands of pre-mitotic cells for a suitable carbohydrate substrate and an adequate supply of oxygen can only be critically demonstrated in vitro. Within a normal mammal there is probably never any shortage of these raw materials. Even in starved mice the fall in the blood glucose level is not great, and the marked depression in epidermal mitotic activity that occurs is evidently due primarily to the action of adrenalin secreted in response to the stress of the situation (6).

3. Hormones:

It is therefore possible that mitotic activity in adult tissues may depend on the proper function of at least two major complexes of metabolic pathways. First, there is the metabolic complex which leads to the duplication of the various essential structures

of the call and the formation of the spindle protein, and second, the metabolic complex which leads to the production and storage of energy.

The growth hormone has been reported to stimulate mitosis in the islets of Langerhans (10), and in the adrenals of the hypophysectomized rats (7). In addition, thyrotropins stimulate mitoses in the thyroid (1). It is also possible that the gonadotropins may act in the same fashion on the gonads, although in this case there is evidence that the observed increased mitotic rate may in fact be mainly due to the action of the androgens and estrogens which are secreted by the gonads in response to the stimulus of the gonadotropins (7).

Other hormones which may have some limited effect on cellular mitoses in normal adult mammals are thyroxin (56) and insulin. With an insufficiency of insulin, there may be a subsequent lowering of intracellular glucose with the result that cells may lack sufficient energy and the mitotic rate may fall. There is evidence that in the normal mouse an increase in insulin concentration may lead to a raised epidermal respiratory rate (46) and a raised epidermal mitotic rate (5).

C) HISTOCHEMISTRY OF THE RESIDUAL BODY OF THE MOUSE TESTIS

It has long been known that a considerable amount of the

cytoplasm of the spermatid is lost during the maturation phase of spermiogenesis. This exfoliated or detached cytoplasm has been reported to form a conspicuous ring of acidophilic bodies referred to as the "residual bodies" (48). Studies indirectly concerned with the residual body have demonstrated that during maturation, processes are occurring within the cytoplasm of the spermatids which result in an increasing RNA-positive granularity of the cytoplasm. This increasing granulation continues until at the moment of release of the mature spermatozoa, one large RNA-positive body is evident within the center of this mass of detached cytoplasm (15). More recently (29), it has been demonstrated, by way of light and electron microscopy, that in addition to the well documented RNA-positive component within the residual body, appreciable amounts of lipid and mitochondria are evident. Little information exists at the present time elucidating the ultimate fate of the residual body.

Although numerous histological and histochemical studies have been performed on the mammalian testis with regard to the precise rhythmic pattern or "wave" of spermatogenesis, the regulating mechanism or mechanisms responsible for this phenomenon have remained obscure. It has been postulated that if such a mechanism does exist, it would most probably exercise its influence at the moment of release of the mature spermatozoa from the seminiferous epithelium (52).

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

Although numerous cytological studies have been carried out on the meiotic behavior of chromosomes, very little is known about protein synthesis occurring in cells undergoing meiotic division (49). 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 the recombination of chromosomal segments during the crossing over phase of meiosis provides for constant changing of the cell's genotype. The biochemical events associated with the transition from somatic mitosis to meiosis are almost entirely unknown. However, the definition by Leblond and Clermont (33) of the cycle of the seminiferous epithelium of the rat in relationship of the differentiation of the spermatids offers an excellent opportunity to investigate this problem by means of radioautographic techniques.

Recent data have indicated that the incorporation of L-Lysine- $U-C^{14}$ into protein of slices of rat testis is stimulated by exogenous glucose to a greater extent than into protein of a large number of other tissues of the rat (16). In addition, it was found that experimental cryptorchidism in the rat is characterized by a marked increase in the incorporation of L-Lysine- $U-C^{14}$ into testicular protein as compared to the normal, scrotal testis (19).

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 the radioautographic technique.

CHAPTER II
MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

A) PREPARATION OF TESTIS SECTIONS

The animals used were 35-45 gram male Swiss mice, approximately 20 weeks in age, obtained from the Abrams Small Stock Breeders (Chicago, Illinois).

The animals were sacrificed by decapitation. The testes were immediately removed and placed in cold Carnoy's solution (6:3:1) overnight. Following fixation, the tissue was dehydrated in three changes of cold absolute ethanol, cleared in methyl salicylate and embedded in Tissuemat (M.P. 55.0-58.5°C). Tissues were sectioned at 8 μ and mounted on glass slides. In place of the usual 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 50°C (23). 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 1 hour prior to staining.

B) HISTOCHEMICAL STAINING METHODS

1. NUCLEIC ACIDS

The Feulgen technique (58) was utilized to demonstrate the localization of DNA. As a control, unhydrolyzed sections were placed in distilled water at 60°C for 8 minutes before staining with Schiff's reagent.

The methyl green-pyronin Y method (54) as modified by Taft (61) was employed to demonstrate the presence of both DNA and RNA. Feulgen's technique confirmed the localization of DNA as indicated by the methyl green (National Aniline), and ribonuclease digestion confirmed the localization of RNA as indicated by pyronin Y (National Aniline). Digestion in protease-free ribonuclease (Mann Research Laboratories Inc., New York, N.Y.) was carried out at a concentration of 1 mg/ml at an incubation temperature of 37°C for a period of 90 minutes to 25 hours depending on the amount of RNA to be removed. As a control, unhydrolyzed sections (distilled water) were run simultaneously with the RNAase digestion.

2. GLYCOGEN

The Periodic acid-Schiff reaction (36) was employed to demonstrate polysaccharides. To confirm the presence of glycogen as demonstrated by the PAS reaction, Best's carmine stain (3) was used. As a control procedure for the Best's carmine stain a 20 minute digestion at 37°C was carried out with a 0.5% solution of saline-activated malt diastase (Merek and Co., Rahway, N.J.).

In order to establish the presence of carbohydrates containing 1:2-glycol groups as demonstrated by the PAS reaction, the acetylation technique of Lillie (34) was utilized. In order to localize RNA and PAS-reactive material simultaneously, PAS-toluidine blue staining combination was employed. The localization of RNA by toluidine blue was followed closely and found to be identical to that found with pyronin Y.

3. LIPIDS

For the demonstration of lipoid material, the testis was fixed in cold 10% neutral formalin overnight and embedded in gelatin. The tissues were cut at 10u on a freezing microtome. Visualization of the lipoid material was demonstrated by staining with Sudan IV. Following counter-staining with toluidine blue, the tissue was mounted in glycerine jelly.

