

ULTRASTRUCTURAL STUDIES ON THE  
TESTIS OF THE DOMESTIC FOWL,  
GALLUS DOMESTICUS

A Thesis Submitted By

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SUMMARY

The ultrastructure of the fowl testis from hatch to maturity is compared following immersion fixation using different fixatives with a variety of buffers. Glutaraldehyde buffered with sodium cacodylate is found to give the best fixation over the whole age range. The effect on fine structure of varying the osmolality of the glutaraldehyde/buffer solution is investigated. The best fixation image is obtained with an osmolality higher than that of the blood plasma or semen. Changes in the concentration of the buffer had a greater effect on the fine structure than changes in the concentration of the fixative. A method for obtaining uniform fixation of the testis of the domestic fowl by vascular perfusion is presented.

The differentiation of the germ cells during spermatogenesis and spermiogenesis is described and found similar to that of other vertebrates. Two types of spermatogonia are noted and the number and type of cytoplasmic bridges between spermatogonia and spermatocytes are described. The paucity of cytoplasmic bridges in the fowl compared to mammals is related to the lack of germ cell synchronisation in avian testes.

The relationship between the spermatid acrosome and the Sertoli cell cytoplasm<sup>is</sup> examined and related to the mechanism of sperm release. This thesis presents the first ultrastructural demonstration of spermiation in the domestic fowl. Four main stages are recognised: (1) the swelling of the smooth endoplasmic reticulum of the spermatid to form vesicles, (2) invagination of these vesicles increasing their surface area, (3) coalescence of the vesicles around the spermatid, so it lies free within a large vesicle, (4) release of the acrosome by the Sertoli cell cytoplasm. This /

This mechanism is compared to those described for amphibian and mammal.

Aspects of the morphological pathway taken by substances passing from the blood stream to the seminiferous tubules that have not been previously examined in the fowl testis are described. The organisation and differentiation of the intertubular tissue is studied and compared to the organisation in mammals. The relationship between the blood vessels, lymphatic vessels, Leydig cells and seminiferous tubules is noted.

Five types of Sertoli-Sertoli cell junctions are described : zonula occludens, zonula adherens, desmosomes, interdigitating membranes, subsurface cisternal junctions and tight junctions. The differentiation of the Sertoli-Sertoli tight junction is correlated with the onset of spermatogenesis. There are no gap junctions like those of mammalian testis.

The tracers, lanthanum nitrate and horseradish peroxidase are used to delineate the pathway from the bloodstream to the tubules. Lanthanum perfused intravascularly with the fixative and peroxidase injected intravascularly are seen lying in the endothelial intercellular clefts, in the interstitial spaces, in the intercellular clefts of the peritubular boundary cells and the surrounding intercellular spaces of the spermatogonia. Further penetration towards the lumen is prevented by tight junctions between adjoining Sertoli cells above the spermatogonial layer. Thus the morphological basis of the blood-testis barrier in the fowl appears to be the Sertoli-Sertoli tight junctions. No barrier is present until the differentiation of the pachytene spermatocytes and the appearance of the Sertoli-Sertoli junctions after the onset of spermatogenesis. The possible functional significance of the barrier is discussed.

The testis is a highly complex organ with both an exocrine and endocrine function. It is responsible for the production of large quantities of spermatozoa and for the secretion of steroid sex hormones. These control the development and functional activity of the germinal epithelium, the accessory sexual structures, and the secondary sexual characteristics as well as influencing behaviour concerned with the process of reproduction.

These functions are regulated by the secretion of two gonadotrophic hormones from the anterior pituitary gland; namely follicle-stimulating hormone (FSH), which regulates the production of spermatozoa in the seminiferous tubules and luteinising hormone (LH), also known as interstitial cell-stimulating hormone (ICSH), which regulates the secretory activity of the intertubular Leydig cells. Purified samples of these two hormones have recently been separated from fowl pituitary glands (Stockell-Hartree & Cunningham, 1969) and two anatomically distinct areas of the hypothalamus are known to control their release (Graber, Frankel & Nalbandov, 1967). The endocrinal control of the avian testis has been reviewed by Lake & Furr (1971) and Lofts & Murton, (1973).

Most avian species are seasonal breeders, whose reproductive physiological mechanisms are synchronised by environmental stimuli in order to ensure that the young are produced at the time of year best suited for their survival. In the domestic fowl this ancestral cyclic pattern has been lost and the testes are maintained in a functionally active state all year.

The male gonads originate from a pair of sexually undifferentiated primordia associated with the intermediate mesoderm.

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The primordial germ cells within these structures are derived from the embryonic splanchnopleur (Dubois, 1965). The left gonad receives a greater number of these germ cells and thus establishes an asymmetrical gonadal development which persists throughout life (Witschi, 1935). This is more pronounced in the female bird in which only the left gonad develops into a functional ovary. In the male the gonads develop into paired testes.

The pair of testes are located in the abdominal body cavity adjacent to the anterior end of the kidneys and just posterior to the lungs. They are attached by a short mesorchium to the dorsal body wall in the mid-line. This is a different arrangement to most mammals and marsupials whose testes migrate from the abdomen through the inguinal canal and finally reside in the scrotum. The testes of the fowl are ovoid encapsulated bodies, each surrounded by a fibrous coat, the tunica albuginea and a fragile serous outer sheath, the tunica vaginalis.

The tunica albuginea of the fowl is much thinner than that of mammals (Lake, 1971). In mammals the tunica albuginea gives off numerous very thin septa which radiate down into the testicular tissue. These septa, known as trabeculae, divide the testis into many lobules and form a connective tissue support for the blood vessels. In the fowl there are no trabeculae providing supportive tissue.

Each testis is composed of three cytologically distinct tissues each located in a different zone, namely the seminiferous epithelium lining the seminiferous tubules, the peritubular boundary tissue and the intertubular tissue. The fine structure of the testis has been extensively studied in vertebrates (see review Burgos, Vitale-Calpe & Aoki, 1970).

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In the adult fowl 95% of the testis is composed of seminiferous tubules. These convoluted tubules branch and anastomose freely, unlike those of the mammal which are unbranched and grouped into lobules bounded by the fibrous connective tissue septa.

Compared to scrotal testes the abdominal testes of the fowl are very soft due to the lack of substantial septa, large diameter of the seminiferous tubules and the high fluid content, which becomes evident on excision. The soft nature of the testis makes fixation by perfusion obligatory in order to obtain a good preservation of the intertubular tissue and luminal surface of the tubules. The abdominal position of the testes in the fowl makes localised perfusion as performed for mammals (Dym, 1973) much more difficult. In the present study a perfusion technique suitable for the fowl testis has been developed.

The seminiferous epithelium is composed of two cell-lines, the developing germ cells and the non-germinal sustentacular Sertoli cells. Only the Sertoli cells and spermatogonial stages of the germ cells are in contact with the basal lamina. All other germ cell types are surrounded and held by cytoplasmic processes of the Sertoli cells. This makes the Sertoli cell the anatomical pathway for metabolic exchange between the developing germ cells, and the blood stream (Vilar, Perrez de Cerro & Mancini, 1962). The metabolic function is indicated by the large Golgi complexes, the great development of endoplasmic reticulum (ER), the number of lipid inclusions and the amount of micropinocytosis observed between the Sertoli cell and the germ cells, whereas the mechanical role of support appears to depend on the cytoplasmic bundles of microtubules (Christensen, 1965) and the intercellular junctions (Nicander, 1967).

Spermatogenesis /

Spermatogenesis is the name given to the continuous process of differentiation of the male germ cells. This is a complex process by which a spermatogonial stem cell gives rise to a spermatozoon. It can be divided into three distinct phases. During the first phase the spermatogonia proliferate mitotically and give rise to spermatocytes while simultaneously maintaining their number by stem cell renewal. The second phase is characterised by the series of nuclear events that the primary and secondary spermatocytes undergo during the process of reductional or meiotic divisions that lead to the formation of haploid cells, the spermatids. The final stage involves a complex series of cytological transformations known as spermiogenesis, that lead to the production of spermatozoa. The release of the mature spermatids into the lumen of the tubule has been termed spermiation as the counterpart of ovulation in the female (Van Oordt <sup>& Klomp</sup>, 1946). This thesis presents the ultrastructural features of the germ cells during spermatogenesis in the domestic fowl and a description of the cytoplasmic events that result in spermiation.

Certain features of the spermatogenic process such as the synchronous development of the germ cells are not yet fully understood. The germ cells mature successively from spermatogonia to primary spermatocytes, to secondary spermatocytes and to spermatids, so that the seminiferous epithelium becomes several cells thick. For a given species each step of spermatogenesis has a constant duration so that the differentiation of the cells appears regulated by a rigid time scaled program. Consequently the epithelium is not a mass of independently developing cells, but is characterised by different germ cell generations organised into cell-associations of fixed composition. The kinetics of spermatogenesis in mammals has been extensively studied (see review Clermont, 1972).

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The continuous differentiation of the spermatogonia is maintained by stem cell renewal. Many spermatogonial stem cells enter spermatogenesis simultaneously, consequently large groups of germ cells develop synchronously. In birds the synchronisation of the germ cells is not as apparent as in mammals (Courot, Hochereau-de Reviere & Ortavant, 1970). In this thesis the fine structure of the seminiferous tubules is examined for features that may account for this difference.

The maintenance of spermatogenesis requires a supply of nutrients and hormones from the blood stream, and FSH from the Leydig cells. The Leydig cells, the arterial supply and venous and lymphatic drainage are all contained within the intertubular region, therefore, the nutrients and hormones must pass through the intertubular and boundary tissue of the testis in order to reach the seminiferous tubules.

The fine structure of the peritubular boundary tissue has been studied previously in this laboratory (Rothwell & Tingari, 1973 & 1974; Rothwell, 1975), and is not examined further in this thesis. The Leydig cells of the intertubular tissue have also been described previously (Rothwell, 1973), but no study concerned with the organisation of the cells of the intertubular tissue has been carried out. In this thesis the relationship between the blood vessels, lymphatic vessels, Leydig cells and seminiferous tubules is examined.

The arrangement of blood vessels within the fowl's testis has been described by Nishida (1964). The testicular artery on each side originates from the abdominal aorta as a common trunk with the anterior renal artery. The blood flows directly into the parenchyma of the testis only a short distance from the abdominal aorta and the artery branches immediately to form many arterial loops amongst the seminiferous tubules. Venous drainage runs to superficial veins that /

that unite and run directly into the posterior vena cava. The common vein is extremely short.

Measurements of testicular blood flow by means of the indicator fractionation technique indicate that the blood flow in the fowl's testis is rapid compared to <sup>that of</sup> the mammalian testis. This technique also revealed a compartment in the testis which rejects rubidium (Setchell, 1970). In mammals measurements of the relative rates of transfer of various molecules from the blood into the testicular lymph and the rete testis fluid, which is produced by the seminiferous tubules, indicated the presence of a peritubular permeability barrier (Setchell, Voglmayr & Waites, 1969).

In the rat the use of tracer molecules has demonstrated that the blood-testis permeability barrier has a morphological basis provided by the tight junctions of the peritubular boundary tissue and the apposed Sertoli cells (Dym & Fawcett, 1970).

The abdominal position of the testes of the fowl make physiological measurements of the lymph fluid and rete testis fluid impossible (Lake, personal communication). The morphological basis of the blood-testis permeability barrier of the fowl is investigated in this thesis.

Before any ultrastructural investigation of the testis of the domestic fowl could be undertaken it was felt necessary to establish a fixation procedure that gave a good and reproducible image. This thesis therefore begins with an account of the investigation into the parameters of the fixation procedure. Knowing the effects that alterations in the fixative and/or buffer system can have on the fine structure enables one to evaluate whether an observed change in ultrastructure is a function of the age and development of the bird or an effect of the fixation procedure used.

2.1 INTRODUCTION

The object of these studies was to devise a procedure that gave a good and reproducible fixation image of the fowl testis. Previous work in this laboratory had indicated that there were special difficulties involved in obtaining good fixation of the testis, due to the heterogeneous nature of this organ. There are three cytologically distinct and differently zoned tissues in the testis, namely the seminiferous epithelium, the peritubular boundary tissue and the intertubular tissue. The cells that make up these tissues have quite different constituents, e.g. in the Leydig cells of the intertubular tissue there are large amounts of lipid whereas the peritubular boundary tissue is composed of fibroblast cells and myofibroblast cells. It was often observed that fixatives that gave good fixation in any one of these three tissues gave bad results with any other (Cooksey & Rothwell, 1973; Rothwell, 1973; Rothwell & Tingari, 1974). As one object of the present ultra-structural studies was to look at the overall pattern of organization of the tissue, a fixation procedure that gave a good overall image was required, as well as ones that gave a perhaps superior fixation of defined areas of interest.

The ideal good fixation produces an image in which there is preservation of tissue with the minimum degree of visible alteration from the labile structural organisation of the living state to the stable structural organisation of the fixed state. It is well documented that this is considered best achieved by perfusion fixation (Palay, McGee-Russell, Gordon & Grillo, 1962; Karlsson & Schultz, 1965; Maunsbach, 1966a; Forssmann, Siegrist, Orci, Girardier, Pictet /

Pictet & Rouiller, 1967; Kjaerheim, 1969; Hayat, 1970).

Perfusion by way of the blood vascular system enables rapid and uniform penetration of the fixative into all parts of the tissue and avoids the distortions caused by manipulation that occur with the use of an immersion fixation technique. Artefacts such as the collapse of the seminiferous tubules and the intertubular lymphatic system, and cellular distortions caused by anoxia are also avoided by the use of perfusion fixation (Fawcett, Heidger and Leak, 1969). Standardisation of the fixation quality in all parts of the tissue further eliminates the problem of selection of material that occurs with immersion fixed blocks. Such blocks commonly exhibit gradients of fixation quality. Other methods of application of fixative to the tissue such as dripping the fixative on the exposed surface of the organ in situ (Maunsbach, Madden and Latter, 1962) or subcapsular injection of the fixative (Maunsbach, 1966a) were not considered in this study as preliminary study showed that they limit the region fixed to surface areas.

Immersion fixation was used initially to study different fixative and buffer systems under various conditions of osmolality, time and temperature. This saved both time and money, one bird being used for the study of many fixing agents under a variety of conditions using only small volumes of solutions. The results obtained from these immersion fixation experiments were then used as a basis for the selection of fixatives for the perfusion fixation experiments.

Hayat (1970) stated that the aims of fixation were the rapid preservation of structure with the minimum alteration from the living state, and the protection from disruption during any subsequent preparation and presentation procedures. A negative definition /

definition of fixation was given by Baker (1960) when he suggested that tissue fixation was the prevention of attack by bacteria, of autolysis, and of changes in shape and volume. Hopwood (1969a) added that a definition of fixation should include no loss of tissue constituents.

In many ways fixation can be thought of as protein denaturation, i.e. a stabilization of the proteins resulting in a loss of many of their properties. Chemical changes that occur during fixation are essentially a separation of liquid from solid phase and a chemical reaction of the fixative with the cell substance especially proteins and lipids. Free water is removed during dehydration and as many of the chemical bonds in living tissue depend on the presence of water for their stability, the fixative must supply stable bonds to hold the molecules thus preventing their translocation or extraction during processing procedures.

The first widely used fixative for tissue preservation at the electron microscope level was osmium tetroxide (Palade, 1952). It is a strong oxidising agent that reacts with unsaturated bonds to form addition compounds. The main properties of osmium tetroxide in reaction with tissue constituents (see review, Hayat 1970) are (a) the stabilization of protein sols by cross-linkage, a reaction that is dependent on the content of the amino acids cysteine, methionine, histidine and tryptophan, (b) good preservation of lipid by formation of addition compounds, (c) good preservation of phospholipoprotein (cell membrane) and nucleoprotein, (d) the ability to impart good electron contrast to the tissue. The disadvantages of using osmium tetroxide are the poor preservation of carbohydrates and nucleic acids, and the very slow poor penetration of the fixative into the tissue.

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The use of aldehydes as fixatives was developed by Sabatini, Bensch and Barrnett (1963). Formaldehyde was already in use as a light microscope fixative and Luft in 1959 introduced acrolein as a fixative for the electron microscope, but it was Sabatini et al who demonstrated the usefulness of the aldehydes as a group for electron microscopical fixation and in particular for electron histochemical work.

The main properties of the aldehydes in reaction with tissue constituents (see review, Hayat, 1970) are (a) stabilization of protein sols by formation of inter- and intramolecular cross-links involving condensation reactions between proteins and fixative, and proteins and proteins, (b) good preservation of macromolecular carbohydrates, (c) good preservation of nucleoproteins, (d) good penetration into the tissue. The disadvantages of using aldehydes are the poor preservation of lipid and the lack of electron contrast imparted to the tissue.

When the properties of osmium tetroxide and the aldehydes are compared, it can be seen that <sup>what</sup> is poorly preserved by one fixative is well fixed by the other. Hence the use of a double fixation of aldehyde followed by osmium tetroxide stabilises the maximum number of molecules and is therefore the most effective in reducing loss of cell constituents. Of the aldehydes, glutaraldehyde, is the most effective in preserving fine structure and causes the least protein conformational changes. Consequently a double fixation of glutaraldehyde and osmium tetroxide has been used more than any other fixative system in the present work.

It is well documented that different fixation and processing regimes can produce different fine structural images of the same tissue /

tissue. Smooth endoplasmic reticulum, when fixed with osmium tetroxide, is seen as discontinuous empty-looking smooth surfaced vesicles whereas, when fixed with glutaraldehyde it is seen as an interconnecting narrow tubular network. In toad spinal ganglia osmium tetroxide fixation causes the apposite external cell membranes to break down into chains of vesicles or tubules whereas they remain intact with permanganate fixation (Rosenbluth, 1963). Formaldehyde causes apical protrusions of the plasma membrane of the microvilli in the proximal convoluted tubule of the rat (Trump and Ericsson, 1965). It has been demonstrated that variation in the lattice spacing of rat striated muscle is dependent on whether the tissue was fixed with glutaraldehyde or with osmium tetroxide (Landon, 1970). Because different fixatives can cause alterations in the ultrastructure of a particular tissue, several fixatives have been employed in this study as a check of one fixative with another with respect to image quality and as a check of the validity of observations i.e. that changes noted with age and development were associated with those parameters and not with fixation.

It is known that as well as the method of applying the fixative many other factors affect the quality of fixation e.g. pH, total ionic strength, specific ionic composition, dielectric constant, osmolality, temperature and length of time of fixation (Hayat, 1970). Of these factors specific ionic composition, osmolality, temperature and length of time of fixation were selected for study with different fixatives. In this study the effect of pH on the quality of fixation has not been examined. However, being conscious of the changes that pH can have on the fixation parameters chosen (Maser, Powell and Philpott, 1967) every care was taken to restrict pH to exactly the right /

right range for the particular buffer system employed.

### Specific ionic composition (buffer system)

Palade (1952) using osmium tetroxide was the first to note that acidification of the tissue precedes fixation causing vacuolation of the ground substance and precipitation of the nuclear material and hence the use of buffers was advocated. The acidification is the result of the irreversible dissociation of the protein macromolecules releasing ionisable carboxyl groups. The aldehydes do not exhibit such a strong acidification because they mainly cross-link proteins, and therefore the effect of specific ionic composition of buffers on tissue is less critical when aldehyde fixatives are used. The alteration in fine structure as a result of using different buffer systems with different fixatives has been studied by several workers (Luft and Wood, 1963; Wood and Luft, 1965; Trump and Ericsson, 1965; Ericsson, Saldino and Trump, 1965; Maunsbach, 1966b; Gil and Weibel, 1968). These workers were studying the differences in fine structure of a particular cell type e.g. rat kidney proximal tubule cells (Maunsbach, 1966b) or a cell organelle e.g. the electron dense granules on the alveolar surface of rat lung (Gil and Weibel, 1968). In the present study greater attention has been focused on the effect different fixative buffer systems have on the overall fixation of the different tissues of the testis rather than on cellular fine structural details.

### Osmolality

Besides maintaining the correct pH the buffer solution has another function in that it increases the tonicity / osmolarity / osmolality of the fixative solution. The terms tonicity, osmolarity and osmolality tend to be used synonymously in the literature, though they have slightly different meanings.

Tonicity /



Tonicity is a term used to describe the ionic strength of a solution as evinced by the response of cells immersed in it. A living cell can be considered as a collection of various solutes ranging from small ion species to large molecules surrounded by a semi-permeable membrane. When the external medium is altered, as in fixation, the cell as a whole becomes subject to osmotic stress. The internal medium (the cell) and the external medium (the fixative solution) are isotonic if the cell neither shrinks nor swells, when immersed in the fixative solution. An isotonic solution is not necessarily the same as an isosmotic solution. Two solutions are isosmotic with each other if they both exert the same osmotic pressure. The osmotic pressure refers to pressures arising as a result of concentration differences across the cell membrane. A concentration difference of 1 mOsm will generate a pressure of 17 mm Hg. It is the particle concentration rather than the molecular concentration which is important in determining osmotic pressures, hence the use of the terms osmolarity and osmolality. A synonym for these terms is osmotic concentration. In this work the osmotic concentration has been determined by means of freezing point depression as recommended by Tahmisian (1964). This is a method of measuring osmotic concentration that uses molal solutions and therefore the term osmolality is used in preference to osmolarity to express osmotic concentration in this thesis. This is in agreement with Fahimi and Drochmans (1965), Maunsbach (1966b) and Maser, Powell and Philpott (1967). For dilute aqueous solutions the molality, the number of moles of solute per 1 Kg of solvent and the molarity, the number of moles of solute per litre of solution are approximately equal. Concentrations of fixative solutions are therefore defined in terms of molarity as is the normal practice in the literature.

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There is disagreement in the literature as reviewed by Hayat (1970) and Bone and Ryan (1972) about whether fixative solutions yield the best results when made isotonic with the body fluids. For glutaraldehyde fixatives some workers consider that the total osmolality of the fixative and buffer should be equivalent to the body fluids i.e. the buffer osmolality is lower than the body fluids. This can be achieved by altering the concentration of the fixative and buffer or by addition of electrolytes or non-electrolytes e.g. NaCl or sucrose (Busson-Mabillot, 1971). Other workers make the fixative vehicle isosmotic with the body fluids e.g. Tilney & Goddard (1970) used sea-water as buffer for the fixation of Arbacia eggs ; Gil and Weibel added NaCl to various buffers for fixation of rat lung. A large number of workers favour a slightly hypertonic fixing solution in which the fixative vehicle is hypotonic to body fluids (Fahimi & Drochmans, 1965; Schultz & Karlsson, 1965; Maunsbach, 1966b; Bone & Denton, 1971). For osmium tetroxide fixation the osmolality appears not to be as significant because the cell membrane becomes freely permeable to small ions and molecules (Wood & Luft, 1965; Elbers, 1966) consequently some workers have obtained reasonable results using distilled water (Malhotra, 1962; Chuang, 1968; Busson-Mabillot, 1971).

After glutaraldehyde fixation the tissue remains quite labile and susceptible to change (Schultz & Karlsson, 1965). It is therefore important that the osmolality of the buffer wash solution is also considered. Fahimi & Drochmans (1965) obtained the best results with moderately hypertonic solutions with or without sucrose. If a buffer is used with the osmium post-fix then optimum results are obtained using the same osmolality as for the initial fixing /

fixing solution and buffer wash (Busson-Mabillot, 1971).

7 Maunsbach (1966b) remarked that certain observations made on one tissue may not be applicable to other tissues as well, so in the present study the effect of changing the osmolality has been examined using glutaraldehyde with the buffers Millonig's phosphate (Millonig, 1961), s-collidine (Bennett & Luft, 1959) and sodium cacodylate (Gormori, 1955).

#### Temperature and Length of Time of Fixation

The temperature and length of time of fixation are two factors that are closely linked. A low temperature decreases the diffusion rate of the fixative into the tissue and hence a longer fixation time should be required. However low temperature also slows down autolysis and the extraction of cell material, so that a longer fixation time increases the extraction of material. The rate of penetration of different fixatives has been reviewed by Hopwood (1969<sup>a</sup>) and it has been shown that the diffusibility constant for glutaraldehyde increases with increase in temperature. Higher temperatures also increases the speed of reaction between the fixative and the cell substance and this compensates for the increased rate of extraction. The correct balance for these two factors must be found for each fixative with a particular tissue. In this work tissue has been processed either at 4°C or at room temperature throughout the procedure without a change in temperature. Ito (1961) and Rothwell (personal communication) reported that changes even during dehydration can affect the fine structure.

#### The Choice of Fixatives and Buffer Systems

The choice of fixatives and buffer systems for this study was determined partly on the basis of what other workers had used for testis fixation and partly on the basis of knowledge gained in this /

this laboratory working with avian tissues. It is very easy when starting to work on the chicken to think of it as a mammal probably because it is warm blooded, and to forget it is a bird. A great deal of scientific research is carried out on mammals and a lot of the literature quoted in this thesis deals with this group, particularly in this section on fixation. A favourite tissue for fixation experiments is the kidney proximal tubule (Wood & Luft, 1965; Maunsbach 1966b). The fixation of this tissue has also been studied in detail in this laboratory (Rothwell, 1974; Rothwell, 1975.). The knowledge gained about the differences in fixation properties between mammalian and avian tissue has provided a useful starting point for the setting up of fixative and buffer systems to be used for avian testis rather than for mammalian testis.

Some early work on the ultrastructure of chicken spermatids (Nagano, 1962) used either s-collidine buffered osmium fixation or an acrolein double fixation. Later workers used a cacodylate buffered glutaraldehyde primary fix followed by a phosphate buffered osmium post-fix (McIntosh & Porter, 1967). In this laboratory a Millonig's phosphate buffered glutaraldehyde double fixation or a modified Karnovsky (4% pHCHO, 1% GTA) were standard fixatives (Cooksey & Rothwell, 1973). A modified Dalton's osmium fixative (3% potassium permanganate, 2.6% sodium chloride), developed from fixation studies on the avian kidney had also been found useful for the testis. For these working on mammalian testis a glutaraldehyde double fixation is the most common fixation procedure buffered with either phosphate ( $PO_4$ ) (Black & Christensen, 1969; Gordon, 1972a), cacodylate (Nagano, 1966; Nicander, 1967; Gordon, 1972b) or s-collidine (Nagano, 1966; Fawcett & Philips, 1970; Dym, 1973). A modification of Bouin's fluid (PAF) for ejaculated spermatozoa has also been /

been tried on mammalian testis (Fawcett & Philips, 1970). In this thesis these fixatives have been tried on avian testis and the results compared. The effect of different buffer systems has been assessed first using those buffers mentioned above and in addition Palade's veronal buffer for osmium tetroxide (1952), which Bawa (1963) used for human testis fixation.

#### Age of bird

From hatch to sexual maturity the testis of the fowl undergoes alterations in size, structure and physiological function. It changes from a firm compact tissue of which 65% of the volume is composed of lumenless seminiferous tubules and the remainder composed of tightly packed polygonal steroid-secreting cells, to a soft tissue of which 95% of the volume is composed of large open seminiferous tubules producing large quantities of spermatozoa in secretions of rete testis fluid. The remaining 5% consists of steroid-secreting cells in a loose connective tissue with abundant interstitial fluid. Previous work had already indicated that this organ required different fixatives at different stages of development. In this work birds of 1, 9 and 21 weeks of age were thus selected for immersion fixation trials. These ages are representative of prepubertal, pubertal and adult testicular development respectively.

For perfusion fixation trials birds of 14-17 weeks of age were selected as this age group provides reasonable sized birds between 1.5-2 Kg in weight for learning the surgical techniques and reliable methods of anaesthesia. These skills could then be applied to birds of all ages.

## 2.2 MATERIALS AND METHODS

### 2.2.1 IMMERSION FIXATION

#### General Procedure

Male fowl of a light-weight laying strain (Shaver) of approximately 1, 9 and 21 weeks of age were killed by a lethal dose of pentobarbitone sodium (Nembutal, Abbott Laboratories). Their left testes were excised, cut into  $1.0\text{mm}^3$  blocks and placed in one of the experimental fixatives (Table 2.1). Each fixative had been adjusted to pH 7.3 - 7.4 and its osmolality measured by means freezing point depression using an Osmette S. The tissue was subsequently washed in the corresponding buffer (Table 2.1) for  $3 \times \frac{1}{2}$  hour, post-fixed in buffered 1% osmium tetroxide (Table 2.1) for 1 hour, dehydrated in a series of graded ethanols and embedded in Araldite (Luft, 1961) or Epon (Luft, 1961). Silver/silver grey sections were cut on an LKB Ultratome III, mounted on copper grids, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined in a Philips 300 electron microscope operating at 60 KV.

#### Experimental Factors Investigated

##### 1. Buffer Systems

Two main experimental series were carried out to investigate the effects of different buffers using two fixation procedures. In series A testis was fixed by osmium tetroxide ( $\text{OsO}_4$ ) buffered with either Palade's veronal acetate, Millonig's phosphate, s-collidine or modified Dalton's buffer (Table 2.2) for 1 hour at  $4^\circ\text{C}$ . For series B buffers were chosen on the basis of the results of series A. Testis was fixed at  $4^\circ\text{C}$  or at room temperature using a double fixation of glutaraldehyde (GTA) and osmium tetroxide buffered with either/

Table 2.1 Fixative/buffer systems for Immersion Fixation Trials

<u>Initial Fixative</u>	<u>Buffer Wash</u>	<u>Post-fix buffer</u>
1. OsO <sub>4</sub> /Millonig's PO <sub>4</sub>	-	-
2. OsO <sub>4</sub> /s-collidine	-	-
3. OsO <sub>4</sub> /modified Dalton's buffer	-	-
4. OsO <sub>4</sub> /Palade's buffer	-	-
5. GTA/Millonig's PO <sub>4</sub>	0.2M Millonig's PO <sub>4</sub>	0.1M Millonig's PO <sub>4</sub>
6. GTA/s-collidine	0.2M s-collidine	0.1M s-collidine
7. GTA/modified Dalton's buffer	modified Dalton's buffer	modified Dalton's buffer
8. GTA/Na cacodylate	0.075M Na cacodylate/ 0.2M sucrose	0.175M Na cacodylate 0.1M PO <sub>4</sub> modified Dalton's 1.5% K <sub>4</sub> Fe(CN) <sub>6</sub>
9. acrolein/PO <sub>4</sub>	0.2M PO <sub>4</sub>	0.1M PO <sub>4</sub>
10. GTA/acrolein/PO <sub>4</sub>	0.2M PO <sub>4</sub>	0.1M PO <sub>4</sub>
11. GTA/pHCHO/Millonig's PO <sub>4</sub>	0.2M Millonig's PO <sub>4</sub>	0.1M Millonig's PO <sub>4</sub>
12. Picric acid/pHCHO/ PO <sub>4</sub> (PAF)	PO <sub>4</sub> (PAF)	PO <sub>4</sub> (PAF)

Blocks of testis were fixed in each of the initial fixatives, washed in the corresponding buffer and post-fixed in buffered 1% osmium tetroxide. The concentrations and molarities used are detailed in Tables 2.2 and 2.3.

References for buffer source

- modified Dalton's - Rothwell (1973)  
 Millonig's PO<sub>4</sub> - Millonig (1961)  
 PO<sub>4</sub> - Mercer & Birbeck (1966)  
 PO<sub>4</sub>(PAF) - Stefanini et al (1967)  
 s-collidine - Bennett & Luft (1959)  
 Na cacodylate - Mercer & Birbeck (1966)

either Millonig's phosphate, s-collidine, or modified Dalton's buffer. Sodium cacodylate buffer was also used (Table 2.2).

## 2. Fixatives

A series of experiments was carried out in which testis was processed at room temperature using several different fixatives or fixative combinations. (Table 2.3)

## 3. Osmolality

A series of experiments were carried out to investigate the effects of differing the osmolalities of the fixatives by altering the concentrations of fixative and buffer. A double fixation of glutaraldehyde and osmium tetroxide was used with three buffers, Millonig's phosphate (Table 2.4), s-collidine (Table 2.5) and sodium cacodylate (Table 2.6). Different combinations of concentration of glutaraldehyde and molarity of buffer were made up to give osmolalities that covered the range of 330 mOsm (the osmolality of the fowl's plasma) to 440 mOsm (the osmolality of the fowl's semen) and higher to 832 mOsm. The buffer wash was made equiosmolal with the plasma by altering the molarity of the buffer for all three buffers and by addition of sucrose for sodium cacodylate buffer. For the post-fixative the buffer was used at the same molarity as the initial fixative except for 0.075M cacodylate fixatives when a plasma equiosmolal buffer or a phosphate buffer were used.

## 4. Temperature and Time

The effect of processing tissue at either 4°C or room temperature for various lengths of time was investigated at the same time as the effect of osmolality on the GTA/Millonig's phosphate buffer fixation. Testis fixed in all combinations of fixative/buffer concentration was processed at either 4°C or room temperature for 1, 3 or 24 hours (Table 2.4) and likewise for the GTA/s-collidine fixation for



Table 2.2 Buffer Systems used for ultrastructural comparison

<u>Series A</u>		
	<u>Single Fixation</u>	<u>Time</u>
1%	OsO <sub>4</sub> / Palade's veronal acetate	1 hour
"	/ 0.1M Millonig's phosphate	"
"	/ 0.1M s-collidine	"
"	/ modified Dalton's buffer	"

Testis blocks were fixed by immersion in one of the above fixatives.

<u>Series B</u>		
	<u>Double Fixation-Initial Fixative</u>	<u>Time</u>
2.5%	GTA/ 0.1M Millonig's phosphate	3 hours
4%	GTA/ 0.1M s-collidine	1 hour
4%	GTA/ 0.075M Na cacodylate	2 hours
2.5%	GTA/ modified Dalton's buffer	1 hour

Testis blocks were fixed by immersion in one of the above fixatives followed by the corresponding buffer wash and post-fixative (see Table 2.1). A cacodylate post-fix was used for the GTA/cacodylate fixative.

Table 2.3 Fixatives used for Immersion Fixation Trials

	<u>Fixative</u>	<u>Time</u>
1.	1% OsO <sub>4</sub> /0.1M Millonig's phosphate	1 hour
2.	2.5% GTA/0.1M Millonig's phosphate	3 hours
3.	5% acrolein/0.1M phosphate	1 hour
4.	6% GTA/2% acrolein/0.1M phosphate	1 "
5.	4% GTA/1% pHCHO/0.1M Millonig's phosphate	3 hours
6.	Saturated sol <sup>n</sup> picric acid/2% pHCHO/PO <sub>4</sub> (PAF)	1 hour

Testis was fixed by immersion in one of the above fixatives, followed by the corresponding buffer wash and post-fixative for fixatives 2 - 6.

Table 2.4 Osmolality of GTA/Millonig's Phosphate Buffer in milliosmols

<u>GTA conc</u>	<u>Molarity of Millonig's PO<sub>4</sub></u>				
	0.3M	0.2M	0.15M	0.1M	0.05M
5%	-	-	-	853	612
4%	-	-	-	707	531
3%	-	832	720	616	-
2.5%	-	-	680	552	-
2%	-	710	550	515	-
1%	726	615	442	-	-

Table 2.5 Osmolality of GTA/s-collidine buffer in milliosmols

<u>GTA conc</u>	<u>Molarity of s-collidine</u>	
	0.2M	0.1M
5%	-	672
4%	-	598
3%	600	504
2.5%	-	448
2%	492	370
1%	366	249

Table 2.6 Osmolality of GTA/sodium cacodylate buffer in milliosmols

<u>GTA conc</u>	<u>Molarity of sodium cacodylate</u>		
	0.15M	0.1M	0.075M
4%	-	676	560
3%	-	558	510
2.5%	600	-	432

1 or 3 hours (Table 2.5).

5. Post-fixative buffer system

Testis was fixed at room temperature with 4% GTA/0.075M sodium cacodylate, washed with 0.075M cacodylate/0.2M sucrose and post-fixed with 1% osmium tetroxide buffered with either 0.175M sodium cacodylate, 0.1M phosphate buffer, modified Dalton's buffer or 1.5% potassium ferrocyanide.

2.2.2 PERFUSION FIXATION

Surgical Procedures

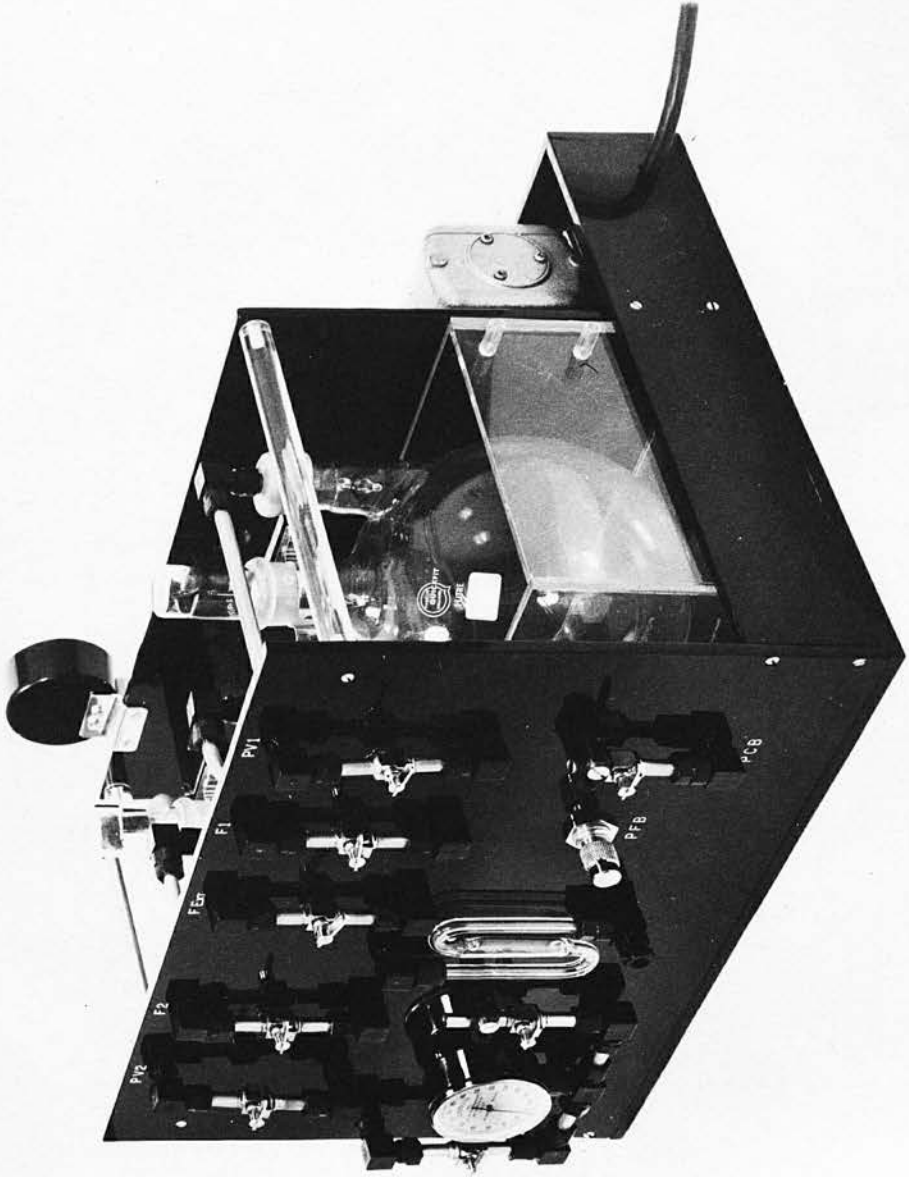
Experimental procedures were standardised around 9 - 12 and 15 - 17 week old male Shavers. The bird was held firmly with its ventral surface uppermost and lightly anaesthetised by slow intravenous injection of sodium pentobarbitone (Nembutal, Abbott Laboratories) into the alar (wing) vein. A bird was judged to be anaesthetised when it made no response to pinches of the comb. No artificial respiration was used. A transverse incision was made in the abdominal wall anterior to the vent and was carried dorso-laterally round through the rib-cage on either side, stopping immediately prior to the brachial arteries. The breast was pulled back exposing the heart and systemic arch. The viscera were moved aside to the right exposing the left testis and abdominal blood vessels.

Perfusion Fixation using a perfusion apparatus

A perfusion apparatus previously described by Rothwell, Burns and Ralph (1973) was used to provide a controlled pressure of flow of perfusate through a hypodermic needle.

Five routes of administration of perfusate were used (Diagram 2.1)

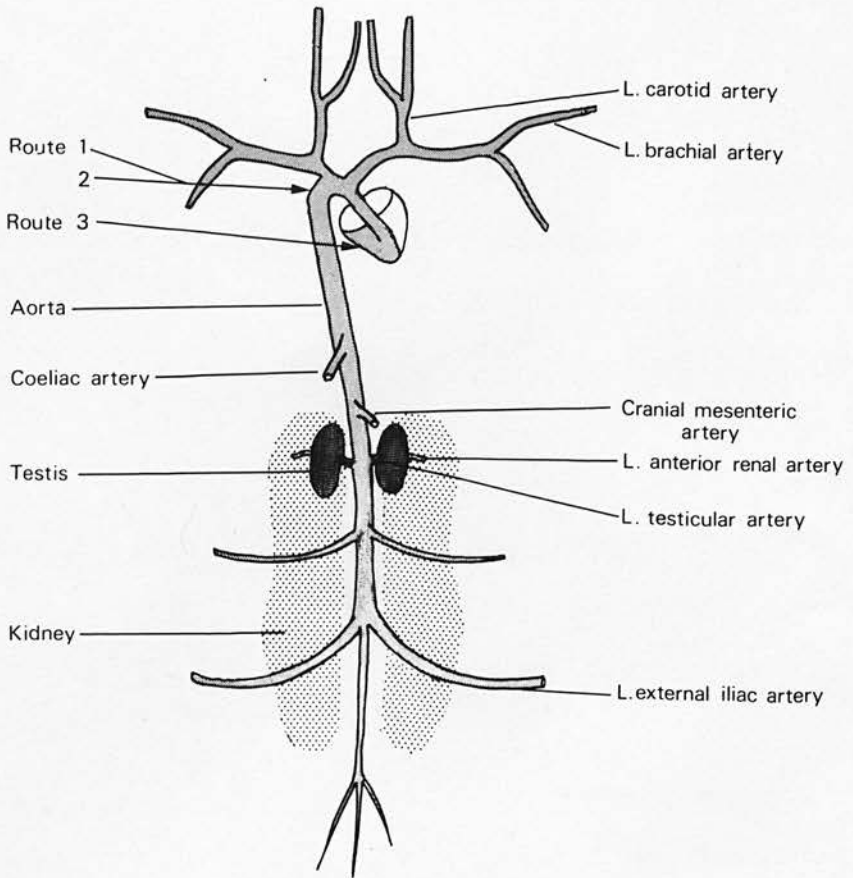
1. /



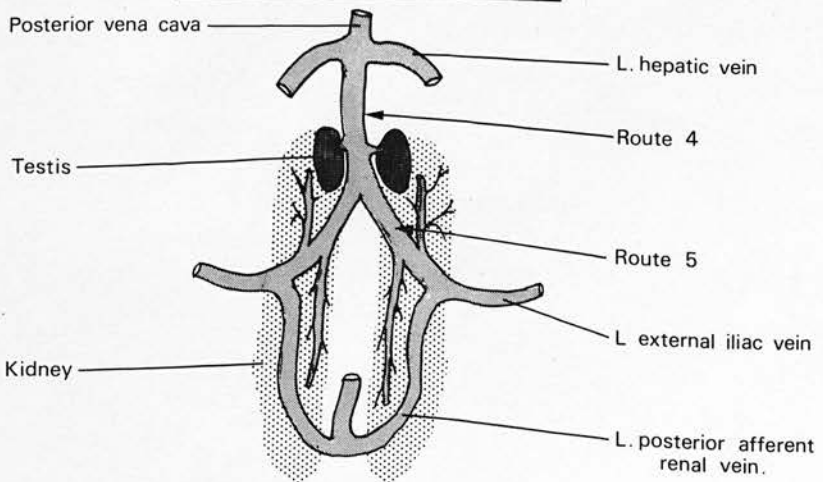
PERFUSION APPARATUS

Fig. 2.1

ARTERIAL BLOOD VASCULAR SYSTEM



VENOUS BLOOD VASCULAR SYSTEM



1. Direct perfusion into the descending aorta. The needle was bent to match the curve in the aorta and the stopcocks opened to allow perfusate to flow before insertion. This avoided infusion of air bubbles. The needle was inserted into the aorta and clamped in position. Free flow was affected by cutting the external iliac veins outside the body cavity or the right auricle. Clamps were placed on the carotid arteries, coeliac and cranial mesenteric arteries.

2. As in (1) above but without clamps.

3. Direct perfusion into the right ventricle. The needle was inserted into the right ventricle, clamped in position and the arteries clamped off as in (1) above. Free flow was affected by cutting the external iliac veins.

4. Direct perfusion into the posterior vena cava anterior to the testicular vein bifurcation. The needle was inserted into the posterior vena cava, clamped in position and the aorta clamped anterior to the testicular artery bifurcation. Free flow was affected by cutting the aorta below the bifurcation.

5. Direct perfusion into the left iliac vein anterior to the renal portal valve. The needle was inserted into the left iliac vein and clamped into position. Clamps were applied to the right iliac vein and posterior vena cava above the bifurcation and free flow affected by cutting the aorta anterior to the testicular artery bifurcation.

A series of perfusions were carried out using 150-200ml of one of the initial fixatives (Table 2.7, Part I) as perfusate. A 24G needle was used with a pressure of 250mm Hg for arterial perfusions (routes 1-3) and 150mm Hg for venous perfusion (routes 4-5), which gave flow rates of 6.5ml/min and 4.5ml/min respectively (Table 2.9).

1.5mm<sup>3</sup> blocks /

Table 2.7 Fixing Solutions used for perfusion fixation

<u>Perfusate and Initial Fixative</u>	<u>Time of Intial Fixation</u>
<u>Part I For 7-21 week old birds</u>	
1. 2.5% GTA/0.1M Millonig's PO <sub>4</sub>	24 hours
2. 4% GTA/0.075M Nacacodylate	2 "
3. 4% GTA/0.1M s-collidine	1 hour
<u>Part II For 1-6 week old birds</u>	
1. 2.5% GTA/0.1M Millonig's PO <sub>4</sub>	24 hours
2. 2.5% GTA/0.075M Na cacodylate	2 "
3. 4% GTA/0.075M Na cacodylate	2 "
4. 4% GTA/0.1M s-collidine	1 hour
5. 2% acrolein/2% GTA/0.1M PO <sub>4</sub>	1 "
6. 1% OsO <sub>4</sub> /modified Dalton's buffer	1 "

150mls-200mls of the above fixatives were perfused into the vascular system of the bird. The left testis was excised and small blocks placed in the same fixing solution used for perfusion. Following initial fixation blocks were washed and post-fixed in the corresponding buffer and fixative (Table 2.1).

Table 2.8 Prewash Solutions Investigated

<u>Prewash</u>	<u>Osmolality</u>	<u>Fixation Procedure</u>
1% saline	320mOsm	4% GTA/0.075M Na cacodylate
1.4% saline	434 "	0.075M Na cacodylate/0.2M sucrose
avian Ringer	310 "	1% OsO <sub>4</sub> /0.075M Na cacodylate
0.075M Na cacodylate	140 "	

100mls of prewash were perfused into the vascular system of the bird followed by 200mls of the above fixing solution. Blocks of excised testis were fixed in the fixing solution for 2 hours followed by the corresponding buffer wash and post-fixative (Table 2.1).