C) ISOLATION AND INCUBATION OF TISSUES

The animals used in these experiments were 60 day old, 185-220 gram male Sprague-Dawley rats obtained from the Abrams Small Stock Breeders, Chicago, Illinois and fed Rockland Rat diet ad libitum. The animals were sacrificed by decapitation and the testes rapidly removed. The outer capsule was cut with a scissors and the testicular tissue gently expressed by manual pressure. Slices of testis which averaged 0.5mm in thickness were obtained with the aid of a Stadie-Riggs microtome at 4°C. The slices from a single

animal, 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. 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, resulting in separated intact tubules having essentially equal diameters. Inasmuch as the depth of the seminiferous tubules 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 was therefore independent of the depths of the counted sections from the surface of the seminiferous tubule. The main chamber of the flask contained 200mg wet weight of tissue in 3.0ml of Krebs-Ringer bicarbonate buffer at pH 7.4. The side-arm contained 100µc of tritiated Lysine in a volume of 0.2 ml. L-Lysine-H³ was obtained from the Nuclear-Chicago Corp. and had a specific activity of 91 mc/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.

D) PREPARATION OF TISSUES FOR HISTOLOGICAL STUDY

At the end of 1 hour incubation period, the flask was removed from the manometer and the flask contents poured through 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 buffer and gently placed in cold Carnoy's solution by means of forceps. In one experiment, the tissue was washed with buffer and then left in buffer containing 0.1% non-radioactive lysine for 30 minutes at 40°C in order to dilute any trace amounts of L-Lysine- H^3 not removed by the buffer washings alone. Following fixation in Carnoy's solution overnight (26), the tissue was dehydrated in alcohol, cleared in methyl salicylate, and embedded in Tissuemat. Tissues were sectioned at 5 μ and mounted on glass slides. The sections were deparaffinized and immersed in water and allowed to air dry prior to dipping in nuclear emulsion.

E) PREPARATION OF SLIDES FOR RADIOAUTOGRAPHY

The slides 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 tape and then placed in the refrigerator for 4 days. 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. After being stained in hematoxylin solution (Harris), the slides were mounted in

Permunt and examined. 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 in order to classify properly the 14 stages of the cycle of the seminiferous epithelium in the rat according to the description of Leblond and Clermont (32).

F) COUNTING RADIOAUTOGRAMS

Grain counting was carried out under oil-immersion using a Whipple micrometer eyepiece grid. Grains were counted within 4 squared 50μ area 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 the present studies. After the proper classification

of a 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 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.

CHAPTER III

RESULTS

CHAPTER III

RESULTS

A) HISTOCHEMISTRY OF THE RESIDUAL BODIES OF THE MOUSE TESTIS

The morphological classification of spermiogenesis in the mouse as proposed by Oakberg (45) has been used in this investigation. During the development of the spermatid, a considerable amount of cytoplasm is lost. The remnant of the cytoplasm detached from the spermatid has long been known as "the residual body" (48). The data of this present study indicates, however that the residual body is but the final stage in a complex series of morphological changes occurring in the discarded cytoplasm of the spermatid. Consequently, the cytoplasm released from the spermatid has been redesignated as the cytoplasmic body and the morphogenesis of this cytoplasmic body (CB) divided into the following four major phases as summarized in Plate 1.

Phase CB-1 in the morphogenesis of the cytoplasmic body

Plate II, figure 1 demonstrates Stage 10 of spermiogenesis in the mouse. Only a small amount of RNA-staining material occurs in the cytoplasm of the spermatid during the development of the cytoplasmic tag. However, in the further course of spermiogenesis, the RNA-positive material appears to coalesce into discrete granules (Plate II, Fig. 2). This coalescence occurs at Stage 11 of spermiogenesis which has also been designated in

the present investigation as phase CB-1 in the morphogenesis of the cytoplasmic body. In addition, the entire cytoplasmic tag in phase CB-1 stains with PAS reaction. Two distinct types of granulations were observed with this stain: 1) Numerous very small PAS-reactive granules scattered uniformly throughout the cytoplasm of the spermatid, and 2) larger, lightly-staining PAS-reactive granules, also found in the cytoplasm of the spermatid. When sections were stained with Best's carmine and digested with malt diastase, only the larger PAS-reactive granules were found to contain glycogen. Moreover, sections which were originally stained with PAS and then subjected to toluidine blue counter-staining indicated that only the larger PAS-reactive granules also stained with toluidine blue.

Plate II, figure 3 indicates the increase in size of the RNA-positive granules during late phase CB-1 in the morphogenesis of the cytoplasmic body. At this stage of development, each cytoplasmic tag was found to contain from 8 to 15 RNA-positive granules. Frozen sections stained with Sudan IV demonstrated finely dispersed lipid droplets throughout the spermatid cytoplasm. By late CB-1, these lipid droplets were found to have migrated toward the distal end of the spermatid.

Phase CB-2 in the morphogenesis of the cytoplasmic body

When the cytoplasmic tag becomes detached from the maturing

spermatid, this structure has been designated as the cytoplasmic body in this investigation. Plate II, figure 4 illustrates phase CB-2 in the morphogenesis of the cytoplasmic body following its liberation from the spermatid at Stage 16 of spermiogenesis. The remnant of the cytoplasmic membrane of the maturing sperm becomes the cytoplasmic body membrane and is PAS-reactive. At this period of development, there is an increase in both the size of the RNA-positive granules found in the cytoplasmic body along with an increase in their staining intensity with pyronin Y. There is also a decrease in the total number of RNA-positive granules in each cytoplasmic body. These RNA-positive granules continue to coalesce and will ultimately form the RNA plaque at phase CB-3 in the morphogenesis of the cytoplasmic body. On the other hand, the smaller PAS-reactive granules continue to coalesce into larger granules which become located along the periphery of the cytoplasmic body in CB-3 (Plate II, Fig. 5 and 6), and which will ultimately form the PAS-reactive satellites seen at this phase of the development of the cytoplasmic body. The larger PAS-reactive granules located within the cytoplasmic body and which stained positively with Best's carmine were also found to ultimately fuse to form part of the plaque area visible in phase CB-3. In addition, the lipid droplets were observed to further coalesce into larger globules which were located in a random

fashion throughout the cytoplasmic body. By late phase CB-2, these lipoid globules have migrated toward the cytoplasmic body membrane. A fusion of two or more cytoplasmic bodies was also observed in many instances, producing a syncytial-like appearance of these structures.

Phase CB-3 in the morphogenesis of the cytoplasmic body

Plate II, figure 5 demonstrates early phase CB-3 in the morphogenesis of the cytoplasmic body. The coalescence of the RNA-positive granules has continued until at this point of development, a single large RNA-plaque is visible within the cytoplasmic body. It is of interest to note that the large RNA-positive plaque also contains a PAS-reactive component since the plaque stains with both toluidine blue and PAS-reaction. The coalescence of the smaller PAS-reactive granules during their peripheral migration has also resulted in the formation of larger PAS-satellites which are now in apparent contact with the cytoplasmic body membrane. Utilizing the acetylation technique of Lillie (34), it was found that both the PAS-satellites and the cytoplasmic body membrane are composed of polysaccharides containing 1:2-glycol groups.