1.5mm<sup>3</sup> blocks of tissue were taken from the left testis on completion of the perfusion, fixed for 1, 2, 3 or 24 hours in the initial fixative, washed and osmicated in the corresponding buffers and fixatives (Table 2.7). The tissue was processed and examined as described for immersion fixed material.

#### Prewash Requirements

A series of perfusions were carried out using 4% GTA/0.075M sodium cacodylate ( 2, Table 2.7) with a variety of prewashes (Table 2.8) at room temperature. Saline solutions were made up to give osmolalities that were similar to that of the fowl's plasma and the fowl's semen. Other prewashes were avian Ringer and cacodylate buffer with the same molarity as that used with the fixative. 100ml of prewash were perfused through the birds followed by 200 ml of fixative/buffer. Tissue was then taken and processed in a similar manner to the above ( 2, Table 2.7).

#### Needle size / Pressure / Volume / Time Variables

Two series of perfusions were carried out using 4% GTA/0.075M sodium cacodylate as the perfusate with no prewash and arterial perfusion into the aorta with clamps (route 1). The needle size and pressure were varied and in one series a constant volume of 150ml of perfusate was used, and in the other series a constant perfusion time of 20 minutes was used (Table 2.9).

#### Perfusion fixation for 7 - 21 week old birds

A series of perfusions were carried out with the perfusion apparatus on 9, 12, 15, 18 and 21 week old male Shavers by the arterial perfusion route into the aorta with and without clamps (routes 1 and 2) using 21G and 23G needles at 150mm Hg and 200mm Hg pressure respectively. 200ml of initial fixative (Part I Table 2.8) at room temperature was used as perfusate for each experiment. No prewash was used.

#### Perfusion /



Table 2.9 Flow Rates for Perfusion Apparatus

<u>Needle Size</u>	<u>Pressure mmHg</u>	<u>Flow rate mls/min</u>
24G	150	4.5
"	200	5.3
"	250	6.5
23G	200	11.5
"	250	13
21G	150	27
"	200	33.7
"	250	35

<u>Series I Perfusions</u>		<u>Fixed Volume - 150mls</u>
<u>Needle size</u>	<u>Pressure mmHg</u>	<u>Time mins</u>
24G	150	34
"	200	28
"	250	23
23G	200	13
"	250	11.5
21G	180	5.5

<u>Series II Perfusions</u>		<u>Fixed Time - 20 minutes</u>
<u>Needle size</u>	<u>Pressure mmHg</u>	<u>Volume mls.</u>
24G	150	90
"	200	106
"	250	130
23G	200	230
"	250	260
21G	150	540

Perfusion fixation for 1 - 6 week old birds

A series of perfusions were carried out with the perfusion apparatus on 1 - 6 week old male Shavers by arterial perfusion into the aorta or ventricle without clamps (routes 2 and 3) using a 23G needle at 200mm Hg pressure. 50ml of initial fixative (Part II, Table 2.8) at room temperature were used for each bird. No prewash was used.

Another series of perfusions were carried out using a 20ml syringe with a 23G needle. 40ml of initial fixative (Part II, Table 2.8) were perfused at hand pressure into the right ventricle.

## 2.3 RESULTS

The following parameters were used for assessment of fixation quality.

1. The integrity of the general tissue, i.e. no marked separation between the intertubular tissue and the seminiferous tubules and boundary tissue.
2. The integrity of the individual tissues, i.e. no marked cell separation or no loss of cellular outline in any one tissue.
3. Good general preservation of cellular organelles especially mitochondria, endoplasmic reticulum ( ER ), nuclear membrane interspace and cell membrane.
4. General image quality, i.e. the amount of cytoplasmic leaching.

For perfusion fixation two other parameters were also considered.

1. The patency of the seminiferous tubule.
2. Preservation of open blood capillaries, lymph vessels and extravascular spaces.

### 2.3.1 IMMERSION FIXATION

The results of varying the buffer system, fixative, and osmolality are described in detail for 9 week old birds first. At this stage the testis has approximately three quarters seminiferous tubule to a quarter intertubular tissue, which means it is a fairly firm compact organ (at this stage) and is able to be better preserved over a wider range of conditions. Alteration of the fixing solution to produce good fixation of 21 week old birds (when the proportion of seminiferous epithelium is higher and the tissue much softer with the presence of semen) and 1 week old birds (when the proportion of Leydig /

Leydig cells is greater) are described later. In general the intertubular tissue was found to be the most difficult part of the testis to fix well, but, as the fine structure of Leydig cells is not examined in this thesis, good fixation of the intertubular tissue refers to the preservation of the relationship between Leydig cells, lymph spaces, blood vessels and boundary tissue. Most of the observations at the subcellular level are confined to the germ cells and Sertoli cells whose ultrastructure is examined in more detail later.

### Buffer System

A good general preservation of all tissues is obtained with series A osmium tetroxide fixatives. Figures 2.1 to 2.10 are representative micrographs of the fixation effects of the four buffers used. The relationship between tubular and intertubular tissue is well preserved by s-collidine and Millonig's phosphate buffers (fig. 2.1 & 2.2), but there is a tendency for the intertubular tissue to separate from the boundary tissue for modified Dalton's and Palade's fixed material (fig. 2.3 & 2.4). These last two buffers are not particularly good for Leydig cells, producing a hypotonic image of swollen mitochondria and vesiculated ER (fig. 2.5 & 2.6). Intercellular material is well preserved with the Millonig's phosphate and s-collidine buffers (fig. 2.1 & 2.2).

S-collidine buffered fixed tissues differ most markedly from the other three in that here the cytoplasm and nucleoplasm has a generally leached appearance (fig. 2.2) and the mitochondria have a pale matrix (fig. 2.7). This leached appearance means that the cell membranes and mitochondrial cristae are well defined, clear and distinct. For the other buffers the mitochondrial matrix is of medium /

medium density (fig. 2.8, 2.9 & 2.10) with the exception of mitochondrial matrix of modified Dalton's fixed Sertoli cells, which is very dense (fig. 2.3). Mitochondrial granules are present with all buffers. Cell plasma membranes are best preserved by Millonig's (fig. 2.8) and modified Dalton's buffer (fig. 2.9). Tissue fixed with Palade's buffer has indistinct and broken membranes (fig. 2.10).

Other cell organelles also differ somewhat from buffer to buffer. Endoplasmic reticulum (ER) tends to be vesicular rather than tubular for all buffers, though longer profiles are observed with Millonig's phosphate buffer (fig. 2.8). This and modified Dalton's are also good for the preservation of rough ER (fig. 2.8 & 2.9). With the other buffers the ribosomes are more often observed loose in the cytoplasm (fig. 2.7 & 2.10) and in the case of s-collidine buffer, aggregated in clumps (fig. 2.7). Lysosomes vary from buffer to buffer in their matrix density and clarity of the limiting membrane. Millonig's, modified Dalton's and Palade's buffers give a dark matrix with distinct limiting membranes (fig. 2.9 & 2.10) whereas s-collidine lysosomes have a pale matrix but distinct membranes (fig. 2.7).

Chromatin is generally evenly dispersed except for s-collidine nuclei, which show coarse peri-nuclear clumping of the chromatin (fig. 2.7). Interchromatin granules were most prominent in Palade's fixed nuclei (fig. 2.6).

In series B, which used a double fixation of glutaraldehyde and osmium tetroxide, good overall preservation of the three tissues is obtained for all buffers except the modified Dalton's. This buffer causes a hypertonic image of the tissue to be obtained, all cells /

cells having dense mitochondria and many cells being dark and contracted in appearance with peri-nuclear vacuoles (fig. 2.11).

Variation in the fixation image for the other three buffers is less marked than with series A. S-collidine again gives a pale leached appearance (fig. 2.12) with patches of cell membrane and cytoplasm missing (fig. 2.13). The mitochondria are, however, well preserved with a medium dense matrix and clear cristae. This buffer is excellent for preservation of the extracellular material of the boundary tissue and gives good contrast to the collagen (fig. 2.13).

Millonig's phosphate buffer also gives good overall preservation (fig. 2.14) though the cytoplasm of the Leydig cells tends to give a leached stretchy appearance (fig. 2.15). Larger amounts of smooth ER are preserved and the ground substance of the cytoplasm is darker than with the other buffers (fig. 2.16). Good preservation of the testis is obtained with sodium cacodylate buffer (fig. 2.17). There is good clarity of the cellular membranes including those of the mitochondria and the Golgi bodies (fig. 2.18 & 2.43) the only exceptions being those of the ER, which are indistinct (fig. 2.18).

### Fixatives

In order to examine the effect of six different fixatives (Table 2.3) on the fine structure of the testis it is easiest to divide them into groups for comparison. Of the six five were used in a double fixation schedule involving at least one aldehyde as a primary fixative followed by a post fixation with osmium tetroxide, whereas the sixth involved a primary fixation with osmium tetroxide alone. The most notable difference between osmium fixed tissue (fig. 2.19) and aldehyde/osmium fixed tissue (fig. 2.20) is the fine structure of the ER. In osmium fixed tissue the rough ER tended to be /

be vesicular and no lengths greater than 0.5 $\mu$  can be seen (fig. 2.21). In aldehyde fixed tissue the RER is tubular and 1.5 $\mu$  lengths can be common (fig. 2.22). The preservation of cell membranes is better with glutaraldehyde and microtubules are not preserved by osmium tetroxide alone. The distribution of chromatin differs being evenly dispersed in the nucleus with osmium (fig. 2.21) and clumped along the nuclear membrane with glutaraldehyde (fig. 2.22). Myelin whorls are observed in glutaraldehyde fixed tissue. This was also found to be the case in any other aldehyde fixed tissue.

Of the aldehydes used for double fixation glutaraldehyde and acrolein are used singly and in combination with each other. Differences in structure between glutaraldehyde fixed material (fig. 2.20) and acrolein fixed material (fig. 2.23) are slight, more a question of the quality of preservation than real differences in ultrastructure. Acrolein fixed testis has a leached appearance particularly in the intertubular tissue where parts of the cytoplasm are completely missing (fig. 2.23). This is also observed in the tubule cells (fig. 2.24). ER tends to be more vesicular and single ribosomes rather than polysomes are observed in testis fixed with acrolein. Microtubules are not preserved by acrolein but the preservation of mitochondria is better than by glutaraldehyde. When glutaraldehyde and acrolein (fig. 2.25) are used in combination there is less leaching than for acrolein (fig. 2.23), but the preservation is not as good as for glutaraldehyde alone (fig. 2.20). Tubular ER and microtubules are retained (fig. 2.26) and the mitochondria are better preserved than in glutaraldehyde (fig. 2.20).

When glutaraldehyde is used with formaldehyde as a modified Karnovsky fixative (fig. 2.27) there is less density of the cellular ground substance and of the mitochondrial matrix than was the case with /

with glutaraldehyde fixed testis (fig. 2.20). The cell outline and membranes of all organelles thus became clear and distinct *becomes* (fig. 2.28). There is a tendency for the nuclear membranes to form intranuclear membrane spaces or protrusions (fig. 2.27, 2.29). The intertubular and boundary tissues display a slightly hypertonic image of denser cytoplasm and wider than normal perinuclear space (fig. 2.29). When formaldehyde is used with picric acid there is formation of large intracellular spaces in the cytoplasm (fig. 2.30). Mitochondria appear swollen (fig. 2.31 & 2.32) and ER vesicular in the Leydig cells (fig. 2.32). There is excellent preservation of lysosomes showing variation in the density of the matrix (fig. 2.33).

### Osmolality

In the first series of experiments designed to examine the effects of osmolality on fixation quality using glutaraldehyde with Millonig's phosphate buffer, the best image is obtained with 2.5%-3% GTA/0.1M Millonig's phosphate (2.34). This fixing solution has an osmolality of 552-616 mOsm.

Fixing solutions of a similar osmolality, which are made up with different concentrations of glutaraldehyde and molarity of buffer e.g. 1% GTA/0.2M Millonig's phosphate and 5% GTA/0.05M Millonig's phosphate (Table 2.4) produce images showing hypertonic and hypotonic effects respectively (fig. 2.35 & 2.36). The hypertonic image displays a shrinkage of tissue forming dark contracted cells and dense mitochondria. Extracellular spaces and perinuclear blisters are also produced as a result of the contraction (fig. 2.37). Hypotonic images show a leached effect due to cytoplasmic swelling. The mitochondria are distended, and swollen ER gives the cells a vesiculated look (fig. 2.38).

Fixing solutions of a different osmolality to the best fixing solution, which are made up with the same molarity of buffer, namely /



namely 0.1M Millonig's phosphate, produce better fixation images with less hypertonic or hypotonic effects (fig. 2.39) than the fixing solutions that have the same osmolality but a different molarity of buffer. The results of these experiments therefore indicated that changes in the buffer concentration have more effect on the quality of the preservation than changes in the concentration of the glutaraldehyde.

These results are confirmed with the other buffer systems used. 4% GTA/0.1M s-collidine (598mOsm) (fig. 2.40 & 2.41) and 4% GTA/0.075M sodium cacodylate (560mOsm) (fig. 2.42 & 2.43) give the best preservation. Note that the osmolalities of these two fixing solutions are similar to that of the best Millonig's phosphate fixing solution. Again alteration of the molarity of these buffers has more effect than changes in the glutaraldehyde concentration. 0.2M s-collidine fixatives (fig. 2.44 & 2.45) and 0.1M and 0.15M cacodylate fixatives (fig. 2.46, 2.47 & 2.48) give hypertonic images of dark contracted nuclei with enlarged perinuclear spaces and shrunken cytoplasm causing vacuole formation and in the case of 0.15M cacodylate fixatives, resulted in loss of cell outline (fig. 2.48).

#### Temperature and Length of Time of Fixation

Testis that is fixed at room temperature is generally better preserved than testis fixed at 4°C. There is greater reproducibility of results for room temperature fixed tissue.

The effect of difference in the length of time of GTA/ Millonig's phosphate fixation are negligible over the 1-24 hour range used. If the fixative/buffer combination produced a bad fixation image, neither increasing nor decreasing the time of fixation improved the preservation. If a 1 hour fixation time produces a good fixation image /

image, there is no deleterious effect if the time is increased to 24 hours. For GTA/s-collidine the leaching effect of the buffer is more pronounced after 3 hours; therefore a 1 hour fixation time is preferred.

#### Post-fixative Buffer

Variation in the post-fix buffer of glutaraldehyde/cacodylate primary fixed tissue alters the quality of the preservation. The leaching of the ground substance observed with 0.175M cacodylate buffered osmium (fig. 2.49) is reduced by using 0.1M phosphate buffer (fig. 2.50), but made worse by modified Dalton's buffer (fig. 2.51). Potassium ferrocyanide did not retain the ground substance of the cytoplasm either (fig. 2.52), but it does enhance the cell membranes and makes the trilaminar structure of the unit membrane more visible (fig. 2.53).

#### Effect of Age

Puberty starts at about 9 weeks of age so that for this age of bird the testes consist of firm compact lumenless seminiferous tubules in a three to one proportion with the intertubular tissue. This means it is easier to produce good overall fixation and with a wider range of fixatives for 9 week old birds than for older or younger birds. Glutaraldehyde buffered with Millonig's phosphate (fig. 2.20), s-collidine (fig. 2.41) or cacodylate (fig. 2.50) and modified Karnovsky (fig. 2.27), acrolein/phosphate (fig. 2.23) or osmium/Millonig's phosphate (fig. 2.19) all gave reasonable fixation. The best image overall was obtained with GTA/sodium cacodylate and a phosphate buffered post-fix (fig. 2.50).

95% of the testes of 21 week old birds consist of seminiferous tubules containing a population of Sertoli cells and developing germ cells. There are small areas of Leydig cells in the intertubular tissue/

tubular tissue. Fixation problems with this age of bird are (1) the separation of cells in the tubules often involving loss of the spermatid population as is observed with osmium tetroxide/modified Dalton's buffer fixed testis (fig. 2.54); (2) the separation of the boundary tissue into layers as with osmium tetroxide/modified Dalton's buffer and acrolein/phosphate (fig. 2.54 & 2.55); (3) the loss of cell integrity due to breakdown of cell membranes in the intertubular tissue as seen with PAF (fig. 2.56). The effect of the paraformaldehyde on fine structure is more pronounced for these older birds when larger vacuoles are formed by the protrusion of cell or nuclear membranes (compare fig. 2.27 & 2.28 with fig. 2.56 & 2.57). The best overall fixation of the three tissues and preservation of their relationship for this age of bird is obtained with glutaraldehyde and the buffers Millonig's phosphate, sodium cacodylate and s-collidine (fig. 2.58, 2.59 & 2.60).

The testes of 1-6 week old birds have a much higher proportion of Leydig cells with 65% of their volume composed of small lumenless seminiferous tubules containing Sertoli cells and early spermatogonia. Modified Karnovsky gives a very hypertonic image for this age of bird (fig. 2.61) Acrolein/phosphate and GTA/s-collidine cause loss of cell integrity with a hypotonic image (fig. 2.62 & 2.63). GTA/Millonig's phosphate produces a good preservation of the tubule cells (fig. 2.64), but causes cell separation in the intertubular tissue (fig. 2.65). Modified Dalton's osmium preserves the tissue and cell relationships (fig. 2.66), but gives a hypotonic image for the tubule cells (fig. 2.67). GTA/acrolein/phosphate and GTA/sodium cacodylate give the best overall fixation of all three tissues (fig. 2.68 & 2.69), but acrolein gives /

gives better preservation of the Leydig cells (fig. 2.68 & 2.70). GTA/sodium cacodylate is considered to give the best overall fixation of all three tissues for 1-6 week old birds.

2.3.2 PERFUSION FIXATION

Anaesthesia

Birds of 9-12 weeks of age that were used initially for the perfusion fixation trials were found to differ greatly in the amount of Nembutal required to anaesthetise them because of their variation in size and development at puberty. Often their hearts stopped beating immediately the body cavity had been opened up. Therefore, anaesthetic procedures were standardised on the 15-17 week old birds which gave more consistent results. Some birds still died suddenly although they had been given less Nembutal than the average. This was thought to be caused partly by the emotional state of the bird before anaesthesia and therefore every care was taken to keep the bird calm before starting the procedures.

Route of administration of perfusate

The surgical techniques required in order to expose the venous system were easier to perform than those required to expose the aortic arch i.e. the pulling back of the sternum and breast sometimes resulted in a rupture of the brachial arteries. Venous perfusions were more often successful than the arterial ones as judged by the setting up of a good flow. These factors favoured the venous route of administration of perfusate, but when tissue perfused in this manner was examined at the ultrastructural level, the blood capillaries were often seen to be filled with red blood cells (fig. 2.71) despite the perfusion working well. Therefore, arterial perfusion was chosen in preference to venous perfusion.

Of /

Of the different arterial perfusion routes the one into the aorta was preferred to those into the ventricle. It was easier to clamp the needle in position in the aorta than the heart. In the initial experiments route 1 (see page 24) with clamps was used with the intention of increasing the flow past the testicular artery bifurcation. However, later when greater expertise in the method had been developed it was found to be easier done without clamps and no difference was experienced in gaining a good flow of perfusate past the testis.

#### Prewash requirements

The use of a prewash solution increased the number of successful perfusions by preventing blockages of fixed clotted blood in the blood vessels. When the tissue was examined in the microscope the prewashes were found to have affected the fixation (figs. 2.72, 2.73, 2.74 & 2.75). A 1% saline prewash increased the loss of extracellular material particularly collagen (fig. 2.72) and 1.4% saline prewash increased the leaching of the ground substance of the cytoplasm (fig. 2.73). Avian Ringer prewash in loss of part of the cytoplasm (fig. 2.74) and a cacodylate prewash caused aggregation of the nucleoplasm (fig. 2.75). As greater technical skill was achieved and the number of successful perfusions increased it was felt that the advantages of a prewash were outweighed by the increase in leaching of cell material.

#### Needle Size

Use of a 24G needle almost invariably resulted in a blocked perfusion because the low pressure flow did not clear the blood from the system quickly enough to prevent it fixing and clotting in situ. When a slow flow was established, however, it resulted in excellent fixation with wide lumina of blood capillaries, lymphatics and seminiferous /

seminiferous tubules (fig. 2.76). Using a 24G needle it took a relatively long time to perfuse 150-200 ml of fixing solution and when the time was set at 20 minutes not enough fixative reached the tissue to preserve it properly. A 23G needle allowed a good flow to be established more often than in the previous case and resulted in enough fixing solution reaching the tissue in 20 minutes to give good fixation. A 21G needle gave a fast enough flow to always clear the blood quickly but this sometimes resulted in testes that looked well fixed but when the fine structure was examined the intertubular tissue was disrupted and the spermatids and sometimes the spermatocytes of the seminiferous tubules were missing (fig. 2.77). It would appear that when using a 21G needle the pressure of perfusate was too great. Therefore a 23G needle at a pressure of 200mm Hg was selected as giving the best results. Under these conditions 18 minutes were required to perfuse 200ml of fixative.

#### Perfusion fixation for 7-21 week old birds

It was found that the best fixation images were obtained with the fixatives that gave the best results for immersion fixation. 2.5% GTA/0.1M Millonig's phosphate perfusate gave good fixation of the seminiferous tubules and boundary tissue (fig. 2.78), but sometimes caused separation of these tissues from the intertubular tissue. 4% GTA/0.1M s-collidine perfusate gave excellent general preservation of all tissues (fig. 2.79), but it was found to result in blocked perfusions more often than did the other perfusates and it has an unpleasant smell if the perfusions are not carried out in a fume cupboard. For these reasons it was not used as often as the other fixatives. 4% GTA/0.075M sodium cacodylate gave excellent general preservation (fig. 2.76) and was considered to be the best fixative for general everyday use.

Perfusion /

Perfusion fixation for 1-6 week old birds

Birds of this age group were more difficult to anaesthetise because of their small size. One drop of Nembutal was enough for a 1 week old bird. Perfusions often did not flow properly because the heart stopped beating too soon, probably because the bird was over anaesthetised.

Because the birds tended to die quickly, the initial perfusions were carried out using a syringe and hand pressure. The pressure of the perfusion cannot be controlled accurately by hand and the inter-tubular tissue showed signs of too high a pressure in the separation of cells and loss of intercellular material, as is seen by comparing the GTA/acrolein/phosphate immersion fixed and perfusion fixed 1 week old testis (fig. 2.68 & 2.80). As greater skill in anaesthetic and surgical techniques for smaller birds was gained the perfusion apparatus was able to be used to give better control of flow rate, and thus better preservation of the relationship between the three tissues (fig. 2.81).

Perfusions of osmium tetroxide with Dalton's buffer did not appear to work well as judged by the testes remaining almost totally yellow instead of turning black. This was confirmed by the fine structure which showed blood capillaries filled with red blood cells (fig. 2.82). In areas where the osmium had penetrated the fixation image was similar to the immersion fixed material showing separation of the boundary tissue from the intertubular tissue but good clear membranes (fig. 2.83). The effect on the fine structure of a failure in perfusion was not as pronounced for the younger hand perfused birds as for the older birds perfused with the apparatus (compare fig. 2.83 & 2.71). Again GTA/sodium cacodylate gave the best overall preservation of the tissue (fig. 2.84).

## 2.4 DISCUSSION

In this chapter fixation of the chicken testis is discussed in terms of obtaining a good overall fixation of the three tissues of the testis and in terms of obtaining good quality fixation of germ cell and Sertoli cell organelles especially cell membranes.

### 2.4.1 IMMERSION FIXATION

The effect of the buffer system on fine structure has been demonstrated with osmium tetroxide and with a glutaraldehyde/osmium tetroxide double fix. Differences in the density of the matrices of the mitochondria and lysosomes, which were observed only with osmium fixed testis, can be largely attributed to extraction phenomena. Osmium tetroxide does not react with polysaccharides or nucleic acids (Bahr, 1954) and cross-linking with proteins depends on the content of amino acids with double bonds. Dallam (1957) demonstrated a 22% loss of protein from rat liver mitochondria during fixation in osmium tetroxide. Loss of protein has also been demonstrated by labelling rat tissue protein with methionine-S<sup>35</sup> (Luft & Wood, 1963). S-collidine buffer is a methylated pyridine which does not react or form strong complexes with osmium tetroxide (Bennett & Luft, 1959). The greater pallor of the matrices of the organelles and leached appearance of the cytoplasm with s-collidine buffer could be explained as extraction of protein (Wood & Luft, 1965). Luft and Wood (1963) demonstrated greater extraction of protein with s-collidine buffered fixatives compared to differently buffered fixatives.

The relative density of organelles and cytoplasm can also be related to the total ionic strength (Trump and Ericsson, 1965). The solubility of proteins at a constant pH is affected by the total ionic strength, the specific ionic composition and the dielectric constant. /



constant. Phosphate buffer has a high ionic strength whereas s-collidine has a low ionic strength and this can be correlated with the findings of this report, where phosphate buffered fixatives gave greater density to the ground substance of the cytoplasm.

Variation in the image is less pronounced for the glutaraldehyde/osmium tetroxide fixed testis because the aldehyde actively cross-links proteins and therefore holds the tissue components better preventing extraction. This is in agreement with the findings of Ericsson, Saldino and Trump (1965) and Maunsbach (1966b) on the proximal tubule cells of rat kidney. However, the increased solubility of proteins with a buffer of low ionic strength still produces a paler image of the cytoplasm in glutaraldehyde/s-collidine fixed testis as compared to glutaraldehyde/Millonig's phosphate fixed testis.

Trump and Ericsson (1965) thought that some of their observed differences in configuration of mitochondria and ER of the cells of convoluted tubule of rat kidney with different buffer systems might be accounted for by differences in osmolality. Hayat (1970) has suggested that the published data on effects of different buffers on ultrastructure should be interpreted with caution because a change in buffer system invariably involves a change in osmolality. He suggested more meaningful information could be obtained if osmolality, pH and other properties of the buffer were kept constant. However, it can also be argued that buffer systems are better compared when their properties are selected in order to produce the best fixation image of the particular tissue being examined.

Fixatives and buffers were selected on that basis for this thesis in the osmium/buffer series (A). Palade's buffer was the only one selected with no prior knowledge of its suitability for chicken /

chicken testis and it was included for comparison because of its widespread use in other laboratories. Some of the effects of this buffer, such as the vesiculated cytoplasm of the Leydig cells and the separation of the boundary tissue from the inter-tubular tissue, can be attributed to osmotic effects. Veronal acetate was not used to buffer glutaraldehyde because it reacts with the fixative and loses its buffering capacity (Sjöstrand, 1967).

In the glutaraldehyde/buffer series (B) the effect modified Dalton's buffer had on the testis seemed to be an osmotic one. This buffer had been previously modified in this laboratory for use with osmium in order to produce the best fixation image for chicken kidney. In this present work used with osmium it produced good fixation for chicken testis, but it was of no use for comparison in the glutaraldehyde/buffer series. Separation of the boundary tissue from the intertubular tissue when modified Dalton's was used with osmium indicates that the osmolality of this buffer was not quite correct for the intertubular tissue.

There is no agreement in the literature on which buffer is the best. Each one has advantages and disadvantages e.g. phosphate buffer causes artefacts in rat lung tissue (Gil & Weibel, 1968). For the chicken testis different buffers are preferred for different aspects of cell or tissue fine structure. Glutaraldehyde or osmium tetroxide buffered with phosphate would be the choice for the preservation of ground substance, intercellular material, and cytomembranes. Osmium buffered with modified Dalton's would also give good preservation of cell membranes. S-collidine buffer with a primary osmium fix gave too leached an appearance to be generally useful, but with glutaraldehyde would be chosen for preservation of intercellular material such as collagen and for the overall fixation of all three tissues. Glutaraldehyde with cacodylate would be preferred /

preferred to the other buffers for the best general cell fixation and preservation of the relationship between the three tissues of the testis. The cacodylate buffer did produce a more leached appearance of the testis than phosphate buffered fixative but this problem was reduced by the use of a phosphate buffered post-fix. The combination of a glutaraldehyde/cacodylate primary fix and a phosphate buffered post-fix preserved the relationship of the three tissues and improved the preservation of the cell substance.

A comparison of the effects of the different fixatives used on the chicken testis does not show any great morphological changes in structure such as demonstrated by Franzini-Armstrong and Porter (1964) with the T-system in fish skeletal muscle. The greatest differences are in the quality of preservation and these differences can be attributed to extraction phenomena. Each fixative has a slightly different chemical reaction with the different molecules of the tissue, so that extractability and solubility of tissue constituents varies from fixative to fixative and this influences the contrast and general appearance of the fine structure.

Differences in chromatin distribution between osmium primary fixed and glutaraldehyde primary fixed testis could be attributed to the different reaction between these fixatives and the nuclear material. Osmium tetroxide has been shown to have no reaction with native DNA and RNA (Bahr, 1954). Glutaraldehyde is thought to react with DNA because purified DNA can reduce silver nitrate-methanamine solution after glutaraldehyde treatment (Thiery, see Millonig & Marinozzi, 1968). Also the thermal transitions of DNA and RNA are altered after exposure to glutaraldehyde and formaldehyde (Hopwood, 1973).

Ericsson, /

Ericsson, Saldino and Trump (1965) found perinuclear clumping of the chromatin fixed with Dalton's buffered osmium. This resembled the clumping along the nuclear membrane with their glutaraldehyde fixed nuclei. They thought this was due to chromium salts and water forming a complex, which combines with reactive groups of proteins to create a binding effect similar to formaldehyde. In the chicken testis the nuclei of Dalton's fixed testis appeared similar to the Palade's and Millonig's fixed testis. The s-collidine buffer was the only buffer that produced coarse aggregates of chromatin and this is considered to be a coalescence of DNA fibres during ethanol dehydration. Ethanol acts as a coagulant type of fixative and this type of clumping has been demonstrated with intramitochondrial DNA fibres in a wide variety of species (Nass, Nass & Afzelius, 1965).

Osmium tetroxide is a non-coagulant type of fixative and able to stabilise some proteins by the formation of gels (Hopwood, 1969b) without destroying many of their structural features. The best established reaction of osmium tetroxide is the oxidation of unsaturated bonds. Unsaturated fatty acids react with osmium tetroxide to form diesters of osmic acid in which one molecule of osmic acid links two molecules of fatty acid in vitro (Korn, 1966a). Korn (1966b) has also shown that these bridged compounds are formed in amoeba and has reviewed the ultrastructure of membranes in relation to osmium fixation (Korn, 1966c).

Membrane reorganisation is one of the best known changes that occur with fixation. In certain tissues osmium tetroxide causes the breakdown of the plasma membranes into chains of vesicles or tubules e.g. toad spinal ganglia (Rosenbluth, 1963); ciliary epithelium (Tormey, 1964); The T-system of fish skeletal muscle (Franzini-Armstrong /

! re-  
phrase

(Franzini-Armstrong & Porter, 1964); and prawn nerve sheath (Doggenweiler & Heuser, 1967). Prefixation with glutaraldehyde preserves the continuity of the T-system, but it does not prevent vesiculation in prawn nerve sheath. In chicken testis no major reorganisation of the fine structure of plasma membranes was observed, but the rough ER had a tendency to form vesicles and smaller wider cisternae when fixed with osmium as opposed to glutaraldehyde. Christensen (1965) concluded from a study of the interstitial cells of guinea pig testis using glutaraldehyde or osmium perfusion fixation, that fenestrated cisternal and tubular forms of smooth ER were the most normal, and disconnected vesicles a result of faulty fixation. A similar situation was found in immersion fixed rabbit lutein cells where osmium fixed cells resulted in irregular diameter smooth ER vesicles (Blanchette, 1966).

Another disadvantage of osmium tetroxide is its slow rate of penetration into tissues. The maximum rate is reached after one hour with a penetration depth of 0.6mm (Burkl & Schiechl, 1968). After this period the rate is slowed because the fixed outer layers then impede further penetration, so that for most cells and tissues including chicken testis blocks of tissue should be no more than 1mm<sup>3</sup>. A fixation time of 1 hour as used throughout this work allows sufficient penetration time. This slow penetration time probably accounts for the inability of osmium tetroxide to fix microtubules in a recognisable state (Hayat, 1970). Microtubules are very labile cell organelles and were not observed in osmium fixed chicken testis.

The major advantage that osmium tetroxide has over most other fixatives is its ability to act as an electron stain as well as a fixative. For this reason it has been used in each fixation procedure /

procedure of this present work either as a primary or as a post-fixative.

The use of an aldehyde prefixation improves the tissue preservation because aldehydes are able to form both intra- and inter-molecular crosslinks with proteins. Polypeptides are crosslinked at reactive side groups by the formation of methylene bridges (Richard & Knowles, 1968). In a comparative study of the aldehydes Sabatini, Bensch and Barnett (1963) decided glutaraldehyde, a dialdehyde, gave the best ultrastructural tissue preservation. This could be a result of the size of the glutaraldehyde molecule, which as Bowes and Cater (1966) suggest seems particularly suitable for binding the gap between amino acids of polypeptide chains. In this thesis it has been shown that glutaraldehyde gives better preservation of the microtubules and ER than either acrolein or osmium tetroxide.

Another point in favour of glutaraldehyde over the other common fixatives is that it causes the least protein conformational changes. This means tissue can be left in the fixative for hours without apparent deterioration as in the case of chicken testis fixed in GTA/Millonig's phosphate.

One disadvantage in the use of an aldehyde/osmium double fixation is that it causes myelinization of lipids (Curgy, 1968). Myelin figures do not appear in tissue that is fixed with aldehyde alone (Trump & Ericsson, 1965). Formaldehyde is known to extract some lipids and Fawcett (1964) has shown that hydration of lecithin and other phospholipids in vitro results in the formation of myelin like figures. Thus, Trump and Ericsson considered that myelin whorls and other membrane structures associated with cell membranes in post-osmicated tissue are the result of a reaction between osmium tetroxide and /

and compounds extracted by the aldehyde in aqueous solution.

Glutaraldehyde has a slightly faster penetration time than osmium tetroxide and reacts very quickly with tissue constituents. This may explain its ability to retain microtubules. Acrolein, an unsaturated monoaldehyde, is one of the most reactive organic substances (Luft, 1959) and penetrates faster than glutaraldehyde. For chicken testis acrolein fixation resulted in the loss of the cytoplasmic tubules. Schultz and Case (1968) have shown a similar effect with acrolein perfused neurons, but otherwise found it gave good preservation of the tissue. In the chicken testis acrolein caused too great a leaching of the cytoplasm to be very useful as a general fixative.

Sandborn (1966) recommended the use of a glutaraldehyde/acrolein mixture for the fixation of neuronal microtubules claiming it gave better preservation than either aldehyde alone. For the chicken testis the preservation of microtubules with this fixative was no better than when glutaraldehyde alone was used. This fixative mixture did give good fixation of the testes of 1 week old birds. This may be a reflection of the higher proportion of Leydig cells (and consequently a greater amount of lipid) in the prepubertal bird. Acrolein has been shown to react with unsaturated fatty acids (Jones, 1969) and causes less extraction of lipid than formaldehyde from rat adrenals (Norton et al, 1962).

The use of a glutaraldehyde/formaldehyde fixative was first suggested by Karnovsky (1965). In a comparative study of the fixatives, glutaraldehyde, formaldehyde, and a mixture of the two, Beauvillain (1970) found the mixture gave the most reproducible results for mouse median eminence. For the chicken testis the preservation after Karnovsky fixatives was very varied. Some very good /

good fixation was obtained with 9 and 21 week old birds but more often the tissue showed fixative diffusion gradients.

Formaldehyde was not used by itself because it has been shown with collagen that the number and stability of the crosslinks introduced are inferior to those formed by glutaraldehyde or acrolein (Bowes & Cater, 1966). Formaldehyde penetrates tissue faster than glutaraldehyde, but it reacts slowly and many of its reactions with proteins are reversible. The advantage of Karnovsky's fixative over glutaraldehyde alone is in the ability of the formaldehyde to penetrate quickly and stabilise the protein until the glutaraldehyde reaches the tissue. It is, therefore, particularly useful for dense tissues such as bone. In the chicken testis this fixative mixture often resulted in variation in the fixation image particularly between the spermatogonial and spermatocyte cell layers, as if it caused a diffusion gradient within the seminiferous tubules.

Formaldehyde in combination with picric acid is the basis of the light microscope fixative, Bouin's fluid. This fixative was adapted for ultrastructural work by Zamboni and De Martino (1967) and used by Stefanini et al (1967) specifically for the preservation of spermatozoa. As a fixative for testicular tissue it causes too many artefacts, such as cytoplasmic vacuoles, to be considered generally useful. Picric acid is a coagulant-type of fixative and thus causes considerable change in protein structure resulting in distortions of the fine structure. This is not acceptable at the electron microscope level of magnification. ? for EM studies

The results of the osmolality experiments have shown that for pubertal and post-pubertal birds the best overall preservation of testis is obtained with a fixing solution that has a total osmolality of /



of between 550-600mOsm e.g. 2.5% GTA 0.1M Millonig's phosphate, 4% GTA/0.075M sodium cacodylate and 4% GTA/0.1M s-collidine. These fixing solutions are hyperosmotic to both the blood serum and semen.

Different tissues vary in the degree of their response to differences in the ion balance between the fixing solution and normal environment. Changes in osmolality within reasonable limits have little effect on compact tissues, such as liver, but for less compact tissues, such as newborn rat epidermis, a difference of 50mOsm can alter the fine structure (Maser et al, 1967). In the fowl testis the 50mOsm range between 550-600mOsm made little difference to the overall fixation of the testis. Of the three tissues of the testis the Leydig cells would seem to be most intolerant of osmotic changes because their fine structure was more often affected by changes in the osmolality with different fixatives. Well fixed Leydig cells were never observed with badly fixed seminiferous tubules.

The term, osmolality, implies the presence of a membrane. In order for osmolality to be a colligative property the membrane must be ideal. Cell membranes are not ideal and are changed through fixation, therefore, when designing fixing solutions an osmolality can be used only as an arbitrary starting number. The arbitrary starting number often used is that which is isotonic to the natural environment surrounding the cell or tissue of interest. The rationale behind this decision is that hypotonic solutions cause swelling of the tissues and hypertonic solutions cause shrinkage.

One method of adjusting tonicity is the addition of a balanced salt solution to the fixative. Sjostrand (1956) obtained successful fixation of mammalian tissues with osmium tetroxide buffered in /

in a solution isotonic to blood serum. However, fixatives made isotonic to blood serum are not necessarily isotonic to the tissue because of variation in the intracellular osmolality. Also the tonicity of excised tissue that has been cut off from its blood supply and had its temperature lowered cannot be the same as when attached to the animal (Dempster, 1960).

Some work has shown that isotonic fixing solutions fail to prevent swelling (Bahr *et al.*, 1957; Millonig & Marinozzi, 1968), but that fixing solutions that are made slightly hypertonic with the addition of non-electrolytes prevent it (Bahr, 1957; Bohman & Maunsbach, 1970). The effectiveness of non-electrolytes in preventing swelling after osmium tetroxide fixation is thought to be due to the loss of relative impermeability by the cell membrane to small molecules. Larger molecules are still unable to pass through and therefore exert an osmotic pressure that prevents swelling (Wood & Luft, 1965).

Addition of electrolytes such as  $\text{Ca}^{++}$  (Elbers, 1966) and  $\text{Na}^+$  (Millonig & Marinozzi, 1968) also prevents swelling and this is thought to be due to the reduction in the repulsive forces caused by the negative charge of protein chains. Divalent ions are more effective and crosslink the protein chains. As well as preventing swelling addition of electrolytes also lessens the amount of extraction. The various salts combine with the amino acids and alter the solubilities e.g.  $\text{Ca}^{++}$  suppresses the extraction of haemoglobin (Tooze, 1964).

In this present work the osmolality of fixing solutions has not been adjusted by addition of electrolytes or non-electrolytes, because of certain disadvantages in these methods. Electrolytes affect the rate of penetration e.g.  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  decrease the penetration /

penetration rate of osmium and produce granularity and opacity in certain cells (Millonig, 1966). With non-electrolytes the rate of penetration is considerably reduced. Hagstrom and Bahr (1960) found a 50% decrease in osmium penetration with addition of 0.25M sucrose. There is also an increase in the extraction of cell materials during fixation and dehydration. Wood and Luft (1965) demonstrated an increase in protein extraction with sucrose or dextran.

In this work adjustment of the osmolality was carried out by altering the concentration of the fixative and molarity of the buffer as in the method of Fahimi and Drochman (1965). Changes in the buffer molarity were found to have more effect on the fine structure than changes in the concentration of fixative. This is in agreement with the findings of Fahimi and Drochman (1965) for rat liver and rabbit muscle, and Maunsbach (1966b) for rat proximal tubule.

For chicken testis the best fixation was obtained using a fixing solution with moderately higher osmolality than that of the blood plasma or semen. Other studies on the effect of varying fixative osmolality using a wide range of tissues are in agreement with this finding e.g. rat liver and rabbit muscle (Fahimi & Drochman, 1965), rat brain (Schultz & Karlsson, 1965), the proximal tubule of rat kidney (Maunsbach, 1966b), ovarian follicle cells of teleost (Busson-Mabillot, 1971) and the reflecting cells of the scales of teleost (Bone & Denton, 1971).

Glutaraldehyde exerts a considerable osmotic effect (Millonig & Marinozzi, 1968), whereas osmium tetroxide contributes little to the total osmolality of a fixing solution (Wood & Luft, 1965). Changes in the glutaraldehyde concentration have less effect than /

than changes in the buffer molarity despite the large contribution to the total osmolality made by glutaraldehyde. As a result Bone and Denton (1971) neglect the osmotic pressure of the glutaraldehyde and describe the osmolality of their fixing solutions in terms of the vehicle having an osmolality slightly below that of the normal external medium. They found that aldehyde fixed cells come to a given volume in a solution that is hypotonic to that which gave this volume before fixation. They explained this effect by thinking of each cell as enclosed by a semi-permeable membrane with the volume of the cell determined by its content of indiffusible molecules and by the concentration of indiffusible molecules in the solution in which the cell is placed. The effect of the aldehyde is then explained if either the cell content of indiffusible molecules was reduced by their escape from the cell or by their condensation into larger units, or if some of the water normally bound within the cell was released.

The fact that osmium tetroxide makes little contribution to the total osmolality of a fixing solution means that it is the vehicle which is adjusted to make the solution isosmotic with the body fluids, as in this work using modified Dalton's buffer and the 0.175M cacodylate post-fix buffer. It is not always necessary to make the fixing vehicle isosmotic as some workers have obtained results using osmium tetroxide in distilled water that are indistinguishable from the results using an isosmotic buffered solution (Malhotra, 1962; Baker, 1965). ? must be

The importance of considering the osmolality of fixing solutions seems related to the permeability of fixed cell membranes. Wood and Luft (1965) demonstrated that the resting potential of frog sartorius fibres /

fibres fell rapidly on exposure to osmium tetroxide. This implies that membranes probably lose their relative impermeability to  $\text{Na}^+$  with this fixative. Elbers (1966) measured ion loss across the cell membrane of Limnaea eggs as fixatives were applied and found that glutaraldehyde did not appreciably alter the permeability of the membrane whereas osmium tetroxide made the membrane freely permeable to ions. The permeability of cell membranes after osmium fixation was confirmed by Bone and Denton (1971) using the volume changes in the reflecting cells on the scales of teleost fish. More evidence is provided by measurements of the changes in the conformation of red blood cell membrane protein using circular dichroism. The residual helical content of the protein was greatly reduced by osmium tetroxide but there was little change after glutaraldehyde.

In conclusion whatever rationale is used initially to determine the range of osmolality of the fixing solution and whatever method is selected to obtain this osmolality, the end result should be an image as free as possible of osmotic effect. Once the fixing solution enters the blood vascular system, the different environment may alter its osmolality and when it reaches the tissue, the different rates of penetration into the tissue of the various ions and molecules of the fixing solution will mean that the actual osmolality of the fixing solution at the cell membrane is different to the measured value. So what is finally important is that the measured osmolality is the one that results in the right osmolality for the inter- and intra-cellular conditions and gives the best fixation image.

#### 2.4.2 PERFUSION FIXATION /

#### 2.4.2 PERFUSION FIXATION

Preservation of fine structure is affected not only by the properties of the fixing solutions but also by the method of applying the fixing solution to the tissue. The use of vascular perfusion fixation for the fowl testis has demonstrated the superiority of this method of fixation over immersion for the preservation of the spatial relationship of blood vessels and lymph vessels to Leydig cells and seminiferous tubules. This method of fixation brings the cells and tissues into contact with the fixative as rapidly as possible maintaining the blood supply and preventing anoxia until the instant of fixation. It ensures uniform penetration of the fixative and therefore gives a reproducible fixation image.

The use of vascular perfusion fixation means manipulation of unfixed testis is avoided. This is especially important for tissues that are sensitive to physical handling such as the post-<sup>testis of</sup>pubertal 12-21 week old bird in which semen production has started. The presence of semen in the testis makes it a very soft organ easily damaged during the excision and cutting of small blocks, as shown by the separation of the spermatid population in immersion fixed material.

The surgical operation required to expose avian testes is far more traumatic than that required to expose mammalian testes because of the abdominal position of the avian testes. Birds underwent major surgery of a very crude kind that involved cutting all the ribs in order to expose the heart and aorta. Generally speaking vascular perfusion into the local blood vessels of the organ to be examined should ensure a higher percentage of successful perfusions because /

because the fixative does not have to travel so far to reach the organ and there is less chance of a blood clot forming. However, for the fowl testis the testicular blood vessels are only 1-3mm in length (Nishida, 1964) and no attempt was made to cannulate them. The aorta in the region of the testicular bifurcation is underneath the very thin-walled vena cava, embedded in tough connective tissue. Any attempts at reaching this vessel resulted in the rupture of the vena cava.

The only local perfusions attempted were into the venous system. These did not clear the blood from the arteries of the testis. On theoretical grounds the use of clamps to direct the flow to the testis would be expected to increase the number of successful perfusions. In practise it was found that it made little difference once the correct perfusion pressure had been established.

The most common reasons for failure of perfusions are errors in carrying out the method. Perforation of the aorta with the needle and death of the bird before the onset of perfusion either by an overdose of anaesthetic or from a broken brachial artery occurred less frequently as greater expertise was gained.

Success of a perfusion depends in part on complete exclusion of the blood from the vascular system and prevention of vasoconstriction. Vasoconstriction can be avoided by ensuring that the pH, osmolality, ionic composition and temperature are suited to the tissue under study (Hayat, 1970). The results of the immersion study of the fowl testis were used to decide the parameters of the fixing solution for perfusion.

It was fortunate that the osmolality of the perfusate was the same as that required to produce the best fixation image with immersion fixation despite the difference in composition of fixing solution introduced by the blood.

Chilled /

Chilled perfusates can cause arteriolar constriction (Karlsson & Schultz, 1965); (Sjostrand, 1967) therefore the perfusates for this study were used at room temperature. This temperature rather than 4°C had already been shown to give better fixation for immersion fixed testis. Glutaraldehyde penetrates slowly but reacts quickly (Flitney, 1966). Higher temperatures increase the rate of diffusion allowing the deeper cells to be fixed sooner.