Plate II, figure 6 presents late phase CB-3 in the morphogenesis of the cytoplasmic body. The plaque area of the cytoplasmic body at this period in development stains very intensely with pyronin-Y

as well as with the PAS-reaction. Whereas at phase CB-1, a 90 minute digestion with RNAase was sufficient to remove all of the demonstrable RNA, it was necessary to employ a 25 hour enzymatic hydrolysis at phase CB-3 in order to completely remove all of the visible RNA. The shape of the plaque varies from an essentially spherical form to an almost amorphous mass. In addition, the RNA-plaque now demonstrates numerous indentations. Sections stained with Sudan IV at this point of development (Plate III, Fig. 7) reveals the presence of lipoid-positive material in the cytoplasmic body space. These lipoid globules appear to be responsible for the indentations of the plaque area. Plate III, figures 8 and 9 demonstrate that these large lipoid globules are formed within the cytoplasmic bodies which are located along the periphery of the seminiferous tubular lumen. From this position, migration of the cytoplasmic bodies begins toward the basement membrane of the seminiferous tubule. At the onset of this migration, the lipoid material begins to decrease and simultaneously with this decrease, the cytoplasmic body membrane collapses, resulting in a decrease in the cytoplasmic body space as well as a condensation of the PAS-satellites on the now spherical RNA-plaque at the initiation of phase CB-4 in the morphogenesis of the cytoplasmic body.

Phase CB-4 in the morphogenesis of the cytoplasmic body

Two fates of the cytoplasmic body can be demonstrated during its migration from an initial position along the periphery of the seminiferous tubule lumen at phase CB-3 to its final position near the basement membrane of the seminiferous tubule during phase CB-4.

The first fate of the cytoplasmic body involves its phagocytosis by a Sertoli cell (Plate III, Fig. 10 and 11). Further changes in the structure of the cytoplasmic body subsequently occur within the Sertoli cell cytoplasm. The cytoplasmic body membrane becomes approximated to the plaque, resulting in the obliteration of the cytoplasmic body space. This obliteration would appear to be due to the removal of the lipid globules from the cytoplasmic body space by the Sertoli cell. This transfer of lipid material from the cytoplasmic body space to the Sertoli cell results in the accumulation of finely-dispersed lipid granules which ultimately aggregate in the basal regions of the Sertoli cell cytoplasm. When the cytoplasmic body space is no longer visible, the cytoplasmic body can now be designated as the residual body. Further development of the residual body within the Sertoli cell cytoplasm includes the loss of its RNA content followed by a gradual loss of its glycogen content. In addition, the PAS-satellites appear to spread over the surface of the plaque which now has decreased in size until its apparent disappearance in the cytoplasm of

the Sertoli cell.

The second fate of the cytoplasmic body involves its migration toward the basement membrane of the seminiferous tubule without undergoing phagocytosis by a Sertoli cell. During this migration of the cytoplasmic body, similar morphological changes occur as previously described for the cytoplasmic bodies found within the cytoplasm of the Sertoli cells. These changes include the loss of RNA (Plate III, Fig. 12) as well as the loss of its lipid granulations in the region of the spermatogonia and primary spermatocytes. Following these morphological changes, the residual bodies continue to migrate toward the level of the spermatogonia (Plate III, Fig. 13) and ultimately occupy a final position against the basement membrane of the seminiferous tubule (Plate III, Fig. 14). In a similar fashion to that which was observed within the Sertoli cell cytoplasm, the residual bodies decrease in size while continuing to stain with Best's carmine but not with toluidine blue. Eventually, the residual bodies can no longer be demonstrated by the staining procedures employed in this investigation.

B) THE EFFECT OF GLUCOSE ON THE UPTAKE OF L-LYSINE- H^3 IN CELLS
OF THE SEMINIFEROUS EPITHELIUM

Plate IV 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 . The 14 stages of the cycle of the seminiferous epithelium in the rat have been classified according to the description of Leblond and Clermont (32). It was found that the seminiferous epithelium of the rat testis retains its morphological integrity during the incubation period of the slice. In addition, 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 radioautographs inasmuch as the grains could be seen more easily without it and the hematoxylin alone was found to give sufficient background stain.

Plate V 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. 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 fewer 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 VI 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 over 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 transitional 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.

Plate VII 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 transitional primary

spermatocytes with little or no labeling 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 1 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 the 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.

Plate VIII summarizes the effects 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 three-fold stimulation of protein from L-Lysine- H^3 in the spermatogonia and the resting leptotene, zygotene and transitional primary spermatocytes. However, glucose enhanced protein 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.

CHAPTER IV

DISCUSSION

CHAPTER IV

DISCUSSION

A) HISTOCHEMISTRY OF THE RESIDUAL BODIES OF THE MOUSE TESTIS

Numerous attempts have been made to formulate a "trigger mechanism" which would regulate the periodicity of the spermatogenic cycle. Roosen-Runge (52) has speculated that the release of older spermatids from the seminiferous tubular lumen is in some unknown manner associated with the regulation of the development of earlier generations of the germinal epithelium. Lacy (30) demonstrated that the bulk of the lipid observed in the Sertoli cells was derived from the phagocytosis of the residual bodies and that following 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.

It has been known since Kolliker (26) first established the cellular evolution of spermatozoa, that the oldest generation of germ cells lies in the center of the seminiferous tubule and the youngest generation of germ cells lies at the periphery of the seminiferous tubule. von Ebner discovered that successive stages of spermatogenesis were arranged next to one another along

the length of the tubule, and immediately recognized that this arrangement was a fortunate circumstance making possible the investigation of the 'genealogy of germ cells'. The "wave-like" character of the longitudinal arrangement of stages was further studied in the rat by Regaud (48) who contended that the wave progressed in a spiral arrangement along the tubule. Curtis concluded that in the mouse, and even more so in the rabbit, the spermatogenic waves varied in length, in continuity, in incidental course, and in their general direction along the tubule. The spermatogenic wave was found to reverse its course and general direction in the rabbit. In the mouse the spermatogenic wave was found to descend from the rete testis; that is, the older stages were always closer to the rete, the younger stages farther away.

The phenomenon of the spermatogenic wave is the result of (a) synchronous development of groups of germ cells and (b) of the progressive spread of this cellular development to adjacent areas of the tubule. When the groups of cells are very large and the spread is regular, as is apparent in the rat, a fairly regular spermatogenic wave results. When the groups are very small, as in man, it becomes difficult to determine whether contiguous groups are in successive stages of development and the wave is no longer manifest even if it exists in rudiments. In most mammals intermediary conditions are found, in which the wave pattern is

quite obvious.