There is disagreement in the literature about the necessity of a prewash and infusion of a vasodilator. Forssmann et al (1967) consider the use of the vasodilator, procaine, to be important for ensuring free flow and homogeneous fixation for whole body rat perfusion, whereas Karlsson and Schultz (1965) considered its use to be detrimental to the successful perfusion of the rat central nervous system. Kjaerheim (1969) found the use of procaine made little difference to the perfusion fixation of fowl adrenal gland, consequently it was not used for fowl testis perfusions.

For 7-21 week old birds glutaraldehyde was the only fixative used because of the large volumes of perfusate required and its relative lack of noxious effects. For the younger smaller birds it was possible to use osmium tetroxide and acrolein. Palay et al (1962) consider it is necessary to use a vasodilator with osmium, but because of the small size of the birds and the relatively short distance the fixative needed to travel to reach the testis no vasodilator was used. However, none of these perfusions worked well as judged by the amount of blood observed in the blood vessels, therefore, a vasodilator should probably have been used.

It /



It is necessary to exclude the blood from the vascular system as soon as possible in order to prevent clot formation and blockage of the blood vessels. Citrated blood is rapidly clotted by glutaraldehyde (Kjaerheim, 1969) and fowl blood clots much faster than mammalian blood (Maxwell, personal communication). Many investigators use a prewash to exclude the blood before perfusing the fixative (Palay et al, 1962; Torack, 1965; Forssmann et al, 1967; Kjaerheim, 1969). Maunsbach (1966a) found a prerinse was only necessary for rat kidney when glutaraldehyde concentrations higher than 2-2.5% were used in perfusates. For the fowl testis the prewashes were found to have a detrimental effect on the fine structure. Although use of a prewash did increase the number of successful perfusions the overriding factor for gaining a successful perfusion seemed to be the flow rate of the perfusate. This is in agreement with Karlsson and Schultz (1965) who found the fixative perfusate was adequate to wash out the blood from the vessels.

The flow rate applied must be sufficient to exclude the blood but must not cause testicular damage. The mean blood pressure in the fowl is 135mm Hg (Kjaerheim, 1969), but as there are narrower points in the perfusion apparatus than the diameter of the aorta, the pressure in the aorta will be lower than the pressure read on the apparatus gauge. Also fixation causes an alteration in the elasticity of the blood vessels and the perfusates differ in viscosity to the blood (Maunsbach, 1966a) so that it is unlikely the pressure in the aorta will be the normal blood pressure even if the apparatus gauge reads within the normal physiological range. Thus slightly higher perfusion pressures 150-250mm Hg were used. This is in agreement with Kjaerheim (1969). He used a 5mm diameter cannula /

cannula into the hen's aorta and thus produced far higher flow rates of 180-190ml/minute for perfusion of fowl adrenal gland than those used for fowl testis in this thesis. He notes that the perfusion failed for more distal regions including the genital tract and suggests perfusion pressures should be adjusted empirically for each organ. In this study the flow rates were varied by altering the needle size as well as the pressure. A flow rate of 27ml/minute was too high for the testis and caused loss of the developing germ cells from the seminiferous tubules. Therefore a flow rate of 11.5ml/minute was selected as giving the best perfusion results, although this flow rate was not fast enough to always ensure the clearing of the blood. The ideal perfusion would be obtained if a <sup>right</sup> fast flow rate with the 21G needle was used to start the perfusion and clear the blood and then the flow rate slowed by exchanging the needle for a 23G size needle.

For the rat kidney Maunsbach (1966b) found the choice of buffer system made little difference to the fine structure. It is interesting to note that for the fowl testes sodium cacodylate was superior to all others in that it was the only buffer that consistently gave the best image for both immersion and perfusion fixed testis over the whole age range. As previously mentioned the buffer concentration is of more importance than the glutaraldehyde concentration. This may indicate that the buffer penetrates more quickly, as postulated by Palade (1952) for osmium tetroxide. This would suggest that the specific ionic composition of sodium cacodylate is better suited than other buffers to the environment found in fowl testicular tissue.

The immersion and perfusion fixed material obtained during this study has been used in the following chapter for ultrastructural studies of the testis of the domestic fowl.

3.1 INTRODUCTION

The present study is an attempt to describe the changes in fine structure of the germ cells of the domestic fowl from hatch to the start of spermiation, and to relate this development of the germ cells during spermatogenesis to changes in the morphology of the intertubular region and to the differentiation of the Sertoli-Sertoli cell junctions.

Studies of the germ cells of the domestic fowl have been largely confined to the light microscope (Guyer, 1909; Guyer, 1916; Miller, 1938; Zlotnik, 1947; de Reviere, 1971). An early electron microscope study of spermatogenesis in the cock related ultra-structural features to the descriptions of the light microscopists (Nagano, 1959). Since then the advent of glutaraldehyde and epoxy resins has improved the fixation and embedding procedures and a more up to date account of germ cell differentiation was felt to be necessary. Earlier work in this laboratory had examined the differentiation of the other cell populations of the testis e.g. the Leydig cell (Rothwell, 1973) the Sertoli cell (Cooksey & Rothwell, 1973), and the boundary tissue of the seminiferous tubule (Rothwell & Tingari, 1974).

Aspects of fowl spermatogenesis have been studied in detail by other workers, e.g. cytoplasmic bridges between spermatocytes (Nagano, 1961), the spermatid tail complex (Nagano, 1962), the microtubule system in the spermatid (McIntosh & Porter, 1967), the mitochondrial membrane of the spermatid (Nicander & Hellström, 1967) and the fine structure of the spermatozoon (Tingari, 1973; Bakst & Howarth, 1975). These studies are mostly concerned with spermatid fine structure /

02.  
structure or spermiogenesis. Little attention has been paid to the earlier stages of spermatogenesis. In this present work the fine structure of the spermatogonia before and after puberty, and the fine structure of the spermatocyte are described. A brief outline of spermiogenesis is given in order to relate this to a description of the mechanism of spermiation.

The release of the spermatids into the lumen of the seminiferous tubules has been studied in the amphibian (Burgos & Vitale-Calpe, 1967), marsupial (Sapsford & Rae, 1969) and several species of mammal (Fawcett & Phillips, 1969; Vitale-Calpe, 1970; Fouquet, 1974). The main features of spermiation as described in these papers are the formation of a specialised Sertoli/spermatid head junctional complex that holds the spermatid within the recesses of the Sertoli cell cytoplasm, and the swelling of the Sertoli ER and apical cytoplasm that causes the unfolding of these Sertoli crypts and the release of the spermatids. The excess spermatid cytoplasm is phagocytosed by the Sertoli cells to form residual bodies. This thesis presents a description of avian spermiation as observed in the domestic fowl for comparison with the suggested mechanism for release of the spermatids of other classes of animal.

In mammals and birds a cross section through a seminiferous tubule shows a layer of spermatogonia around the basal lamina, one or several layers of spermatocytes inside these, and groups of spermatids adjacent to the lumen. These different germ cell generations are developing simultaneously. The start of a new generation is connected in a definite manner with the development of the preceding ones. Thus the different cell types are not arranged at random but are organised in fixed cell-associations. For example one cell-association /

Figure A

Photomicrograph of a cross section through a seminiferous tubule of a 21 week old bird. The multi-layered seminiferous epithelium lining the tubule demonstrates several different cell-associations. Many different stages of spermatid are seen lying in a luminal position e.g.  $e1_5$ ,  $L_6$ ,  $L_7$ , and  $L_8$  unlike the situation in the rat where only one stage of spermatid development would be seen lining the lumen. Spermatid stages are labelled according to the classification of de Reviere (1971). A full description of these is given later in chapter 3 page 75. The marked segment demonstrates the cell-association described in the text.

B-type spermatogonium - SgB	Resting Spermatocyte - RSc
Pachytene Spermatocyte - PSc	Multi-phase spermatid - $R_2$
elongated spermatid - $L_7$	Lumen - Lu

Araldite embedded, Paragon (Gurr, Ltd.) stained.

X 150



association may be spermatogonia-B with resting spermatocytes, pachytene spermatocytes, Golgi-phase spermatids and elongated spermatids (fig. A). After the elongated spermatids are released the resting spermatocytes begin meiotic prophase, the Golgi-phase spermatids become acrosome stage spermatids and hence a different cell-association is observed. The succession of a complete series of associations up to the reappearance of the first one has been named the spermatogenic cycle (Regaud, 1901).

There are two main methods for classifying the seminiferous epithelial cycle stages of mammals; one based on the development of the acrosomic system of the spermatid (Leblond & Clermont, 1952) and the other on the morphological changes of germ cell nuclei (Roosen-Runge & Giesel, 1950). The former method has been used in an attempt to define cell-associations in the duck (Clermont, 1958) and the latter method has been used in studies of the quail (Yamamoto, Tamate & Itikawa, 1967).

Each cell association has been designated a stage in the spermatogenic cycle. In the rat a regular succession of stages can be found along the axis of the seminiferous tubule. A complete series of stages forms the spermatogenic wave. Thus the phase pictures seen are the result of two well ordered processes; one proceeding in the wall along the tubular axis; the other proceeding from the basal lamina to the lumen. In the rat irregularities or local inversions in the order of the stages do occur, but a complete succession can be found (Perey, Clermont & Leblond, 1961).

In birds a cross section of a seminiferous tubule contains several cell-associations (fig. A) unlike the rat in which only a single cell-association is often visible. Numerous atypical cell-associations /

associations also occur in the seminiferous epithelium of birds. Attempts by different workers to define a seminiferous epithelial cycle in the duck (Schöneberg, 1913; Clermont, 1958) and the quail (Yamamoto, et al, 1967) have resulted in disagreement due to the inaccuracy and variability of the cell-associations. In the domestic fowl the cell associations occupy very variable areas and no arrangement of the stages in a definite order has been found (de Reviere, 1971). No attempt was made by de Reviere to define a seminiferous epithelial cycle. In this thesis the stages of spermatid development during spermiogenesis as defined by de Reviere have been used to classify the spermatid stages as observed in the electron microscope. No attempt has been made to define cell-association stages or the succession of stages in the spermatogenic cycle of the fowl. This aspect of spermatogenesis is better studied with the light microscope when larger areas of tubules can be scanned.

It has been suggested that the synchronisation of germ cell development that produces the cell-associations is the result of inter-cellular bridges between germ cells (Fawcett, Ito & Slautterbach, 1959). So far cytoplasmic bridges in the fowl have only been demonstrated between telophase spermatocytes and interkinetic spermatids (Nagano, 1961), hence in this study the earlier stages of germ cell development have been examined for the presence of intercellular bridges.

Another explanation for the regular and successive replacement of germ cells about a radial axis is the existence of a local controlling mechanism (Roosen-Runge, 1952; Lacy, 1962). This was postulated on the basis of the observation that, in most mammals, the same sequence of events in the spermatogenic cycle takes place after the stage of spermatid release. Lacy (1962) proposed that the phagocytosed /



phagocytosed lipid from the residual bodies stimulates the Sertoli cell to produce a substance which influences spermatogenesis and thus regulates the spermatogenic cycle.

The hormonal control of spermatogenesis is still not fully understood despite a large literature on the subject (see reviews, Steinberger, 1971; Clermont, 1972). It appears well established that in the rat FSH acts on the seminiferous tubules and LH acts on the androgen-secreting Leydig cells (Mancini, Castro & Seiguer, 1967; Castro, Alonso & Mancini, 1972). The assumption has been made that a similar situation exists in the bird testis (see review, Lofts & Murton, 1973).

It has been shown in rats that spermatogenesis can be initiated and proceed to late pachytene spermatocytes in the absence of gonadotrophins or androgens (Steinberger & Duckett, 1965; Steinberger & Steinberger, 1965). Administration of testosterone allows spermatogenesis to proceed to the point of young spermatid formation, but does not allow maturation of the spermatid. This maturation stage required FSH (Steinberger & Duckett, 1967) or a combination of testosterone and FSH (Kalra & Prasad, 1967), thus both testosterone and FSH must pass into the seminiferous tubules to be effective.

FSH is produced in the pituitary and arrives at the testis via the blood vascular system. Testosterone is produced in chick Leydig cells as a response to LH stimulation (Connell, Connell & Eik-Nes, 1966). Thus these two substances have to pass through the intertubular and boundary tissue of the testis to reach their target cells in the seminiferous tubule.

All material entering the seminiferous tubules must first pass through the extracellular fluid of the intertubular tissue that bathes /

bathes the seminiferous tubules. It is not possible to collect this interstitial fluid, but it is assumed that the composition of the lymph, which can be collected in mammals, mirrors the composition of the interstitial fluid (Setchell, 1970). It has been shown that the testicular lymph of the ram contains appreciable amounts of testosterone (Lindner, 1963) and proteins (Cowie, Lascelles & Wallace, 1964). There seems to be little barrier to the passage of a variety of substances from the blood plasma into the lymph (Setchell, Voglmayr & Waites, 1969), but differences in the rate of penetration of various substances from the lymph into the seminiferous tubules have been noted. The permeability of the tubules has been determined by measuring the composition of the rete testis fluid, this is secreted by the seminiferous tubules and collects in the rete testis (the channels into which the seminiferous tubules open).

The morphological pathway that substances take from the bloodstream to the seminiferous tubules has been demonstrated in the rat and monkey by means of electron-opaque substances (Dym & Fawcett, 1970; Dym, 1973). The tracers delineate the extracellular spaces of the testis and were shown lying in the intercellular clefts of the endothelial cells, in the interstitial and lymph spaces of the intertubular region, between the cells of the boundary tissue in the monkey and penetrating the intercellular clefts of the spermatogonia and Sertoli cells. In the rat tracers were partially arrested in the boundary tissue by the tight junctions. No tracer was observed beyond the junctional specialisations between Sertoli cells in either rat or monkey.

These junctional specialisations occur in the epithelium where two Sertoli cells arch over a spermatogonium and meet. They consist of a series of tight junctions followed by a gap junction with /

with subsurface cisternae and associated filaments. Dym & Fawcett (1970) proposed that these tight junctions resulted in the physiological compartmentation of the seminiferous tubules by preventing the passage of substances into the seminiferous tubules beyond the level of the spermatogonia. The Sertoli-Sertoli junctions thus constitute the principal morphological basis of the blood-testis permeability barrier in monkey or rat.

In this chapter the morphology of the pathway taken by the tracers has been studied. Two areas in particular have been examined, the organisation of the intertubular tissue and the nature of the Sertoli-Sertoli junctions. The intervening tissue between these two areas, the boundary tissue, has already been studied in this laboratory (Rothwell & Tingari, 1973 & 1974, Rothwell, 1975). It was found to consist of an inner fibrous lamella of homogeneous dense material, the basal lamina, and collagen fibres, and peripherally to this, concentric overlapping layers of elongated peritubular cells with attenuated processes. Two distinct cells types were observed; inner fibroblastic cells and outer layers of myofibroblast cells. Where the cell processes overlap, membrane modifications for adhesion were observed. This pattern of organisation more closely resembles that of man (Ross & Long, 1966) than that of the rat.

The nature of the testicular lymphatic vessels and their relationship with the blood vessels, Leydig cells and seminiferous tubules has been studied in a wide range of mammals (Fawcett, Neaves & Flores, 1973). In this thesis a study of the organisation of the fowl intertubular region has been carried out in order to relate it to the fowl boundary tissue organisation and the passage of tracers. A comparison with the organisation of mammalian intertubular regions is /

is made.

A preliminary description of Sertoli-Sertoli junctions in the fowl has been given in an earlier paper (Cooksey & Rothwell, 1973). As stated then these differ considerably from those of the rat (Dym & Fawcett, 1970) or mouse (Flickinger & Fawcett, 1967) particularly in the fine structural details of the subsurface cisternal junctions. They also differ from those of the monkey (Dym, 1973). The tight junctions described for the fowl were not stated as being related to any special region of the Sertoli cell membrane and in particular were not stated as being found in conjunction with the cisternal junction.

For the rat (Vitale, Fawcett & Dym, 1973) and the monkey (Dym, 1973) the presence of Sertoli-Sertoli tight junctions was found to be dependent on the age of development of the animal. There was no blood-testis barrier in the prepubertal animal and no junctional complexes. These were only observed once pachytene spermatocytes were present in the tubules.

In this thesis the adjoining Sertoli cell membranes have been examined more closely for the presence of tight junctions particularly in the region above the spermatogonial cell layer. Birds of 1-21 weeks of age have been examined in order to assess when any such junctions differentiate. The morphological knowledge gained has been used in the fourth chapter, where tracer substances have been used to demonstrate the presence of a blood-testis permeability barrier in the fowl testis.

## 3.2 RESULTS

### SPERMATOGENESIS

Spermatogenesis begins around 7-9 weeks of age for the Shaver strain of the domestic fowl. During the preceding post-hatch prepubertal period the seminiferous tubules contain a population of two cell lines, the germ cells and the Sertoli cells (fig. 3.1 & 3.2), both lying in direct contact with the basal lamina.

The columnar Sertoli cells of the seminiferous epithelium lie perpendicular to the basal lamina (fig. 3.3) with their apical cytoplasm filling the centre of the tubule (fig. 3.1) and the apical lateral plasma membranes forming junctional complexes (fig. 3.4). They have ovoid to columnar nuclei with irregular indentations and prominent nucleoli (fig. 3.3). The ER and Golgi complex are well developed and numerous lysosomes and lipid bodies are observed in the basal cytoplasm (fig. 3.3).

The pear-shaped to ovoid prespermatogonia lie with their axes perpendicular to the basal lamina and their round nuclei more towards the centre of the tubule than the Sertoli cell nuclei (fig. 3.1 & 3.2). The nucleoplasm is pale with a disperse distribution of chromatin and a simple nucleolus of varying density (fig. 3.5). The cytoplasm contains small amounts of rough ER and many free ribosomes. The mitochondria show round, dumbbell, rod or cup shapes. They are located in the basal cytoplasm with the Golgi complex (fig. 3.5 & 3.7) and the centriole (fig. 3.7). Micropinocytosis can be observed between the germ cells and Sertoli cells as seen by the formation of coated vesicles (fig. 3.5 & 3.6). There are no lipid inclusions visible in these cells. This distinguishes spermatogonial cytoplasm from basal Sertoli cytoplasm (fig. 3.7).

During /

During the prepubertal stage mitotic Sertoli cells can be observed (fig. 3.8). These can be distinguished from the later spermatogonial mitoses by the continuance of the Sertoli-Sertoli junctional complexes during mitosis.

At 7-9 weeks spermatogenesis begins and spermatogonia are observed undergoing mitosis (fig. 3.9). The metaphase plate is normally perpendicular to the basal lamina consequently both daughter spermatogonia maintain contact with the basal lamina.

Occasionally cytoplasmic bridges between two or three spermatogonia are seen (fig. 3.10). There is a layer of electron dense material similar in density to that of a desmosome at the inner surface of the plasma membrane along the bridge. The remains of the spindle fibres are sometimes seen within the bridge. The frequency of the intercellular bridges varies from tubule to tubule.

Once spermatogonial mitosis has begun differences in the structure of the spermatogonia are seen (fig. 3.11). Those designated A-type spermatogonia following the mammalian classification are slightly larger in volume than B-type spermatogonia. They have an ovoid nucleus with fairly homogeneous chromatin which is not associated with the nuclear membrane (fig. 3.12 & 3.13). The major cell axis lies parallel to the basal lamina giving a long cell surface contact with the basal lamina (fig. 3.12). In these cells the mitochondria tend to lie in clusters (fig. 3.11, 3.12 & 3.13) in the basal region with the Golgi body and centriole. The mitochondria show round, dumbbell or elongated profiles. A dense inter-mitochondrial substance is seen lying in the cytoplasm between clusters of mitochondria (fig. 3.13). There is also a small amount of rough ER and many loose ribosomes in the cytoplasm of these cells.

A-type /

A-type spermatogonia usually occur singly, whereas B-type spermatogonia occur in groups (fig. 3.11) often joined by intercellular bridges (fig. 3.14 & 3.15). B-type spermatogonia are distinguished by their clumps of chromatin particularly along the nuclear membrane and by the more spherical shape of the nucleus (fig. 3.14). Fingers of Sertoli cell cytoplasm are often observed between B-type spermatogonia and the basal lamina, though a small portion of the spermatogonia always remains in contact (fig. 3.14). Mitochondria are few and only spherical and elongated profiles are observed. They are not arranged in clusters (fig. 3.11 & 3.14) which is one of the distinguishing features between A- and B- type spermatogonia. There is little difference in the rough ER and ribosome content of A- and B- type spermatogonia (fig. 3.13 & 3.15).

Division of B-type spermatogonia results in resting or preleptotene spermatocytes very similar in appearance to the B-type spermatogonia (fig. 3.16). The metaphase plate is parallel to the basal lamina so the spermatocyte lies towards the centre of the tubule. Intercellular bridges are observed between spermatogonia and spermatocytes (fig. 3.17) and between two spermatocytes (fig. 3.16A). The bridges are often observed almost completed (fig. 3.16A) with the dense material between the two plasmalemma indentations fused.

In the first stage of the meiotic prophase, leptotene, the size of the nucleus is increased. The chromatin becomes dispersed and chromosomal cores or thin threads are seen (fig. 3.18, 3.19 & 3.20). There is an increase in the ER and tubular smooth ER differentiates (fig. 3.19). There is also an increase in the size of the Golgi body. This enlarges to form a horseshoe complex with two centrioles lying in the curve next to the nucleus (fig. 3.18, 3.20 & 3.21). /

3.20 & 3.21). The mitochondria cluster at this pole of the cell and a dense intermitochondrial substance is observed (fig. 3.20 & 3.21). Another organelle is formed during leptotene consisting of large vesicles lying between the outer nuclear membrane and a long narrow cisternum of ER (fig. 3.19 & 3.20).

During the second stage in meiotic prophase, zygotene, the chromosomal cores pair. The chromosomes become visible as electron dense material massed around the chromosomal cores (fig. 3.22, 3.23). Often these cores are to be seen attached to the nuclear membrane (fig. 3.22 & 3.33). The chromosome pairing is seen as the formation of synaptonemal complexes within the chromatin masses (fig. 3.23). A sex vesicle also forms within the nucleus. This is seen as a patch of chromatin fibrils (fig. 3.22 & 3.23). The pattern of mitochondria, Golgi body and ER distribution is similar to that of leptotene cells, but longer mitochondria with transverse cristae are often seen (fig. 3.23).

The third stage of meiotic prophase, pachytene, is the longest stage (Swiestra and Ford, 1963) so that pachytene nuclei are the most commonly observed stage in the spermatocyte population. The chromatin becomes denser and more compact round the synaptonemal complexes of a pachytene nucleus (fig. 3.24 & 3.25). Long lengths of these complexes characterise the pachytene nucleus. They consist of a medium dense central element running in a lighter region between two very dense parallel filaments surrounded by a dense conglomeration of chromosomal fibrils (fig. 3.26). The cisternae of the Golgi body now form a complete circle with the centrioles lying outside in a juxtannuclear position (fig. 3.25 & 3.27). Round or rod-shaped mitochondria with transverse cristae occur in groups around /



around the Golgi region. Longer lengths of rough ER (fig. 3.25), and some small lengths of smooth ER are seen in close proximity to the mitochondria (fig. 3.27). There are numerous free ribosomes in the cytoplasm (fig. 3.27).

The fourth stage of meiotic prophase, diplotema, is difficult to study because the chromosomes lose definition (Rhoades, 1961). During this stage there is dissociation of the chromosomes into a non-paired state and therefore the loss of the synaptonemal complex.