Developing germ cells are essentially synchronized in most animals. Cleland (11) attempted to develop a hypothesis to explain this synchronization. He discussed his views thoroughly and rejected a "rigidly timed self-limited situation in the life history of the cells" because of evidence from the general field of biology that "when cells are not in close connection with one another, biological variability is sufficient to preclude a high degree of synchronization". On the other hand, he examined the degree of cellular connection within the germinal epithelium and found the histological structure of the tubules well adapted to secure synchronization. He assumed the germ cells to be embedded in a syncytial continuum of Sertoli cells and observed the close proximity of cells. This led to the hypothesis that selective influences may be transmitted from cell to cell being mediated by the Sertoli cells and responsible for the cellular synchronization.

While the syncytial nature of the Sertoli cells must not be rejected, there is convincing evidence that spermatocytes and spermatids are commonly interconnected by cytoplasmic bridges, and that bridges most probably exist between cells attached to different adjacent Sertoli cells. The close approximation of germ cell and Sertoli Cell has been adequately demonstrated in

electron microphotographs, and the transmission of substances from Sertoli cell to germ cell and vice a versa has been inferred with high probability from a variety of histochemical and morphological investigations. It is, therefore, easy to assume that correlative stimuli may travel through large areas of the germinal epithelium and thus affect synchronization. The final limitation of the areas may come about through the rate of speed with which stimuli may pass through germ and Sertoli cells. In fact, the nature of the spermatogenic wave may depend on the speed of transmission along the tubule; thus, the phenomenon may be basically similar to that of a true "wave" than von Ebner (21) originally thought. 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.

The data of this investigation indicates that glycogen is a significant constituent of the cytoplasmic body. Following the development of the cytoplasmic body into the residual body at phase CB-4, two possible fates of this structure have been demonstrated. The residual body can either be phagocytized by a Sertoli cell 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 can contribute appreciable amounts of glycogen to either the Sertoli cell or to the germinal epithelium (44).

One possible function for the glycogen of the residual bodies which are phagocytized by the Sertoli cell may involve the conversion of glycogen to glucose-6-phosphate and then to 6-phosphogluconate via the hexose monophosphate shunt with a resulting increase of TPNH acting as a co-factor for steroid synthesis in much the same manner as has been demonstrated by Hayano and Dorfman (22), Sweat and Lipscomb (60) and Tchen and Elock (62). In this way, the glycogen of the residual bodies may aid in the initiation or acceleration of the production of a Sertoli cell steroid hormone as originally postulated by Lacy (30).

B) THE EFFECT OF GLUCOSE ON THE UPTAKE OF L-LYSINE- H^3 IN CELLS

OF THE SEMINIFEROUS EPITHELIUM

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 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 (52), other than being incorporated into protein. Lysine

does not readily undergo transamination (9) and has been found to yield only extremely small amounts of tricarboxylic acid cycle intermediates during the course of its catabolism (18).

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 (33), 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 the spermatids, known as spermiogenesis.

Five mitotic peaks have been found to occur during the divisions of the spermatogonia in the spermatogenic cycle (32). The first three peaks of spermatogonial mitoses, occurring at Stages IX, XII and I, give rise to type A spermatogonia. The fifth peak of spermatogonia mitoses occurs 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 mitosis 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 (39). 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 involve paring, crossing over, and reduction in chromosome number (49). 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 (57). 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 (35). 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.

CHAPTER V

SUMMARY

CHAPTER V

SUMMARY

1. A histochemical study of the detached cytoplasm of the maturing spermatid has been carried out utilizing the mouse testis. The detached cytoplasm has been designated as the cytoplasmic body and four distinct phases in its morphogenesis have been reported.
2. The cytoplasmic tag of stage 11 of spermiogenesis has been designated as phase CB-1 in the morphogenesis of the cytoplasmic body and was found to contain individual granules of RNA, glycogen, polysaccharides with 1:2-glycol groups and lipid.
3. During phase CB-2 and CB-3, coalescence and fusion of these granules occurs which result in a centrally-oriented plaque of RNA and glycogen, peripheral satellites of 1:2-glycol containing polysaccharides and large globules of lipid.
4. During phase CB-4, the cytoplasmic body membrane becomes approximated to the plaque and at this stage of development the cytoplasmic body can be compared to the "residual bodies" of Regaud.
5. Peripheral migration toward the basement membrane of the seminiferous tubule now occurs with some of the residual bodies undergoing phagocytosis by Sertoli cells while others

eventually occupy a final position at the level of the spermatogonia. At this time a loss of the RNA and lipid components occurs without an accompanying loss of the glycogen content. Eventually the glycogen plaque can no longer be demonstrated by the staining techniques employed in the present studies.

6. 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 the 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.
7. 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.
8. 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.
9. 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.

10. 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.
11. 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.

CHAPTER VI
PLATES AND LEGENDS

PLATE I

Diagrammatic representation of the four phases in the morphogenesis of the residual body of the mouse testis. The abbreviations used are as follows: CB, cytoplasmic body; CT, cytoplasmic tag; GLY-G, glycogen granule; GLY-P, glycogen plaque; LD, lipoid droplet; LG, lipoid globule; M, cytoplasmic body membrane; PAS-g, PAS-reactive small granule; PAS-G, PAS-reactive large granule; PAS-P, PAS-reactive plaque; PAS-S, PAS-reactive satellite; RB, residual body; RNA-G, RNA-reactive granule; RNA-P, RNA-positive plaque; SCM, spermatid cell membrane; SN, spermatid nucleus; Sp, cytoplasmic body space.

MORPHOGENESIS OF THE CYTOPLASMIC BODY OF THE MOUSE TESTIS

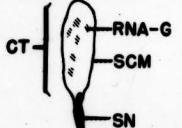

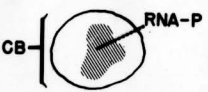

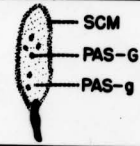
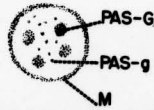
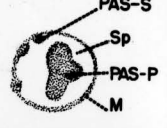

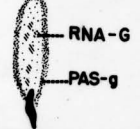
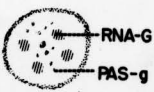
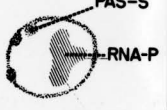
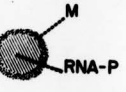
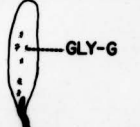



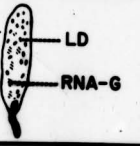
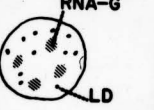


	CB-1	CB-2	CB-3	CB-4
TOLUIDINE BLUE				
PAS				
PAS-TOLUIDINE BLUE				
BEST'S CARMINE				
SUDAN IV-TOLUIDINE BLUE				

PLATE I

PLATE II

EXPLANATION OF FIGURES

All sections were cut at 8 μ

Magnification x 1,900

1. Stage 10 (43) of spermiogenesis in the mouse. Note the lack of granulations in the cytoplasmic tags (arrow). Toluidine blue.
2. Stage 11 of spermiogenesis in the mouse (early Phase CB-1 in the morphogenesis of the cytoplasmic body). Note the appearance of RNA granules (arrow) in the cytoplasmic tag (CT). PAS-toluidine blue.
3. Stage 14 of spermiogenesis in the mouse (late Phase CB-1 in the morphogenesis of the cytoplasmic body). Note the increase in size and the decrease in number of the RNA granules (arrow) in the cytoplasmic tag (CT). Toluidine blue.
4. Stage 16 of spermiogenesis in the mouse (Phase CB-2 in the morphogenesis of the cytoplasmic body). The detached cytoplasm of the maturing spermatids has been designated as the cytoplasmic body (CB) in the present report. Note the further increase in size along with a decrease in the number of the RNA granules. Toluidine blue.
5. Early Phase CB-3 in the morphogenesis of the cytoplasmic body. Note the single large RNA plaque (P) in the cytoplasmic body and the PAS satellites (S). PAS-toluidine blue.

6. Late Phase CB-3 in the morphogenesis of the cytoplasmic body. The RNA plaque (P) now demonstrates indentations. Note the numerous PAS satellites (S) which have also increased in size and lie in close proximity to the cytoplasmic body membrane (M). PAS-toluidine blue.

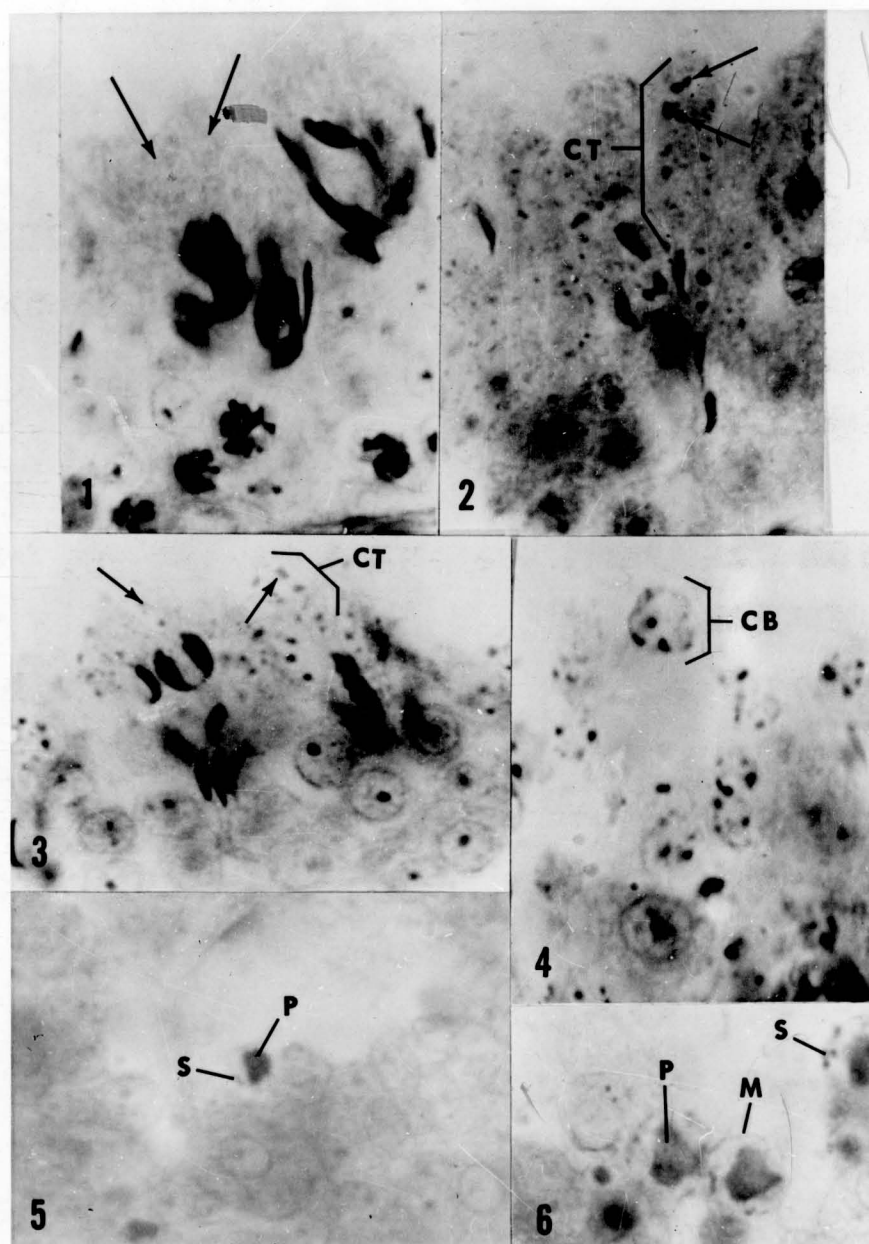


PLATE II

PLATE III

EXPLANATION OF FIGURES

7. Late Phase CB-3 in the morphogenesis of the cytoplasmic body stained with Sudan IV-toluidine blue. The arrow indicates Sudanophilia in the cytoplasmic body space. Note that the lipoid material (arrow) produces the indentations of the RNA plaque (P). Frozen section cut at 10 μ . Magnification x 1,900.
8. Late Phase CB-3 in the morphogenesis of the cytoplasmic body stained with Sudan IV-toluidine blue demonstrating lipoid material (arrows) in cytoplasmic bodies along the periphery of the seminiferous tubule lumen. Frozen section cut at 10 μ . Magnification x 1,900.
9. Lower magnification of the seminiferous tubule stained with Pyronin Y. Note the periluminal localization of the cytoplasmic bodies during phase CB-3. Section cut at 8 μ . Magnification x 600.
10. Phase CB-4 in the morphogenesis of the cytoplasmic body. When the membrane of the cytoplasmic body is no longer visible, the cytoplasmic body has been designated as the residual body (RB) in the present report. Note the Sertoli cell phagocytizing a residual body. Iron hematoxylin. Section cut at 8 μ . Magnification x 1,900.

11. Higher focal plane of Figure 10 demonstrating the Sertoli cell cytoplasm (C) passing over a residual body (RB).
Magnification x 1,900.
12. Decrease in the staining intensity of the RNA plaque with toluidine blue in Phases CB-3 and CB-4 in the morphogenesis of the cytoplasmic body. Section cut at 8 μ . Magnification x 1,900.
13. Migration of residual bodies (arrows) to the level of the spermatogonia. Section cut at 8 μ and stained with iron hematoxylin. Magnification x 1,900.
14. Migration of residual bodies (arrows) to the level of the basement membrane. Sections cut at 8 μ and stained with iron hematoxylin. Magnification x 1,900.