In the last stage, diakinesis, the chromosomal masses migrate to the periphery of the nucleus (fig. 3.28). During this stage the Golgi complex disappears and the nuclear membranes become a circle of vesicles and finally disappear too (fig. 3.29).

Metaphase is characterised by the dense masses of the chromosomes arranged on the equatorial plate of the spindle (fig. 3.30). The cytoplasm is much paler than during prophase and the ER becomes arranged as long strands round the periphery of the cell. The matrix of the mitochondria is very dense and the cristae are dilated and there is a clear intracristal matrix (fig. 3.31). The Golgi body has disappeared but a group of multi-vesicular bodies and lysosomes are seen (fig. 3.32).

During anaphase the cytoplasmic organelles remain the same as in metaphase but the chromosomes separate and migrate to either pole (fig. 3.32). The first meiotic division is completed by telophase with the formation of new nuclear envelopes round each chromosomal mass, and cleavage of the cytoplasm, which is normally incomplete as evinced by the number of cytoplasmic bridges seen during anaphase (fig. 3.32) which persist through to secondary spermatocyte formation (fig. 3.33).

The resulting /

The resulting secondary spermatocytes have nuclei half the size of the prophase nucleus (fig. 3.33). The chromosomes are seen as circular or Y-shaped dense masses often associated with the nuclear membrane (fig. 3.34). The cytoplasm has a similar appearance to that of the metaphase or anaphase stages of the primary spermatocytes with long profiles of smooth ER, loose ribosomes and mitochondria with dilated cristae (fig. 3.34 & 3.35). Some mitochondria resemble those of earlier prophase with transverse cristae and a dense intermitochondrial substance is seen between clusters (fig. 3.34). A Golgi complex and multivesicular bodies are present (fig. 3.34).

Division of the secondary spermatocytes produces interphase spermatids with slightly smaller nuclei. Their nucleoplasm is finely granular with some denser areas (fig. 3.36). Long profiles of smooth ER, loose ribosomes, mitochondria with dilated cristae, a simple Golgi complex and multivesicular bodies can be seen.

Intercellular bridges are more frequently seen between anaphase, telophase primary spermatocytes, secondary spermatocytes and spermatids (fig. 3.32, 3.33, 3.35 & 3.36) than they are between spermatogonia or other stages or spermatocytes. Specialised bridges between telophase cells have also been described by Nagano (1961). In these bridges a number of membranous cisternae are seen orientated at right angles to the original spindle axis lying along the plane of cleavage joining the plasma membrane indentation (fig. 3.36 & 3.37). The cisternae are not continuous with the plasma membranes and the space between cisternae is filled with an electron dense substance (fig. 3.37). The cisternae are straight in the centre of the bridge and curve into each cell on either side (fig. 3.36). The cisternae of the specialised bridges disappear during interkinesis (fig. 3.35 & 3.38).

## SPERMIOGENESIS

De Reviere (1971) working with the light microscope recognised eight stages of development during spermiogenesis in the cock. He described three categories of round spermatids ( $R_1 - R_3$ ), two categories during elongation of the nucleus ( $el_4^1 - el_5^2$ ) and three categories of elongated spermatids ( $L_6 - L_8$ ).

$R_1$  spermatids resemble secondary spermatocytes. At the electron microscope level they are seen as polygonal cells with an oval or spherical nucleus (fig. 3.38). These are the interphase spermatids described earlier.

$R_2$  spermatids are the equivalent of the mammalian Golgi stage spermatids. The proacrosome vesicle is seen within the Golgi complex (fig. 3.39). The nucleus is often slightly irregular in shape at this stage and the nucleoplasm is finely granular with some denser areas. Two stacks of Golgi cisternae lie at right angles to the nuclear membrane with a large dense spherical vesicle, the proacrosome, between them (fig. 3.40). Tubular smooth ER occurs in close proximity to both the Golgi complex and the mitochondria (fig. 3.40). Many mitochondria no longer have the dilated intracristal spaces of  $R_1$  spermatids (fig. 3.39). Groups of single membrane bound multivesicular or multigranular bodies are found near the Golgi zone (fig. 3.40) and also <sup>there is seen</sup> a new organelle which resembles a sunflower in appearance (fig. 3.39). It consists of stacks of parallel cisternae running between large vesicles filled with a flocculent material (fig. 4.43).

During the third stage of spermatid development ( $R_3$ ) the chromatin condenses around the periphery of the nucleus leaving a clear centre (fig. 3.41). The acrosome vesicle enlarges till it touches /

70.  
touches the nuclear membrane (fig. 3.41). The Golgi bodies then disappear and the spherical nucleus invaginates at the point of contact with the convex surface of the acrosome vesicle (fig. 3.41 & 3.42).

During stage four ( $el_4$ ) the nucleus begins to elongate becoming pear-shaped with a wavy nuclear envelope (fig. 3.41 & 3.42). The finely granular chromatin becomes evenly distributed throughout the nucleus (fig. 3.42). Groups of microtubules are observed near the nuclear membranes (fig. 3.42). The pair of centrioles come to lie in a juxtannuclear position often at a  $90^\circ$  radius from the position of the acrosome (fig. 3.43). This is the point of attachment for the spermatid tail, the origin and ultrastructure of which has been described by Nagano (1962).

The ( $el_4$ ) mitochondria begin to take up the characteristic form of the mature spermatid. They are spherical or oval with a medium dense matrix. The cristae are seen running in a transverse, diagonal or longitudinal direction (fig. 3.42).

At stage five ( $el_5$ ) the acrosome flattens against the nucleus and takes a hemispherical shape at the same time making contact with the cell membrane (fig. 3.44). The nuclear membrane invaginates to form a small vesicle below the acrosome and a small dense granule is observed within the cavity of this vesicle (fig. 3.44). This develops during stage six into the perforatorium which attaches the acrosome to the nucleus.

During the development of the spermatid from the spermatogonium to stage ( $el_5$ ) there has been a progressive movement away from the basal lamina so that  $el_5$  spermatids are situated at the luminal surface of the seminiferous tubule (fig. 3.45). The spermatids orientate themselves with the acrosome pointing towards the Sertoli nucleus and the basal lamina. /

lamina. Further elongation pushes the acrosome with the closely applied spermatid cell membrane into the Sertoli cell cytoplasm towards the basal lamina. The spermatid cytoplasm trails behind in a caudal position (fig. 3.44 & 3.46).

Elongation of the nucleus reaches a maximum at stage  $L_6$  (fig. 3.46). The elongation is achieved by means of the left handed double helix of microtubules surrounding the nucleus (McIntosh & Porter, 1967). A longitudinal section through an  $L_6$  spermatid shows rows of microtubules cut in cross section or where the section is cut obliquely through the spermatid, shows a parallel array of microtubules running across the spermatid (fig. 3.47). A transverse section through an  $L_6$  spermatid shows the microtubules surrounding the nucleus (fig. 3.48).

Bunches of long straight  $L_6$  spermatids are seen lying in the mid portion of the Sertoli cytoplasm with a cone-shaped acrosome at the level of the primary spermatocytes (fig. 3.46 & 3.47) and the tail free in the lumen (fig. 3.46). The Sertoli cytoplasm surrounding the acrosome shows patches of an electron dense amorphous material (fig. 3.47 & 3.48).

The  $L_6$  spermatid chromatin is condensed into dense granules (fig. 3.47) and the mitochondria are rod-shaped with longitudinal cristae (fig. 3.47 & 3.48) concentrated around the caudal end of the nucleus.

*by this stage.*

The two centrioles have ~~now~~ differentiated into the neck piece. The proximal centriole lies at right angles to the nuclear axis joined to the nucleus by five non-striated connecting pieces made of a dense homogenous material (fig. 3.49). The distal centriole lies in the plane of the nuclear axis joining the proximal centriole and axonemal complex. The plasma membrane is invaginated around the axonemal complex to the level of the distal centriole (fig. 3.49).

A dense /

A dense homogenous substance is observed in the cytoplasm adjacent to the distal centriole (fig. 3.49).

At stage L<sub>7</sub> a new contraction of the nucleus takes place and the chromatin granules coalesce to form a dense homogenous nucleus (fig. 3.50). The microtubules now run longitudinally down the length of the nucleus forming the manchette (fig. 3.48 & 3.50). These microtubules cause the nucleus to take up its characteristic 7 $\mu$  radius of curvature (McIntosh & Porter 1967). The nucleus begins to move towards the lumen partly through its own cytoplasm so that two wings or a single wing of cytoplasm on either side of the acrosome are formed (fig. 3.50, 3.51 & 3.52). The acrosome remains surrounded by an invagination of Sertoli cytoplasm in which is a series of dense patches around the acrosome tip (fig. 3.50 & 3.51). During stage L<sub>7</sub> the mitochondria arrange themselves around the distal centriole and axonemal complex to form the middle piece (fig. 3.53 & 3.54). The wings of spermatid cytoplasm are filled with multi-vesicular bodies and spare mitochondria not used in middle piece formation. Also during this stage there is an increase in the smooth ER. Short wide cisternae or vesicles are formed (fig. 3.50, 3.52 & 3.54). Many of these vesicles are observed adjacent to the plasmalemma (fig. 3.50).

L<sub>8</sub> spermatids are mature spermatids ready to be released into the lumen (fig. 3.55). They have a completely electron dense nucleus. The neck and middle piece are surrounded by mitochondria. The nucleus lies apart from the remains of the manchette and the cytoplasm is filled with large vesicles of smooth ER, spare mitochondria, lysosomes, multigranular and multivesicular bodies.

SPERMIATION /

## SPERMATION

The release of the mature spermatids into the lumen and the formation of residual bodies from the surplus spermatid cytoplasm is closely related to the action of the spermatid and Sertoli cell cytoplasm.

The process starts at the end of stage L<sub>6</sub> when the fully elongated nucleus begins to retract back to the lumen. As the nucleus moves back through the spermatid cytoplasm the Sertoli cell retains a surrounding layer over the acrosome portion (fig. 3.50 & 3.52). The two wings of residual spermatid cytoplasm that form on either side of the acrosome/Sertoli junction complex contain the spare mitochondria and many multivesicular and multigranular bodies (fig. 3.51). Cisternae and vesicular smooth ER is observed lying at random throughout the cytoplasm (fig. 3.50 & 3.52). As the spermatid moves back to the lumen and the chromatin condenses, the smooth ER increases in amount and becomes mainly vesicular. The vesicles tend to lie around the cell periphery or around the nucleus (fig. 3.56) and a few vesicles are observed opening into the intercellular space (fig. 3.57). All vesicles are single membrane bound. No double membrane bound vesicles formed by invaginations of the Sertoli cell cytoplasm into the spermatid cytoplasm such as seen in the hamster (Fouquet, 1974) are observed in the fowl. Possibly many more vesicles open into the intercellular space than are observed normally, because when the section is tilted through 40° using the goniometer stage of the microscope a vesicle that appears closed at 0° can be seen to be open to the intercellular cleft at 40° (fig. 3.58a & 3.58b).

Once stage L<sub>7</sub> is completed the mature spermatid that is to be released from the residual spermatid cytoplasm is held firmly by the acrosome/Sertoli junction complex. The spermatid cytoplasm remains /

remains surrounded by thin Sertoli cell processes throughout spermiation. The cytoplasm vesicles now increase in size and <sup>become</sup> invaginate (themselves) to form C-shaped vesicles (fig. 3.59), thus increasing their surface area. Some vesicles appear to coalesce around the nucleus so that it lies in a vesicle within the cytoplasm (fig. 3.59 & 3.60). The pressure caused by the expanding vesicles ~~would~~ then allow the spermatid to slip out of the cytoplasm. The last point of attachment before final release is the acrosome/Sertoli junction complex (fig. 3.61). After release long strands of Sertoli cell cytoplasm line the lumen and surround the vesiculated residual spermatid cytoplasm (fig. 3.62).

The remaining cytoplasm is filled with vesicles and one large vesicle where the spermatid was (fig. 3.62). The multivesicular and multigranular bodies break down releasing their contents (fig. 3.63) and the vesicles coalesce into larger vesicles forming double membrane vesicles (fig. 3.64) that come to enclose the spare organelles. A concentration of the cytoplasm occurs (fig. 3.65) until a dark condensed mass is absorbed into the Sertoli cytoplasm at the level of the round spermatids, where the Sertoli cytoplasm contains much tubular smooth ER and lipid droplets (fig. 3.66).

#### INTERTUBULAR TISSUE

The intertubular tissue of the fowl is characterised by a loose arrangement of constituent cell types. This loose arrangement and the abundant interstitial fluid means the tissue organisation is easily destroyed during preparation procedures, but the use of the perfusion technique developed in the present work gives some preservation before excision and enables the different cell elements to be identified.

The /



The Leydig cells have the characteristics of steroid hormone-producing cells namely abundant smooth ER, mitochondria with tubular cristae, a prominent Golgi complex and numerous lipid droplets (Rothwell, 1973) (fig. 2.70). The Leydig cells occur singly (fig. 3.67), in rows (fig. 3.69) or in clumps (fig. 3.70). They may occur clumped round a blood vessel (fig. 3.71) or separated from it by connective tissue and interstitial fluid (fig. 3.67).

Blood vessels are observed within the angle of the tubules usually in association with the boundary tissue (fig. 3.69).

The lymphatic vessels also lie within the angle of the tubules in a central position and take the shape of the intertubular region (fig. 3.69 & 3.71). They are lined with continuous endothelial cells that are supported by bundles of collagen in the surrounding interstitial spaces (fig. 3.68 & 3.69). The lymph fluid can be less electron dense than the interstitial fluid (fig. 3.68).

The other cell constituents are undifferentiated mesenchymal and cells/limited connective tissue with no trabeculae structure (fig. 3.68 & 3.67).

The density of the fine grained precipitate observed in the lymph vessels and interstitial spaces varied from bird to bird. Variations in the density are observed whatever fixative system is used as perfusate. The variation in density seems, therefore, to be a function of the efficiency of the perfusion.

The development of this pattern of organisation of lymphatic vessel/blood vessel/Leydig cell is demonstrated in figures 3.72, which is from a 3 week old bird, to figure 3.79 which is from a 15 week old bird.

In young prepubertal birds the proportion of the intertubular region to seminiferous tubule is far greater than in the 20 week /

week old bird (compare fig. 3.72 & 3.69). In the young bird the intertubular region is filled with polygonal Leydig cells. There are small centrally placed capillaries and small areas of connective tissue filled with interstitial fluid (fig. 3.72 & 3.73). By six weeks of age small lymphatic vessels can be seen amongst the Leydig cells (fig. 3.73).

With the onset of spermatogenesis at 9 weeks the seminiferous tubules increase in size rapidly (de Reviere, 1971) and the proportion of intertubular tissue to seminiferous tubule decreases (fig. 3.74). The interstitial spaces of the connective tissue increase in size (fig. 3.75) and the Leydig cells become more tenuous with interstitial fluid between them (fig. 3.76). During puberty regions that are filled almost entirely by polygonal Leydig cells (fig. 3.77) or by large lymphatic vessels (fig. 3.78) can be found within the same bird at 12 weeks of age. By 15 weeks of age the adult pattern of organisation is established (fig. 3.79).

#### SERTOLI CELL JUNCTIONS

In the prepubertal bird the Sertoli cell is a simple columnar epithelial cell (fig. 3.80 & 3.81) with lateral cell membranes running from the basal lamina to the lumen, which in the prepubertal bird is closed (fig. 3.1). These cell membranes show five types of Sertoli-Sertoli cell junctions, four of which have been described in an earlier preliminary study. (Coodsey & Rothwell, 1973). K

A longitudinal section through the Sertoli cell shows a series of adhesion points at the apical surface similar to the junctional complex of an epithelial cell (fig. 3.80 & 3.82). A series of alternating zonulae occludentes (tight junctions) and zonulae adherens (intermediary junctions) are followed by a macula /

macula adherens (desmosome) (fig. 3.82). The membranes of a zonula occludens fuse together and obliterate the intercellular space. The outer leaflets of the two unit membranes merge to form an intermediate line and give the junction a pentalaminar appearance (fig. 3.83 & 3.84).

The zonula adherens region has an intercellular space of 200 Å and a fibrillar material of moderate density in the subjacent cytoplasm (fig. 3.82 & 3.84). The fibrils can be seen to form a plate running parallel to the cell membrane from which it is separated by a narrow light zone (fig. 3.82).

The macula adherens or desmosome junction is characterised by an accumulation of electron dense material which forms a very dense plate 200 Å from the cell membrane on either side of the opposing cell membranes (fig. 3.82 & 3.83). The cell membranes run straight and parallel through the junction but lack the 'median stratum' line of typical desmosomes (Farquhar & Palade, 1963). The intercellular space is filled with striations of a diffuse moderately dense material (fig. 3.83).

Obliquely cut sections can show long lengths of the zonula occludens and adherens demonstrating the zonula or belt like nature of these junctions (fig. 3.84), whereas oblique sections through the macula adherens region show a series of button-like desmosomes (fig. 3.85).

Further down from the apical region of the cell (membrane) the next Sertoli-Sertoli cell junction type is the interdigitation of the adjoining cell membranes, (fig. 3.80, 3.81, 3.86 & 3.87). The base of the undulation of the inner cell surface has a subjacent layer of amorphous material that is similar looking to that of a coated vesicle.

Below /

Below the interdigitating region are the subsurface cisternal junctions. These lie just above the level of the Sertoli nucleus (fig. 3.81). The apposing cell membranes have hemispherical subplasmalemmal cisternae lying at a distance of 120 Å from the plasmalemma (fig. 3.87). Ribosomes are attached to the inner or cytoplasmic side of the cisternae. The intercellular space remains normal and the cell membranes run straight and parallel throughout the junction.

In the prepubertal bird where two Sertoli cells meet above a spermatogonium there is no constriction of the intercellular space (fig. 3.88 & 3.89). With the onset of spermatogenesis and the formation of pachytene spermatocytes punctate tight junctions are observed in this position above a spermatogonium (fig. 3.90). An amorphous material may be seen subjacent to the cell membrane at the point where a constriction will later develop (compare fig. 3.89 & 3.90). These tight junctions are also observed where two Sertoli cells adjoin spermatocytes (fig. 3.91 & 3.92). Slightly longer tight junctions, showing clearly the pentalaminar structure, are seen in close proximity to the subsurface cisternal junction above the level of the spermatocyte nucleus (fig. 3.93 & 3.94). No narrowing of the cell membranes within the cisternal junction is observed at any age.

Once elongated spermatids are present at the tubule lumen the apical junctional complex disappears.

### 3.3 DISCUSSION

Spermatogenesis, the differentiation of the male germ cells is a complex process that involves initially a stage of cell proliferation, first of the Sertoli cells and then of the spermatogonia, secondly a stage of meiotic division and finally a stage of differentiation of the spermatids into mature spermatozoa.

In the prepubertal stage the testis of the fowl consists of Sertoli cells and prespermatogonia. Prespermatogonia are difficult to distinguish from later A-type spermatogonia, <sup>in fsw</sup> In that both the nuclei show a diffuse distribution of chromatin and clusters of diversely shaped mitochondria occur in the cytoplasm. The main distinguishing features are that prespermatogonia have nuclei which lie above the level of the Sertoli cell nuclei and have only a small area of contact with the basal lamina. No intercellular bridges are observed between prespermatogonia. This is in contrast to the situation found in the postnatal rabbit testis (Gondos, Renston & Conner, 1973) where intercellular bridges are a distinguishing feature of the prespermatogonia. In the fowl prespermatogonia are characteristically separated from one another by several Sertoli cells.

Two types of spermatogonia have been observed once spermatogenesis has begun. This agrees with de Reviere (1971) who described two types of spermatogonia using the light microscope. However, in other birds e.g. the quail (Yamamoto et al, 1967) and duck (Clermont, 1958) three types of spermatogonia (A-type, intermediate-type and B-type) have been observed. Although only A- and B- type spermatogonia have been described in this thesis, it is possible that there is an intermediate-type as well. The chromatin condensation of B-type spermatogonia varied considerably but a graduation of /

of condensation made it impossible to distinguish definitely an intermediate-type spermatogonia. The continuous production of spermatozoa from the seminiferous epithelium must involve a renewal process of spermatogonial stem cell division. In mammals renewal of the stem cells is either from prespermatogonia or by isolation of preferential cells following spermatogonial division. For instance in the rat  $A_1$ -type spermatogonia undergo three divisions before they produce  $A_1$ -type spermatogonia again (the stem cell) and intermediate-type spermatogonia, which divide to produce B-type spermatogonia (Leblond & Clermont, 1952). Analysis of spermatogonial generations in the cock using labelled thymidine injections to spot DNA synthesis has not been able to indicate clearly whether there were 2 or 3 generations of spermatogonia (de Reviere, 1968a, see Courot, Hochereau-de Reviere & Ortatvant, 1970). The number of spermatogonial divisions varies considerably between different classes of animal. This study confirms that it is low in birds compared to mammals where generally there are 4-6 generations (Leblond & Clermont, 1952; Swierstra & Foote, 1963). In fish there can be as many as 13 (Holstein, 1969).

The diversely shaped mitochondria and the dense inter-mitochondrial substance observed in fowl prespermatogonia, spermatogonia and spermatocytes are thought in the rabbit to be a sign of rapid biogenesis of mitochondria in preparation for the cell division and organelle redistribution of the spermatogonia (Nicander & Plöen, 1969). The similar appearance of the mitochondria in the fowl suggests they may also be a sign of rapid biogenesis.

The resting spermatocytes resemble the B-type spermatogonia. They are better described as preleptotene spermatocytes because during this stage it has been shown that the cells are actively synthesising DNA (Monesi, 1962).

During /

During prophase the fowl spermatocytes increase in volume and the cytoplasmic organelles alter in number and complexity. The leptotene stage marks the differentiation of smooth ER. This is considered to be synthesised initially at the rough ER (Dallner, Siekevitz & Palade, 1966). It reaches a maximum amount at metaphase when long peripheral strands are observed. The Golgi body is transformed at leptotene to **become** a large horseshoe-shaped complex of cisternae and vesicles in close association with the centrioles. Multivesicular and multigranular vesicles are first observed near the Golgi apparatus and may be formed in relation to it. The origin and function of these vesicles and strands of rough ER in a juxtannuclear position is unknown.

The structure of spermatocyte mitochondria alters considerably during meiosis. The cristae swell during metaphase and anaphase to form a pseudomatrix so that the mitochondria appear to be reversed (Andre, 1962). The dense intermitochondrial substance observed between clustered mitochondria (see Fig. 3.21) in the fowl is thought by Andre (1962) to be in rat spermatocytes an aggregation or coalescence of ribosomes and he proposed that it marked the site of formation of new mitochondria. Mitochondria have since been shown to arise from pre-existing mitochondria by division (Luck, 1963). (Clerott (1968) was unable to demonstrate the presence of RNA in the dense intermitochondrial substance of frog spermatocytes. Fawcett, Eddy & Phillips (1970) have suggested that this dense substance is the precursor material of the chromatoid body of mammals. Electron histochemical studies have failed to show the presence of RNA in the chromatoid body (Eddy, 1970). The chromatoid body in rat is first observed in primary spermatocytes, persists through /

through spermiogenesis and finally comes to lie round the flagellum in the region of the annulus. It is composed of thin filaments that are consolidated into dense strands that form an irregular network. During stage  $L_6$  in fowl spermatids dense masses are seen lying around the distal centriole and it is thought these may correspond to the chromatoid material of mammalian spermatids (see fig. 3.49).

Comings & Okada (1972) dispute the idea that the chromatoid body originates from the dense intermitochondrial substance. They suggest that in the mouse the chromatoid body is an extrusion of nucleolar material. Such a view is supported by light microscope cyto-chemical studies which suggest it is composed of basic proteins and RNA (Sud, 1961). These authors consider such studies to be more accurate than electron histo-chemical investigations.