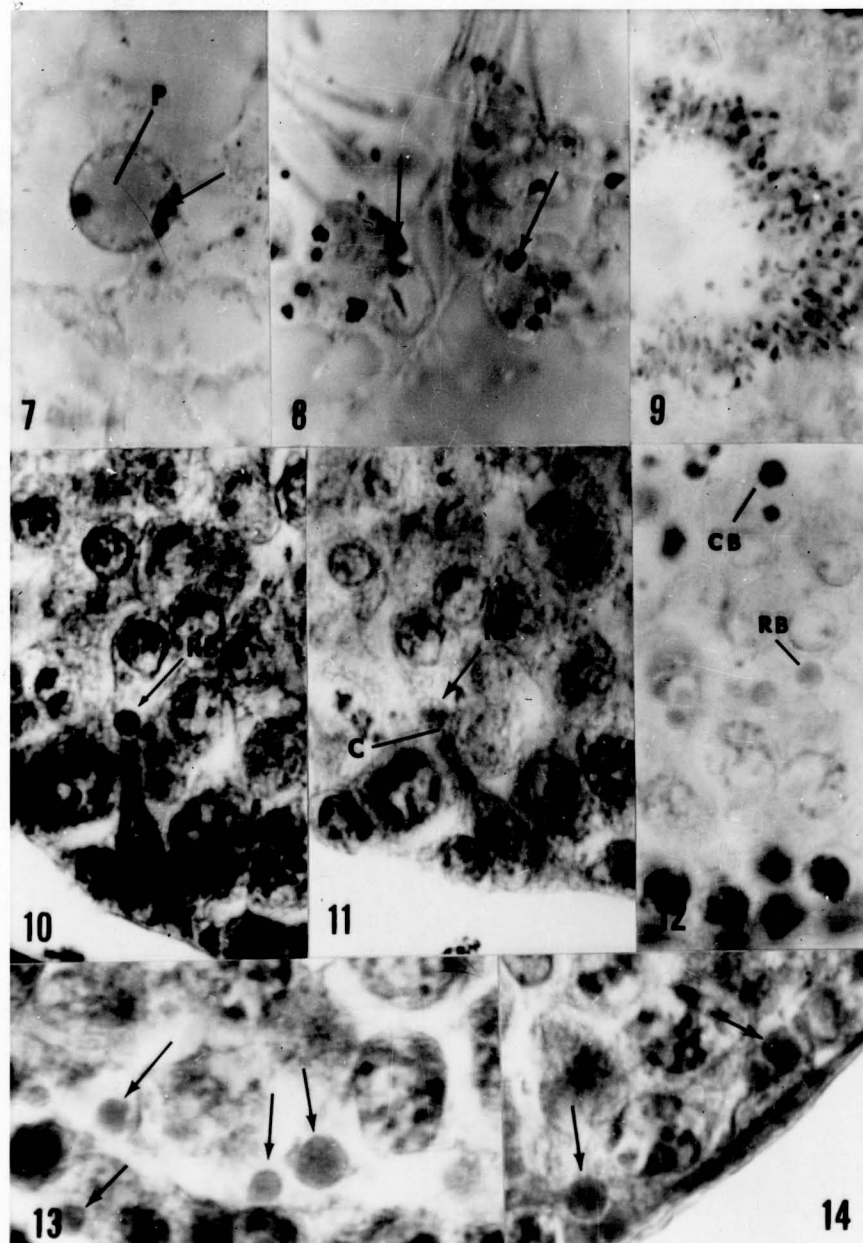


PLATE III

PLATE IV

Histological examination of the seminiferous epithelium following a 1 hour incubation period of slices of rat testes at 37.5°C. (1) to (14) illustrate the 14 stages of the cycle of the seminiferous epithelium in the rat according to the description of Leblond and Clermont (30). Fixation in Carnoy's solution; 5 μ sections stained with hematoxylin. Magnification approximately x 700.

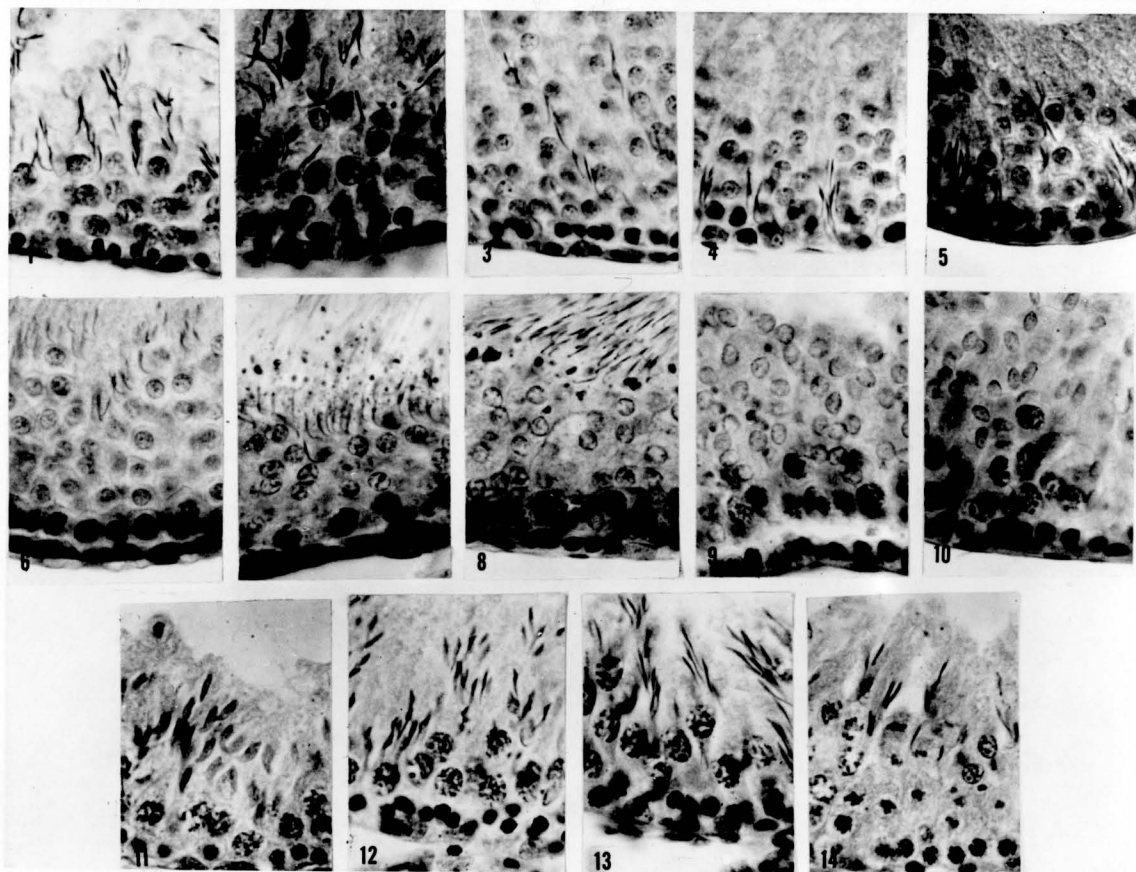


PLATE IV

PLATE V

Radioautographs of sections taken from slices of rat testes incubated for 1 hour with L-Lysine- H^3 in the presence of 0.009 M glucose. (2), (4) and (6) represent stages VII, VIII and XIII, respectively while (1), (3) and (5) represent corresponding alternate serial sections stained without dipping in nuclear emulsion. All sections were cut at 5 μ and stained with hematoxylin. Exposure of the radioautograph was for 4 days. Magnification approximately x 700.

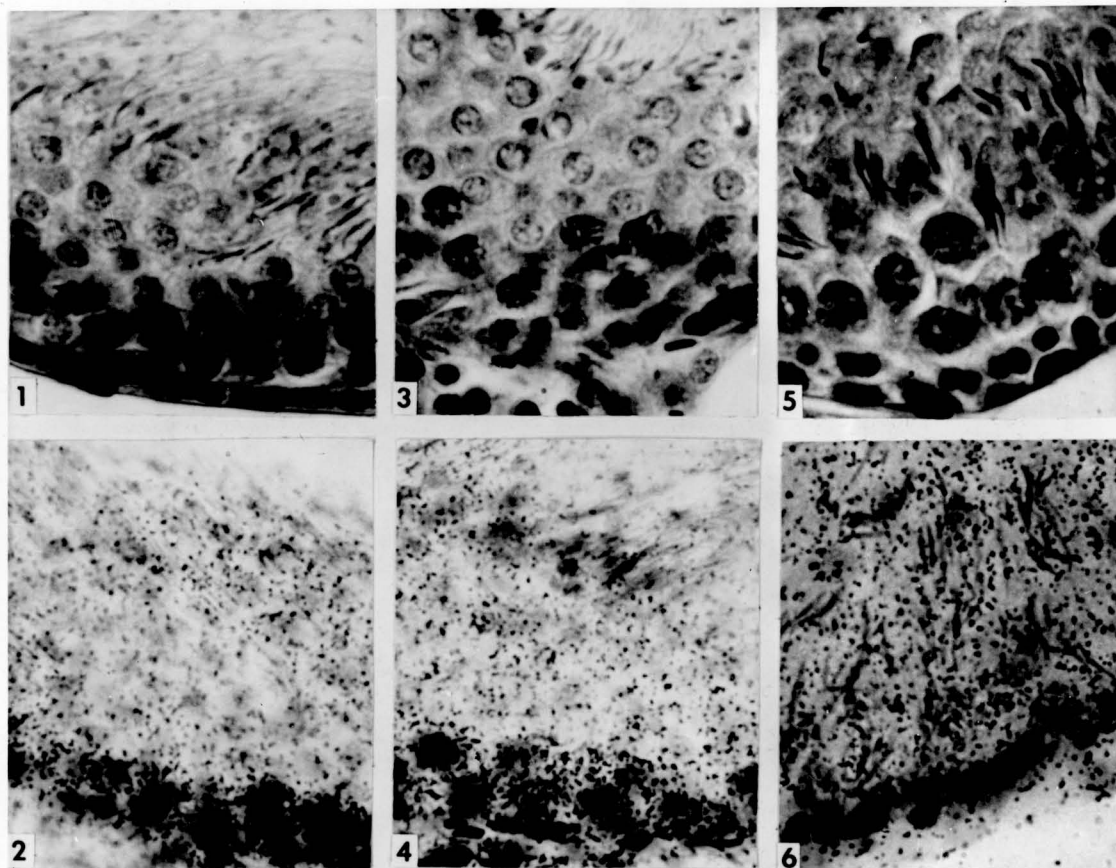


PLATE V

PLATE VI

Radioautograms of sections taken from slices of rat testes incubated for 1 hour with L-Lysine- H^3 in the presence and absence of 0.009 M glucose. (1) and (2) represent stage I in the absence and presence of glucose, respectively; (3) and (4) represent stage V in the absence and presence of glucose, respectively; (5) and (6) represent stage VII in the absence and presence of glucose, respectively; (7) and (8) represent stage IX in the absence and presence of glucose, respectively; (9) and (10) represent stage XIII in the absence and presence of glucose, respectively. All sections were cut at 5 μ and stained with hematoxylin. Exposure of the radioautograms was for 4 days. Magnification approximately x 700.