Nuclear changes during meiotic prophase in the fowl are similar to those in mammals and in the pigeon (Fawcett, 1956). The synaptonemal complex of meiotic prophase has been positively identified as occurring during synapsis of bivalent chromosomes (Moses, 1968). The lateral elements of the synaptonemal complex are considered to be the core of the single meiotic prophase chromosome and the central element is only visible when the chromosomes are paired.

The mechanics of spermiogenesis in the fowl have been beautifully illustrated and explained by McIntosh & Porter, (1967). Briefly the round spermatid is elongated by a left-handed double helix of microtubules surrounding the nucleus. Cross bridges connect consecutive turns of the two helices and force successive turns of the helices to slide over one another thus constricting the nucleus and reducing its diameter. Once elongation is complete the microtubules are replaced by a paraxial array of microtubules, the manchette /



manchette, which determines the curvature of the final sperm head. Spermatids with a malformation of the manchette resulting in a coiled up nucleus after elongation are not uncommon and led Zlotnik (1947) to propose this was a normal part of spermiogenesis.

The formation of the acrosome of the fowl in the Golgi body and its attachment to the nucleus by the formation of a perforatorium has been described in an early electron microscopical paper by Nagano (1962). This author, however, did not describe the relationship between the acrosome and the Sertoli cell. Electron micrographs presented in this thesis (fig. 3.40, 3.42, 3.44, 3.47, 3.51, 3.52 & 3.61) show the development of the acrosome/Sertoli junction complex and demonstrate the role it plays in spermiation.

During the elongation of the spermatid, the acrosome with the closely applied cell membrane protrudes from the body of the cell and takes up its characteristic cone shape. The acrosome is the only part of the spermatid to protrude into the Sertoli cell cytoplasm unlike <sup>the situation for</sup> mammalian spermatids in which both the nucleus and the acrosome lie within the Sertoli cytoplasm (Fawcett & Phillips, 1969).

The acrosome/Sertoli junction complex in the rat (Brokelmann, 1963) and other mammals (Flickinger & Fawcett, 1967) has been shown to consist of a series of dense parallel bundles of filaments lying between the Sertoli plasma membrane that surrounds the acrosome and a layer of subsurface cisternae. In the fowl dense patches of an amorphous substance (fig. 3.47 & 3.48) are observed in the Sertoli cytoplasm subjacent to the acrosome but there are no corresponding cisternae. These acrosome/Sertoli complexes play an important part in both mammalian and avian spermiation.

The present work indicates that spermiation in the fowl occurs in several stages, firstly a swelling of the spermatid smooth

ER to form vesicles (fig. 3.50), secondly an invagination of these vesicles increasing their surface area (fig. 3.56), thirdly a coalescence of the vesicles round the spermatid so that it lies free within a large vesicle in the cytoplasm (fig. 3.59, 3.60) and finally the release of the acrosome by the Sertoli cell resulting in the release of the spermatid into the lumen (fig. 3.61, 3.62). The Sertoli cytoplasmic processes surround the spermatid and its cytoplasm throughout this process so that when the spermatid is released the residual cytoplasm is phagocytosed by the surrounding Sertoli cell without being extruded into the lumen (fig. 3.65).

This differs in several respects to the mechanism of spermiation described for amphibious and mammals (Burgos & Vitale-Calpe, 1967; Vitale-Calpe, 1970) and from the mechanism described for mammals by Fawcett & Phillips (1969). Spontaneous spermiation in the toad (Burgos & Vitale-Calpe, 1967), the hamster (Vitale-Calpe & Burgos, 1970a) and the guinea pig (Vitale-Calpe, 1970) are described as occurring by **the widening** of the Sertoli ER and swelling of the cytoplasmic matrix erasing the apical recess that holds the head (acrosome/nucleus) of the spermatid and thus pushing it into the lumen. ians.

This mechanism was disputed by Fawcett & Phillips (1969) who suggested that simple effacing of the crypts occupied by sperm heads does not account for retention of residual bodies. They suggested there were active movements of the apical Sertoli cytoplasm holding the residual cytoplasm while the heads are progressively extruded. This description is in better agreement with the mechanism proposed for the bandicoot by Sapsford & Rae (1969) and for the hamster by Fouquet (1974).

Fouquet /

Fouquet disagrees with Vitale-Calpe and Burgos' proposal that there is apical swelling of the Sertoli cytoplasm in the hamster. *ascribes this appearance to* He accounts for their image as being an artefact of fixation. He suggests that Sertoli processes penetrate the spermatid cytoplasm that covers the middle piece. These are seen as double membrane bound vesicles containing cytoplasm and act as clamps retaining the cytoplasm as the spermatid is pushed out into the lumen by the obliteration of the Sertoli crypts.

Vitale-Calpe and Burgos (1970b) claimed that the effect of administration of LH on spermiation in the hamster was to cause the release of younger than normal spermatids and postulated that sperm release was under hormonal control. However, Vitale-Calpe has been unable to repeat these results while working in Fawcett's laboratory (Dym, Personal communication).

It would appear from this work that the mechanism of spermiation in the fowl is <sup>A</sup>different essentially because the spermatid nucleus lies within the spermatid cytoplasm at all times. The backward movement of the spermatid through the cytoplasm forms two wings of residual cytoplasm while the Sertoli cell maintains its hold on the acrosome. There are no Sertoli processes within the body of the cytoplasm and the proces is one of loosening the hold of the spermatid cytoplasm by the formation and coalescence of vesicles. The obliteration of the Sertoli cell recess holding the acrosome and the release of the spermatid into the lumen is the same as in the mammal.

The degeneration and phagocytosis of the residual bodies of the fowl have not been dealt with in this thesis beyond observing that the multigranular and multivesicular bodies break down releasing their contents, and that double membraned structures are observed in the /

the vicinity. These observations are in agreement with those of Dietert (1966) who examined the fate of mouse residual bodies and found that the multigranular vesicles were lysosomes with acid phosphatase activity. Dietert also observed that some of the residual bodies were connected by intercellular bridges. In the fowl no intercellular bridges were observed between spermatids once elongation of the nucleus had begun.

The full biological significance of the syncytial organisation of male germ cells is not yet established, but it is thought to play a part in maintaining the synchrony of the germ cell differentiation that results in cell-associations (Fawcett et al, 1959). Pairs of spermatocytes and groups of at least four spermatids were observed in the rat (Fawcett et al, 1959), but Roosen-Runge (1962) does not think the small size of these clones provides a sufficient explanation for the wide area of synchronous development in the rat, where cross sections of seminiferous tubules usually show the whole section at the same stage of the cycle. Later work on mammalian species (Dym & Fawcett, 1971) has indicated that much larger numbers of spermatids are connected and may number in the hundreds.

In the rat Perey et al (1961) suggested that a pair of type A spermatogonia and their progeny occupied a certain area of the tubule which they designated a unit segment and adjacent unit segments made up a particular stage of the cycle. Dym & Fawcett (1971) suggest that a unit segment is made up of conjoined cells in perfect synchrony whereas there is less precise synchrony between the (other) adjacent units of that stage of the cycle.

In the fowl several cell-associations (or stages) are seen in one cross section of the tubule (fig. A) (de Reviere, 1971). This situation is similar to that in the human (Clermont, 1963).

This /

This may indicate that there is little synchrony between unit segments but each unit segment has synchronous cells producing a particular cell-association. In the fowl numerous atypical cell-associations are observed (de Reviere, 1971). This may be accounted for by the lack of cell bridges or completed cell bridges observed in the fowl testis. Intercellular bridges between spermatogonia or between spermatocytes were not as common as those described for mammalian species (Dym & Fawcett, 1971). Also cell bridges were often observed almost completed as in figure 16A, where the two plasmalemma indentations with their electron dense layers have met and the electron dense areas have fused. This is not the same as the electron dense midbody described between the plasmalemma indentations of the intercellular bridges of mammals (Dym & Fawcett, 1971). If more cells are truly separate in the fowl this may account for asynchronous development and atypical cell-associations.

Intercellular bridges were more common between metaphase spermatocytes and between spermatids and in the fowl bridges with multiple cisternae oriented perpendicular to the bridge were seen in metaphase cells. Cell degeneration is a normal occurrence at certain stages of the spermatogenic cycle (Oakberg, 1956) so this type of bridge with transverse cisternae is considered by Dym & Fawcett to prevent cell death spreading to other cells in the syncytium.

Another explanation for the number of cell-associations seen in cross-section of the fowl seminiferous tubule is that the tubules branch and anastomose to a considerable degree so that it would be difficult for these to be an orderly spermatogenic wave as in the rat.

The /

The organisational pattern of the intertubular region that maintains the local androgen concentration required to sustain spermatogenesis has only fairly recently been studied in mammals (Fawcett et al, 1973). Because of the delicate nature of the intertubular lymphatic systems their extent and arrangement have only been realised since the advent of perfusion fixation (Fawcett et al, 1969).

A great deal of variation in the histology of the intertubular tissue is observed in different species of mammal (Fawcett et al, 1973). Three main patterns of organisation were described. Firstly those species which have a small volume of Leydig cells, very little interstitial connective tissue and large peritubular lymphatic sinusoids e.g. rat and mouse. The second category has clusters of Leydig cells widely scattered in a loose connective tissue stroma with conspicuous lymphatic vessels e.g. ram, monkey and man. Thirdly are those species with closely packed epithelioid Leydig cells filling nearly all the intertubular region. In this case the lymphatics are few and small e.g. boar and zebra. The organisation of the fowl intertubular region is similar to the second category. This observation fits in with those for fowl boundary tissue, which has also been found to resemble the pattern in the monkey or man most closely (Rothwell & Tingari, 1973).

The development of the lymphatic vessels, loose connective tissue and abundant interstitial fluid has been shown to be correlated with the onset of puberty. At 7-9 weeks spermatogenesis begins and hence a supply of nutrients for the developing germ cells is required. At this age the interstitial spaces become established.

The fine grained precipitate found in the interstitial spaces of the intertubular tissue is interpreted by Fawcett et al (1973) as being plasma protein. Androgens and nutrients are thought to /

to diffuse to the tubules through the edematous ground substance. The lymphatic vessels which are generally centrally located would <sup>then</sup> be concerned with the return of the proteins to the general circulation.

In the prepubertal fowl a junctional complex has been described that is similar to the typical junctional complex of all epithelial cells (Parquhar & Palade, 1963). The complex in the fowl at the luminal surface consists of a series of zonulae occludentes, which act as diffusion barriers or seals; zonulae adherens which are the insertion sites for the microfilaments of the terminal web, and a desmosome, which provides structural resistance to lateral shearing forces. These junctional complexes are lost once spermatogenesis has begun and spermatocytes are progressing towards the lumen.

Other junction types are also present in a more basal region of the Sertoli cell membrane in the prepubertal bird. The subsurface cisternal junctions have no narrowing of the intercellular space within them or associated with them. These and the interdigitating junction are retained after puberty.

Once spermatogenesis has begun unusual junctional specialisations between adjoining Sertoli cells are seen. These have also been described in many species of mammal (Brokelmann, 1963; Flickinger & Fawcett, 1967; Nicander, 1967; Dym, 1973). In the fowl, where adjoining Sertoli cells meet above a spermatogonium, a focal tight junction is observed with the typical pentalaminar form (fig. 3.92). Subsurface cisternal junctions are also found in a more apical position often above the level of the first layer of spermatocytes and associated with these are another focal tight junction usually in a basal position relative to the cisternal junction (fig. 3.94).

This /

This situation differs somewhat from that in the rat (Dym & Fawcett, 1970) or monkey (Dym, 1973). In these animals there is a series of focal tight junctions followed by extensive regions of subsurface cisternal junctions. The structure of these subsurface cisternal junctions differs from those of the fowl. In the rat the cisternae are separated from the cell membrane by a  $500\text{\AA}$  gap as opposed to a  $120\text{\AA}$  gap in the fowl. In the rat there are also groups of hexagonal packed filaments in the cytoplasm between the cisternae and the cell membrane. These have not been found in the fowl. In the rat the normal  $200\text{\AA}$  intercellular space narrows to  $90\text{\AA}$  and in certain regions to  $20\text{\AA}$  within the junction. These gap junctions have not been observed in fowl testis. The only narrowing of the intercellular space is at the focal tight junctions immediately above the spermatogonia or next to a subsurface cisternal junction.

Gap junctions were described first by Revel & Karnovsky (1967), who demonstrated their difference from tight junctions by the use of tracer substances. Penetration of lanthanum into the gap junction revealed a hexagonal array of subunits in the cell membrane. Gap junctions have been implicated in intercellular communications i.e. cell to cell transfer of ions (ionic coupling) and metabolites (metabolic coupling) (Gilula, Reeves & Steinbach, 1972).

As gap junctions are considered to function as a cell communication network, the lack of them in the fowl testis may be another reason for the large number of different cell-associations found in a cross-section of tubule.

Freeze fracture preparations of junctional areas between Sertoli cells in the rat disclose within the membrane multiple parallel rows /



rows of particles and short rods (Fawcett, 1973). Fawcett believes these underlie the lines of fusion of the outer leaflets of adjoining cell membranes. The length of these images suggest that these tight junctions are linear and not focal. He found that the image of a Sertoli cell freeze-cleaved tight junction was quite different to that of a zonula occludens. Since both types of junction are effective barriers it is suggested that the difference in structure is due to the permanence of the zonula occludens as opposed to the relative mobility of the Sertoli tight junction which must be continually dissolved as a new spermatocyte generation is formed and reconstituted below it.

In the following chapter experiments have been carried out to determine whether the punctate tight junctions between Sertoli cells in the fowl provide an effective barrier to the passage of tracer substances.

THE BLOOD-TESTIS BARRIER IN THE FOWL4.1 INTRODUCTION

The concept of a permeability barrier to the passage of blood-borne substances to the brain was established at the end of the last century by the discovery of the exclusion of dyes from the brain following injection into the blood stream. At the beginning of this century it was also noticed that dyes were excluded from the seminiferous tubules of the testis following intravenous injection (Ribbert, 1904) but for many years this observation was largely overlooked. The blood-brain barrier, however, has been studied extensively and is considered to be located at the level of the capillary endothelium.

Bennett, Luft and Hampton (1959) working on mammalian tissue described the ultrastructure of several distinct types of capillaries, which varied in their permeability to large molecules. One type exemplified by the capillaries of skeletal and cardiac muscle has a continuous endothelium. Adjoining endothelial cells are separated by an intercellular gap 150-200Å wide, but there are small areas where the membranes are closely approximated. These areas maintain cell adhesion without extensive obliteration of the intercellular clefts. Substances are thought to move across the capillary walls either by intercellular vesicular transport or by passing through the narrow cleft between the endothelial cells (Bruns and Palade, 1968; Karnovsky, 1967) and of the two pathways, the intercellular route is believed to be quantitatively the more important.

The fenestrated capillaries that are found in the absorptive intestinal mucosa, the renal glomerulus and nearly all endocrine glands, are the most permeable capillaries (Pappenheimer, Renkin & Borrero, 1951). The cell junctions are similar to those of muscle capillaries, but the endothelium is extremely attenuated and penetrated by numerous pores about  $600\text{\AA}$  in diameter which are closed only by a delicate diaphragm.

The cerebral capillaries are quite different. They have extensive tight junctions between the endothelial cells which seem to seal off the intercellular cleft and prevent the passage of extracellular tracers such as horseradish peroxidase (Reese and Karnovsky, 1967; Brightman and Reese, 1969). These special junctional complexes between endothelial cells appear to be the morphological basis of the blood-brain barrier.

The concept of a blood-brain barrier has become widely accepted but the idea of a blood-testis barrier only began to receive more attention after DeBryn<sup>u</sup>, Robertson and Farr (1950) had noted that fluorescence of testicular nuclei was exceptionally weak following subcutaneous administration of diamino acridine dyes. After intravenous administration other compounds are also excluded from the testis e.g. lissamine green (Goldacre and Sylvén, 1962), actinomycin (Ro and Busch, 1965) and rubidium (Waites and Setchell, 1966). The experiments using intravenously administered substances suggested that the testicular capillary endothelium has a low permeability to a variety of substances. In contradiction to these reports other work (Everett and Simmons, 1958; Cowie, Lascelles and Wallace, 1964; Mancini, Vilar, Alvarez and Seiguer, 1965) has shown

that the capillaries of the testis are highly permeable to serum proteins, which suggests that the blood-testis barrier is not located at the level of the capillary endothelium. This agrees with the results of subcutaneous administration of dyes which suggest the barrier operates at another level within the testis.

The question of testicular capillary permeability was further studied by Kormano (1967) using the light microscope. He found intravenous injections of the dye, light green, failed to stain the testis of adult rats, which suggests there is a barrier at the capillary endothelium level. On the other hand when acriflavine dye was administered subcutaneously to adult rats he found nuclear fluorescence of the endothelial cells and interstitial cells but no fluorescence of the cells of the seminiferous tubules, which again suggests that the barrier mechanism may not operate at the level of the capillary endothelium. When either dye was injected by either method into a prepubescent rat there was staining of the whole testis including the seminiferous tubules. Kormano attempted to relate this establishment of the barrier at the onset of puberty to the concurrent appearance of alkaline phosphatase activity in the capillaries. This enzyme is believed to be concerned with the energy supply for transport phenomena across the capillary walls (Nandy and Bourne, 1963). Kormano therefore assumed the blood-testis barrier to be operative at the level of the capillary endothelium as in the brain.

More recently, the testis, unlike other endocrine glands which have fenestrated capillaries, has been shown to have continuous endothelium capillaries like those found in muscle (Fawcett, Leak and Heidger, 1970). In the rat the intercellular clefts between

endothelial cells are at least  $200\text{\AA}$  wide and areas of closer approximation are of limited extent. Different sized molecules e.g. carbon and horseradish peroxidase were observed throughout these clefts, thus there is no morphological basis for a permeability barrier at this level in the tissue.

Other work (Everett and Simmons, 1958; Lindner, 1963; Mancini, Vilar, Alvarez and Seiguer, 1965) had already shown that the capillaries of the testis were highly permeable to serum proteins and physiological studies of mammalian testis involving cannulation of the testicular lymphatics and rete testis (Voglmayr, Waites and Setchell, 1966; Setchell, 1967; Setchell, Voglmayr and Waites, 1969) have demonstrated that while substances pass readily from the blood plasma to the lymph fluid, only a few pass freely across the seminiferous tubules into the testicular fluid. The site of the permeability barrier was therefore concluded to be in or around the seminiferous tubules.

The abdominal position of the testis precludes the possibility of cannulation experiments to determine any differences in the composition of the blood, lymph or rete testis fluid in the fowl. However, use of the indicator fractionation technique to measure testicular blood flow has indicated a rubidium rejecting compartment in the fowl testis (Lake, personal communication).

Morphological studies using electron opaque extracellular tracers (Fawcett, et al, 1970; Dym and Fawcett, 1970; Dym, 1973) have substantiated the hypothesis that the site of the blood-testis barrier was at the seminiferous tubules. In the rat and guinea pig interstitially injected large particulate markers, carbon and colloidal thorium, were excluded from the seminiferous tubule by

occluding junctions in the peritubular myoid cell layer.

However, in some areas of the tubule the smaller tracer molecules, ferritin and the enzyme, peroxidase, a protein, were able to pass between the cells in the myoid cell layer and enter the intercellular spaces between the spermatogonia and the Sertoli cells of the tubule. Deeper penetration than to the level of pre-spermatocytes was prevented by specialised junctions between Sertoli-Sertoli cells (Fawcett, et al, 1970). Intravascular injection of colloidal lanthanum and peroxidase in the rat (Dym and Fawcett, 1970; Vitale, Fawcett and Dym, 1973) also demonstrated a partial barrier at the peritubular myoid cell layer and a complete barrier at the Sertoli-Sertoli junction.

In the monkey (Dym, 1973) the pattern of organisation of the seminiferous tubule boundary tissue is different to that in rodents. No occluding junctions have been observed in the peritubular myoid cell layer and intravascularly injected lanthanum and horseradish peroxidase penetrate readily between the cells of this layer and into the intercellular spaces between spermatogonia and between Sertoli cells as far as their occluding junctions. The specialised Sertoli-Sertoli junctions are therefore the only component of the blood-testis barrier in the monkey.

The pattern of organisation of peritubular boundary tissue in the fowl testis is more like that of the monkey and man than that of rodents. In the present work colloidal lanthanum and horseradish peroxidase have been used to determine whether or not there is a blood-testis barrier in the fowl and if so, at what level in the tissue it operates.

Lanthanum was first used during fixation as an en bloc stain for the surface coat of cells (Doggenweiler and Frenk, 1965), but Revel and Karnovsky, (1967) found that by making the lanthanum nitrate solution alkaline before adding it to the fixative, they obtained a precipitate of colloidal lanthanum in the intercellular spaces. It was so easily washed out again that they concluded it was not bound to specific molecular components of the cell membranes. They further demonstrated that colloidal lanthanum could be used to distinguish between the permeability of a tight junction and a gap junction.

Graham and Karnovsky, (1966) introduced the use of horseradish peroxidase as a tracer molecule at the ultrastructural level. It is a small protein enzyme with a molecular weight of 44,000, visualised by incubating the tissue with diaminobenzidine, which reacts with the peroxidase to give an electron-opaque product. It gives good localisation and the amplifying effect of enzymatic activity produces great sensitivity, because a few molecules of peroxidase at a site can yield a relatively larger amount of reaction product which can be easily visualised. This technique has been used to localise the blood-brain barrier at the fine structural level (Reese and Karnovsky, 1967; Brightman and Reese, 1969).

The blood-brain barrier is established early in fetal life (Olsson, Klatzo, Sourander and Steinwall, 1968) unlike the blood-testis barrier which does not develop until some time after birth (Korman, 1967). In the rat Vitale et al., (1973) found that the development of the barrier was correlated with the differentiation of the pachytene spermatocytes which also coincided with the development of the Sertoli-Sertoli junctions. In this study as well as

localising the blood-testis barrier in the fowl, experiments have  
been carried out to relate <sup>to</sup> the development of the testis ~~to~~ the  
( development of the barrier. )



## 4.2 MATERIALS AND METHODS

### 4.2.1 Lanthanum Nitrate

#### General Procedure

Male Shavers of 6, 9, 12, 15 and 21 weeks of age were anaesthetised and the heart and testis exposed as described in surgical procedures, Chapter 2. 200 ml of one of the initial fixatives (Table 4.1) at room temperature was perfused into the aorta (route 1) using a 23G needle and a pressure of 200 mm Hg provided by the perfusion apparatus. The left testis was excised and 1.5mm<sup>3</sup> blocks of tissue were fixed in the initial fixative for 1 or 2 hours, washed and osmicated in the corresponding buffer and fixative (Table 4.1). The tissue was dehydrated in ethanol and embedded in Araldite. White/silver sections were cut on an LKB Ultratome III, mounted on copper grids and examined either unstained or stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) in a Phillips 300 electron microscope.