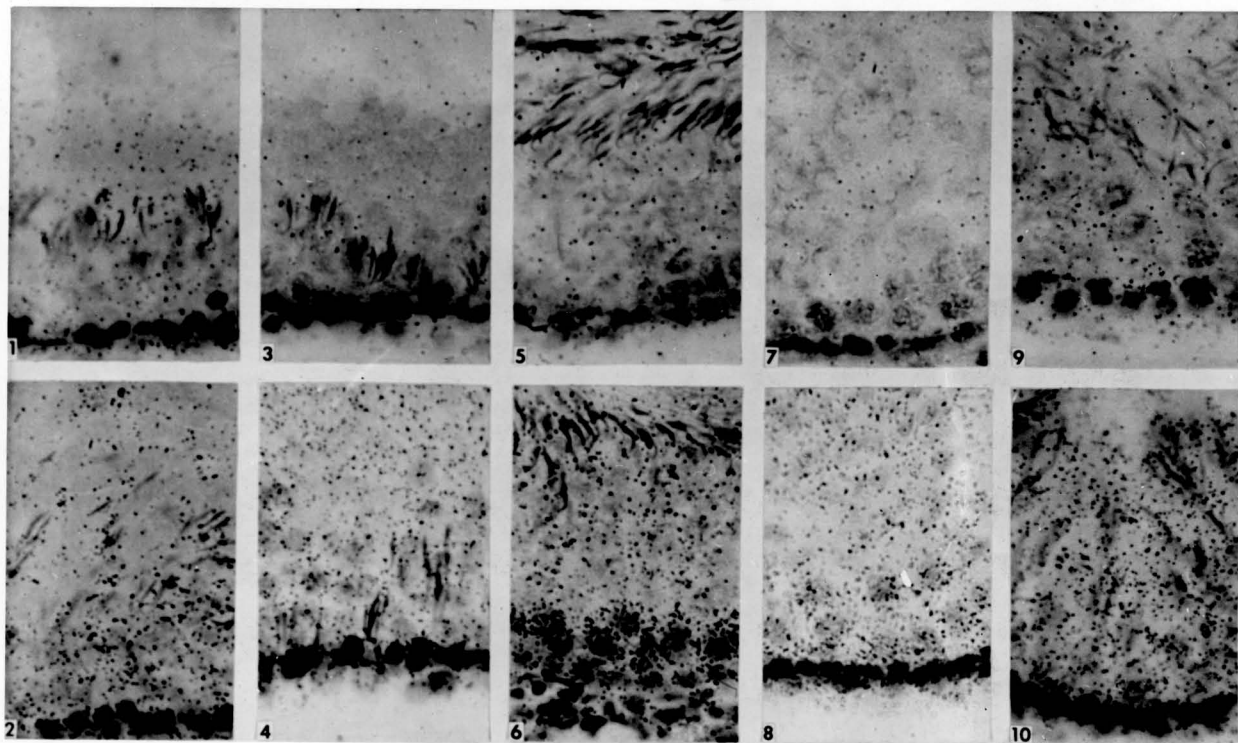


PLATE VI

PLATE VII

Incorporation of L-Lysine- H^3 into protein of cells of the seminiferous epithelium of the rat testis. Flask contents: L-Lysine- H^3 ($3.4 \times 10^{-4} M$), 100 μc in 0.2 ml; 0.009 M 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 curves represents the average of four individual experiments involving 20 determinations for each of the designated cells of the cycle of the seminiferous epithelium. The standard error for each value in the control system was ± 3 grains/200 μ^2 above background while the standard error for each value in the glucose-supplemented system was ± 7 grains/200 μ^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 (30). Numbers 1-19 refer to spermatids at various steps 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; M, primary spermatocytes in metaphase; DI, diplotene primary spermatocytes; SM, secondary spermatocytes in metaphase.

PLATE VIII

Effect of 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. For experimental details see legend for Fig. 4. Each point on the graph represents the average of the designated cells of the cycle of the seminiferous epithelium as obtained in Fig. 4 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 (30). 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. For additional abbreviations, see legend for Fig. 4.

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

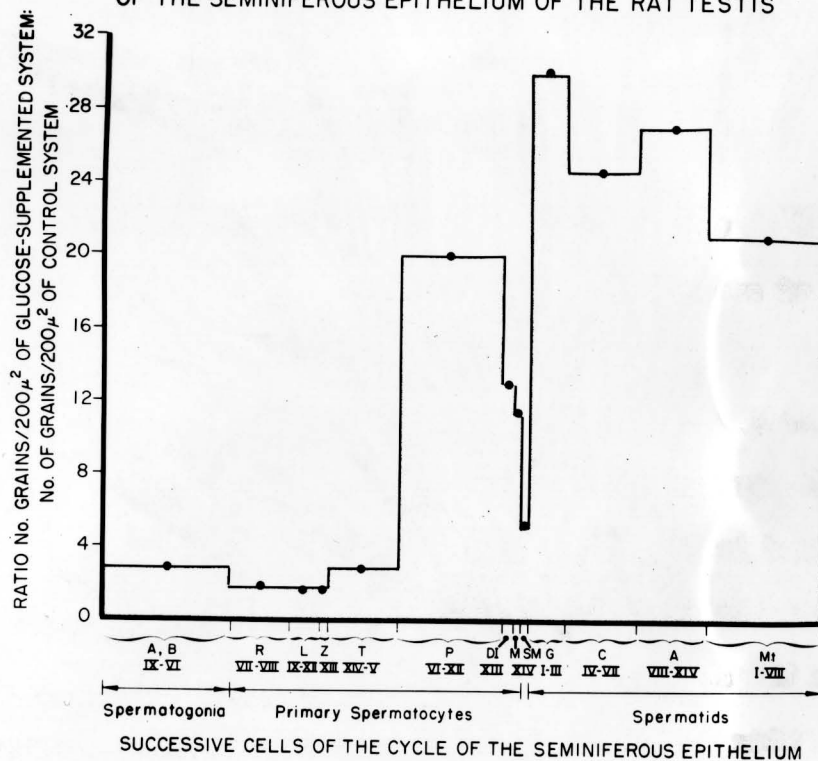


PLATE VIII

CHAPTER VII

TABLES

*

The individual values presented for each animal represent the average of the following successive cells of the cycle of the seminiferous epithelium: type A and type B spermatogonia in stages IX-VI; resting 1° spermatocytes in stages VII-VIII; leptotene 1° spermatocytes in stages IX-XII; zygotene 1° spermatocytes in stage XIII; transition 1° spermatocytes in stages XIV-V; pachytene 1° spermatocytes in stages VI-XII; diplotene 1° spermatocytes in stage XIII; metaphase 1° spermatocytes in stage XIV; metaphase 2° 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.

**

Expressed in grains/200 μ^2 above background

TABLE 1

INCORPORATION OF L-LYSINE-H³ INTO PROTEIN OF CELLS OF THE

Animal No.	Spermatogonia		Resting 1° Spermatocytes		Leptotene 1° Spermatocytes	
	Control	Glucose	Control	Glucose	Control	Glucose
1*	11	40	39	70	37	68
2	18	38	41	68	50	52
3	11	43	44	71	37	67
4	17	44	35	72	41	73
Mean**	14	41	40	70	41	65
S.D.	2.8	2.0	3.0	1.2	4.4	6.4
t	11.6		13.2		13.7	
P	0.001		0.001		0.001	

Animal No.	Pachytene 1° Spermatocytes		Diplotene 1° Spermatocytes		Metaphase 1° Spermatocytes	
	Control	Glucose	Control	Glucose	Control	Glucose
1	4	84	8	65	1	56
2	3	74	7	67	4	54
3	3	77	7	66	5	58
4	7	89	7	77	8	60
Mean	4	81	7	69	4	57
S.D.	1.4	4.8	0.4	4.0	2.0	1.8
t	69.0		22.3		27.0	
P	0.001		0.001		0.001	

Animal No.	Cap phase Spermatids		Acrosome phase Spermatids	
	Control	Glucose	Control	Glucose
1	0	38	1	49
2	5	58	3	55
3	2	46	2	53
4	11	53	2	59
Mean	4	49	2	54
S.D.	3.2	6.2	0.6	3.4
t	9.2		24.5	
P	0.001		0.001	

SEMINIFEROUS EPITHELIUM OF THE RAT TESTIS

Zygotene 1° Spermatocytes		Transition 1° Spermatocytes	
Control	Glucose	Control	Glucose
35	58	18	74
34	68	32	60
40	58	13	66
39	57	31	74
37	60	24	68
2.0	3.8	6.8	4.8
7.7		23.7	
0.001		0.001	

Metaphase 2° Spermatocytes		Golgi phase Spermatids	
Control	Glucose	Control	Glucose
2	25	2	57
3	21	4	66
6	22	0	56
13	33	3	61
6	25	2	60
3.6	3.8	1.0	3.2
16.4		24.0	
0.001		0.001	

Maturation phase Spermatids	
Control	Glucose

1	36
4	52
2	39
1	39
2	41
1.0	5.0
34.8	
0.001	

CHAPTER VIII

BIBLIOGRAPHY

CHAPTER VIII

BIBLIOGRAPHY

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APPROVAL SHEET

The thesis submitted by Casimir F. Firlit has been read and approved by three members of the faculty of Loyola University.

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

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 21, 1965

Date

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

Signature of Advisor