#### Lanthanum perfusion fixation studies

A series of perfusions were carried out using the fixative systems in Table 4.1. The initial fixative was made up according to a modification of the method of Dym and Fawcett (1970). A 2% lanthanum nitrate solution was slowly adjusted to pH 7.6 using 0.1N NaOH with vigorous stirring. This solution was added to an equal volume of the buffer at pH 7.4 prepared in such a way that the final concentration was equal to that ordinarily used for perfusion fixation without lanthanum (Table 2.7). The buffered lanthanum solution was then made up with glutaraldehyde to give

Table 4.1 Fixative/buffer systems used for lanthanum perfusion

<u>Initial Fixative</u>	<u>Buffer Wash</u>	<u>Post-fixative Buffer</u>
1. 4% GTA  0.075M Na cacodylate  1% La (NO <sub>3</sub> ) <sub>3</sub>  Time: 1 hour	0.075M Na cacodylate  0.2M sucrose  1% La (NO <sub>3</sub> ) <sub>3</sub>  3 x 30 minutes	a) 0.175M Na cacodylate  1% La (NO <sub>3</sub> ) <sub>3</sub>  b) 0.1M PO <sub>4</sub>  c) 1.5% K <sub>4</sub> Fe(CN) <sub>6</sub>  1 hour
2. 4% GTA  0.1M s-collidine  1% La (NO <sub>3</sub> ) <sub>3</sub>  Time: 1 hour	0.2M s-collidine  1% La (NO <sub>3</sub> ) <sub>3</sub>  3 x 30 minutes	0.1M s-collidine  1% La (NO <sub>3</sub> ) <sub>3</sub>  1 hour
3. 2.5% GTA  0.1M Millonig's phosphate  1% La (NO <sub>3</sub> ) <sub>3</sub>	* -	-

200ml of initial fixative were perfused into the bird.

The tissue was processed in the fixative, washed in buffer and post-fixed for the times stated at room temperature. \* This fixative was not used because the phosphate precipitated the lanthanum.

the final fixative solution. The Millonig's phosphate fixative was discarded before use because the mixing of the phosphate buffer and lanthanum solution caused precipitation of the lanthanum.

#### Assessment of prewash requirement

A series of perfusions were carried out using 200ml of initial fixative preceded by a 100ml perfusion of a prewash (Table 4.2). Control birds were perfused without a prewash (treatments 1 & 6, Table 4.2). Blocks of tissue were fixed for 2 hours in cacodylate buffered fixatives and for 1 hour in s-collidine buffered fixatives, washed and osmicated in the corresponding solutions. (Table 4.2).

#### Assessment of other methods of applying lanthanum fixatives

Two 17 week old Shavers were anaesthetised and the body cavity opened to expose the testes. Clamps were placed on the aorta and on the posterior vena cava anterior and posterior to the testicular artery bifurcation. These blood vessels were then cut anterior to the anterior clamps and posterior to the posterior clamps and the testes with associated clamped blood vessels were dissected out. The anterior clamp on the aorta was released and a 23G needle inserted into the aorta. 40ml of initial fixative (Table 4.1) were perfused by means of a syringe attached to the needle using hand pressure. The posterior clamp on the vena cava was released to allow free flow. 1.5mm<sup>3</sup> blocks of testis were taken and processed as described in general procedure.

1.0mm<sup>3</sup> blocks of testis from a 15 week old Shaver were fixed by immersion in fixative 1 (Table 4.1) for 1½ hours at room

Table 4.2 Prewash and Lanthanum fixative/buffer systems

<u>Prewash</u>	<u>Initial Fixative</u>	<u>Buffer Wash</u>	<u>Post-fixative</u>
1. -	4% GTA 0.075M Na cacodylate 1% La(NO <sub>3</sub> ) <sub>3</sub>	0.075M Na cacodylate 1% La(NO <sub>3</sub> ) <sub>3</sub>	1% OsO <sub>4</sub> 0.1M PO <sub>4</sub>
2. 4% GTA 0.075M Na cacodylate	"	"	"
3. 1% saline	"	"	"
4. Avian Ringer	"	"	"
5. 0.075M Na cacodylate 4% Dextran	4% GTA 0.075M Na cacodylate 2% Dextran 1% La(NO <sub>3</sub> ) <sub>3</sub>	0.075M Na cacodylate 2% Dextran 1% La(NO <sub>3</sub> ) <sub>3</sub>	1% OsO <sub>4</sub> modified Dalton's 1% La(NO <sub>3</sub> ) <sub>3</sub>
6. -	4% GTA 0.1M s-collidine 1% La(NO <sub>3</sub> ) <sub>3</sub>	0.2M s-collidine 1% La(NO <sub>3</sub> ) <sub>3</sub>	1% OsO <sub>4</sub> 0.1M s-collidine 1% La(NO <sub>3</sub> ) <sub>3</sub>
7. 4% GTA 0.1M s-collidine	"	"	"

100ml of prewash followed by 200ml of initial fixative were perfused into the bird. The tissue was processed in the initial fixative, washed and osmicated in the corresponding buffer washes and post-fixatives.

temperature and processed as above.

#### The effect of different concentrations of lanthanum

A series of perfusions were carried out with fixative 1 (Table 4.1) using 0.1%, 0.5% and 1% lanthanum concentrations and a phosphate buffered osmium post-fixation (1(b) Table 4.1).

#### Assessment of the use of potassium ferrocyanide in post-fixation

A series of perfusions were carried out with fixative 1 (Table 4.1) followed by a post-fixation using 1% osmium tetroxide in 1.5% potassium ferrocyanide (1(c) Table 4.1).

#### 4.2.2. Horseradish Peroxidase

A dose of 1mg per 20gm body weight of horseradish peroxidase (Taab Laboratories, Type II) dissolved in 2.5ml of distilled water was injected into the wing (alar) veins of 8, 12 and 21 week old male Shavers. After 15 minutes the birds were killed by a lethal dose of pentobarbitone sodium (Nembutal, Abbott Laboratories). The left testis was excised, cut into 1.5 x 1.5 x 10.0mm<sup>3</sup> strips, fixed at room temperature in 4% glutaraldehyde in 0.075m cacodylate buffer pH 7.4 for 2 hours and then washed overnight in 0.075m cacodylate/0.2m sucrose buffer. 50-75  $\mu$ m sections were cut on a Smith and Farquhar tissue chopper and stored in 0.05m Tris-HCl buffer pH 7.6. The tissue was then incubated in a diaminobenzidine tetrachloride solution for 15 minutes either at room temperature or at 37°C. The incubating medium was made by dissolving 5mg of diaminobenzidine tetrachloride in 10ml of 0.05m Tris-HCl buffer pH 7.6. 0.1ml of 1% hydrogen peroxide was added just before use. After incubation the sections were washed with 0.05m Tris-HCl buffer and post-fixed with 1%

osmium tetroxide in 0.1m phosphate buffer. The tissue was dehydrated in ethanol and embedded in Araldite (Luft, 1961). White/silver sections were cut on an LKB ultratome III and mounted on copper grids. They were examined unstained in a Phillips 300 electron microscope.

8, 12 and 21 week old male Shavers were injected with 2.5ml of distilled water as controls. After 15 minutes the birds were killed, their left testes excised and processed in a similar manner to above.

### 4.3 RESULTS

#### 4.3.1. Lanthanum Nitrate

##### Fixation Studies

The fixative systems 4% GTA/0.075M sodium cacodylate and 3% GTA/0.1M s-collidine (1 & 2, Table 4.1) when used with colloidal lanthanum, gave adequate fixation of the testis, but there was a lessening in the general quality of the image (compare fig. 4.1-4.9 with fig. 2.76 and 2.79) and the formation of many cytoplasmic and nucleoplasmic vacuoles. It was not possible to use a GTA/Millonig's phosphate fixative because during preparation, when the lanthanum solution and phosphate buffer were mixed, the lanthanum precipitated out. For the same reason, when a phosphate buffer was used for the post-fix after a cacodylate perfusion no lanthanum was included in the solution.

##### Assessment of prewash requirements

If no prewash was used the lanthanum precipitated out on contact with the blood, so that unless a good fast flow was established immediately, any precipitate that formed was not washed out of the body and blocked the blood vessels preventing access of further lanthanum and fixative. Because of the small diameter of the testicular blood vessels in the fowl, the testes were particularly prone to localised blocking of the perfusion.

All four prewashes tried with the cacodylate fixative (2,3,4 and 5, Table 4.2) were equally effective in clearing the blood and increasing the number of successful perfusions obtained. However, <sup>where</sup> the tissue was examined in the microscope differences in effect were noticed. It was found that Dextran (5, Table 4.2)

increased the precipitation of the lanthanum on the capillary endothelial walls and little or no lanthanum reached the testis (fig. 4.1). The cacodylate fixative-without-lanthanum prewash (2, Table 4.2) was more effective in preserving the fine structure than the saline or avian ringer prewash (compare fig. 4.2 with figs. 4.3, 4.4). The blocking of the perfusion when using the s-collidine fixative (6, Table 4.2) was not improved by the use of the fixative-without-lanthanum prewash (7, Table 4.2). As soon as the lanthanum entered the blood vessels the perfusions blocked (fig. 4.5). It seems that s-collidine buffered lanthanum precipitates very easily with the smallest amount of blood, therefore in all further lanthanum perfusions using the perfusion apparatus a cacodylate fixative was used preceded by a fixative-without-lanthanum prewash (2, Table 4.2).

#### Assessment of other methods of applying lanthanum fixatives

When hand perfusion of dissected out testes were carried out in order to give easier access of the lanthanum to the testis, it was found that the uncontrolled pressures of the perfusion caused disruption of the tissue and loss of the intertubular area (fig. 4.6). Immersion fixation gave a better preservation of the fine structure of the seminiferous tubules than any lanthanum perfusion method (fig. 4.7), but the intertubular and boundary tissue organisation were destroyed. Therefore the perfusion technique using the apparatus gave the best overall results.

#### The effect of different concentrations of lanthanum

When the concentration lanthanum was decreased with the hope of reducing the blocking of the blood vessels, it was found



that concentrations lower than 1% were not visible in the micrographs.

#### Assessment of the use of Potassium ferrocyanide in post-fixation

Use of osmium tetroxide/potassium ferrocyanide post-fix after an initial cacodylate fixation greatly enhanced the preservation of the fine structure especially of the membranes. This made observations on the relation between the cell junctions and lanthanum deposits possible (fig. 4.8, 4.9).

#### 4.3.2. Horseradish Peroxidase

There was no observable difference between the intensity of deposition of electron opaque reaction product in sections that had been incubated at 37°C rather than at room temperature. The control birds demonstrated there was no endogenous peroxidase present in the testis except in the red blood cells (fig. 4.10).

#### 4.3.3. Observations of the blood-testis barrier

The lanthanum nitrate perfused with the fixative is observed in the intercellular clefts of the capillaries and filling the interstitial spaces of the intertubular tissue (fig. 4.11, 4.12). The tracer lies in the intercellular spaces between the peritubular myofibroblast cells (fig. 4.13, 4.14) and penetrates the space between the spermatogonia and the surrounding Sertoli cells. The spermatogonia are seen clearly outlined by the electron opaque lanthanum filling the intercellular clefts (fig. 4.8, 4.15, 4.17).

In birds that have passed puberty (15 and 21 week old males) the lanthanum can be seen extending into the intercellular

space between two Sertoli cells for about  $0.5\mu$  before it stops (fig. 4.8, 4.15, 4.17). Where two Sertoli cells meet above a spermatogonium tight junctions are observed. Beyond the tight junctions lie the sub-surface cisternal junctions of the Sertoli cells. In figures 4.16 and 4.18 lanthanum stops just prior to two tight junctions and a cisternal junction.

In the prepubescent 6 week old male and the 9 week old bird in which spermatogenesis has begun (i.e. the number of spermatogonia are increasing and a few resting spermatocytes are present), lanthanum penetrates the intercellular clefts around the spermatogonia and those between Sertoli cells as far as the centre of the tubule (fig. 4.19, 4.20, 4.21). Here the lanthanum does not extend through the zonula occludens junctions between Sertoli cells (fig. 4.20, 4.21 insets). The tracer is seen within the intercellular space between the plasma membranes of a sub-surface cisternal junction of two Sertoli cells (fig. 4.22).

In the 12 week old male spermatogenesis has begun but there is variation between tubules in the degree of development of the spermatogenic cycle. Once any spermatocytes with pachytene nuclei have been produced no lanthanum is observed beyond more than a few micra in the Sertoli-Sertoli intercellular clefts. Along with the appearance of pachytene nuclei is the formation of the lumen and the loss of the occluding junctions at the luminal surface.

The intravenously injected horseradish peroxidase is distributed in essentially the same way as the lanthanum nitrate perfused with the fixative. The peroxidase reaction product is observed in the intercellular clefts of the capillaries and filling

the interstitial spaces of the intertubular tissue (fig. 4.23), and of the boundary tissue (fig. 4.24). The 21 week old birds the spermatogonia and resting spermatocytes are seen clearly outlined by the peroxidase in the intercellular spaces (fig. 4.24, 4.25, 4.26). The peroxidase extends for only a short distance or not at all into the intercellular space between two Sertoli cells (fig. 4.26, 4.27, 4.28).

In order to observe the peroxidase reaction product the sections were examined unstained. This makes the unit membrane structure difficult to observe (fig. 4.28) and no tight junctions can be demonstrated at the point where the peroxidase stops. It can be said though, that this is the point where the tight junctions are observed in stained material.

In the 6-9 week old birds the peroxidase penetrates the Sertoli-Sertoli cell intercellular space to the centre of the tubule (fig. 4.29, 4.30).

#### 4.4 DISCUSSION

The concept of the blood-brain barrier and its location in the walls of the capillaries led Kormanó (1967) to assume that the blood-testis barrier in the rat was also located in the capillary endothelium. Morphological studies (Fawcett et al, 1970) established that in the guinea pig the testicular capillary endothelium was lacking in extensive areas of tight junctions and was therefore readily permeable to extracellular tracers. This has been confirmed in the rat (Dym and Fawcett, 1970) and the monkey (Dym, 1973). Mammalian physiological studies also show the lack of a barrier at the testicular capillary endothelium as demonstrated by the high permeability of the capillaries to serum proteins (Everett and Simmons, 1958), testosterone (Lindner, 1963) and various ions (Setchell, 1967). In the present study lanthanum and peroxidase have been observed within the intercellular cleft of the endothelial cells, therefore in the fowl as in mammals the blood-testis permeability barrier is not at this level of the tissue.

In mammals the blood-testis barrier has been located in or around the seminiferous tubules. In the rat a partial barrier has been found at the peritubular myoid cell layer and a complete barrier at the specialised Sertoli-Sertoli junctions (Dym and Fawcett, 1970). The rat has a single continuous sheet of peritubular myoid cells, which meet edge to edge or overlap each other. For the <sup>major part</sup> ~~great~~ majority of its length the intercellular space between these myoid cells in the rat is about 200Å wide, but at one or more places it narrows to form occluding junctions which prevent the

penetration of lanthanum. Occasionally, however, the intercellular cleft does retain its  $200\text{\AA}$  width throughout allowing the passage of lanthanum. In the monkey Dym (1973) found the barrier only operated at the Sertoli-Sertoli junctions, there being no barrier at all at the peritubular myoid cell layer. The monkey has several layers of peritubular myoid cells, which overlap each other, but are always separated by intercellular spaces of at least  $300\text{-}400\text{\AA}$ . No occluding junctions were observed between these cells. In the fowl there are also several overlapping layers of peritubular myofibroblast cells separated by intercellular spaces of at least  $200\text{\AA}$ , and there are no regions of closer apposition of membranes. Hence in the fowl, as in the monkey, there is no blood-testis barrier at the peritubular layer.

In the rat and monkey Sertoli-Sertoli tight junctions are observed where two Sertoli cells meet above a spermatogonium or resting spermatocyte. These are followed by extensive junctional complexes with associated subsurface filaments and cisternae, and  $90\text{\AA}$  (in the rat) or  $70\text{\AA}$  (in the monkey) and  $20\text{\AA}$  interspaces, i.e. gap junctions.

In the fowl tight junctions with a pentalaminar structure also occur where two Sertoli cells meet above a spermatogonium or resting spermatocyte. Further up the lateral Sertoli-Sertoli cell membranes are subsurface cisternal junctions that differ from the mammalian ones in lacking filaments in the space between the subsurface cisternae and the plasma membrane. There is also no associated narrowing of the appositional plasma membranes that form gap junctions within the subsurface cisternal region, the plasma

membranes remaining  $200\text{\AA}$  apart throughout this junction. Tight junctions with a pentalaminar structure, (Farquhar and Palade, 1963) are often observed immediately prior to the subsurface cisternal junctions. In this study the lanthanum and peroxidase have never been observed more than a few micra within the Sertoli-Sertoli intercellular cleft, therefore the blood testis barrier in the fowl is considered to be located at the Sertoli-Sertoli junctions.

Often the lanthanum stops with a spluttering effect (fig. 4.16) as if there is a build-up of resistance to its passage. This suggests that there could be a chemical change in the properties of the Sertoli cell membranes once they surround spermatocytes undergoing meiosis, which results in an impermeable barrier to intercellular substances. By this it is not necessarily meant that the lanthanum is acting as a stain, though it is possible that the electron opacity is due to staining of the cell surfaces (Shea, 1971) rather than or in addition to the precipitation of lanthanum in the intercellular spaces (Revel and Karnovsky, 1967). However, most workers suggest that it is the dimensions of the tight junction that normally blocks the access of the tracer or stain to the intercellular space or cell surfaces beyond, and in general the observations of this work agree with this.

The role of the tight junction as a barrier has been assessed by means of lanthanum in many tissues, e.g. mouse heart and liver (Revel and Karnovsky, 1967), rat liver (Schatzki, 1969) mouse liver (Goodenough and Revel, 1970) rat kidney glomerulus (Gang, 1970), frog skin (Martinez-Palomo, Erlij and Bracho, 1971), sheep rumen (Henrikson and Stacy, 1971) and rabbit gall bladder and

intestine (Machen, Erlij and Wooding, 1972). A barrier to the movement of lanthanum has been found at most tight junctions, but in some salt-transporting epithelia there are also "leaky" junctional complexes which are permeable to lanthanum (Machen et al., 1972). There is also evidence that lanthanum can be stopped where there are no tight junctions, e.g. in the stratified epithelium of the rumen (Henrikson and Stacy, 1971). These workers suggest it is the twisting plasma membranes and their mucopolysaccharide coat which gradually prevent the passage of lanthanum in the outer strata of the epithelium. Fowl Sertoli-Sertoli cell plasma membranes do not twist, although they are more irregular than germ cell-Sertoli cell membranes, and although it is thought all cells have a mucopolysaccharide coat (Overton, 1969), it is not known whether the mucopolysaccharide coat of the Sertoli cell plasma membranes is one that is particularly impenetrable, but if this was so, it could be a possible reason why lanthanum sometimes stops with a spluttering effect prior to the tight junctions (fig. 4.16).

The difficulty experienced during this study in developing a perfusion fixation procedure that resulted in the lanthanum in the first place reaching the testicular blood capillaries and secondly diffusing from the capillaries to the tubules, suggests that the passage of lanthanum in the internal environment is not easy.

The use of lanthanum in assessing permeability of tight junctions must take into account the manner in which the tracer is administered to the tissue (Neaves, 1973). Administration of ionic lanthanum in vitro without any fixative present results in

the penetration of the tight junction by lanthanum (Machen, et al, 1972). Colloidal lanthanum injected into the common bile duct in vivo also penetrates tight junctions (Schatzki, 1969;1971). If, however, the colloidal lanthanum is administered simultaneously with the fixative then the tight junctions are impermeable (Dym and Fawcett, 1970; Goodenough and Revel, 1970). The present work using colloidal lanthanum in the fixative would agree with that of the latter workers.

Schatzki thought that as lanthanum is a substance foreign to the body it cannot be considered to be an inert tracer, and he therefore suggested it possibly can cause leakage across the tight junctions without morphological change. Matter, Orci and Rouiller (1969) and Goodenough and Revel (1970) reported that tight junctions could be made leaky to lanthanum by pretreatment with acetone, although there was no recognisable morphological change. This evidence would seem to suggest that in vivo the tight junction acts as a barrier and if it is fixed in its natural state it will be impermeable to lanthanum, but if the lanthanum is allowed access before fixation, the junction becomes leaky because of the damage caused to it by the lanthanum.

In this study lanthanum was found to be a difficult substance to use, because it precipitated out on contact with the blood and was therefore difficult to introduce into the tissue. It also had a considerable affect on the fixation with the formation of vacuoles and in some areas total disruption of the tissue (fig. 4.6).

Only the use of a potassium ferrocyanide buffered osmium post-fix enabled the unit membrane structure to be observed after



lanthanum perfusion. So although lanthanum is considered to be useful as a relatively cheap tracer substance, it was felt that results obtained from its use should be checked by use of a biological tracer such as the enzyme, horseradish peroxidase.

In this study the peroxidase is clearly located within the intercellular clefts of the endothelial cells of the capillaries, in the interstitial spaces of the intertubular and boundary tissue and within the intercellular clefts around the spermatogonia. Peroxidase is observed for only a short distance into the Sertoli-Sertoli intercellular space. These results confirm those obtained with the lanthanum. Although it was not possible to distinguish tight junctions in unstained material, the point at which the peroxidase stopped was where tight junctions between Sertoli cells are normally positioned. This observation is in agreement with the suggestion that the blood-testis permeability barrier is located at the level of the Sertoli-Sertoli tight junctions in the fowl.

Peroxidase has been used to study the blood-testis barrier in several species e.g. guinea pig (Heidger, 1969), rat (Vitale et al, 1973), monkey (Dym, 1973) and dog (Heidger, 1974). Peroxidase, being a naturally occurring enzyme, would seem to be a better assessor of tight junction permeability as it should cause no damage. However, Matter et al, (1969) noted that oxygen bubbles, formed by catalytic action of the tissue during incubation with hydrogen peroxide, could disrupt the junctions. Such an effect was not observed during this study. Peroxidase is a more sensitive tracer than lanthanum, since (only) a few molecules of enzyme are able to generate more and more electron opaque reaction

product as incubation proceeds. It is a low molecular weight protein of 44,000 (Nicholas and Bailey, 1960) and therefore comparable in size to egg albumen which has a molecular weight of 42,000 and is known to be greater than  $30\text{\AA}$  in diameter (Goodenough and Revel, 1971).

It has been reported that peroxidase is taken up by the Sertoli cell and transported to the lumen (Reddy and Svoboda, 1967; Aragon, Lustig and Mancini, 1972). In the present study no large uptake of peroxidase by spermatogonia or Sertoli cells was observed. This agrees with the findings of Dym (1973) and Vitale *et al.*, (1973). It has been suggested that the difference between these has arisen because of the difficulty in determining cellular outlines at the light microscope level. There was much discussion by light microscopists over whether Sertoli cells existed as a syncytium before Bawa (1963) using the electron microscope presented conclusive evidence that Sertoli cells existed as individual cells. It is often difficult to tell in light microscope studies whether material is inter- or intra-cellular in the Sertoli cell regions.

The demonstrations of a barrier in the seminiferous epithelium has interesting implications in relation to the physiology of the testis. Dym and Fawcett (1970) have put forward the hypothesis of the compartmentation of the seminiferous tubule from work using the rat. As a blood-testis barrier has been found in the fowl at the spermatocyte level of the Sertoli-Sertoli plasma membranes, this hypothesis could apply equally well to the fowl. They suggested the seminiferous epithelium is partitioned into a basal compartment containing spermatogonia and early spermatocytes

and an adluminal compartment containing more advanced germ cells. The Sertoli cells are the only cells that extend from the basal lamina to the lumen with cytoplasmic processes surrounding all the different stages of the germ cells. Any blood-borne substances can pass directly to only the spermatogonia, early spermatocytes (resting and early leptotene spermatocytes) or Sertoli cells via the intercellular spaces of the intertubular tissue and boundary tissue, thus the only access to the later spermatocytes and spermatids is via the Sertoli cell cytoplasm. Meiotic prophase, two maturation divisions and spermatogenesis may require a special environment which is provided for by the Sertoli cells and blood-testis barrier (Vitale et al., 1973).

Evidence of a special environment in the tubules has been provided by physiological studies. The rate of penetration of various substances from the blood plasma to the testicular lymph and rete testis fluid has been examined by Setchell (1967), Johnson and Setchell (1968) and Setchell et al., (1969). The substances fell into three groups, those that passed readily into the lymph and rete testis fluid e.g. tritiated water, urea, ethanol and bicarbonate; secondly those that passed readily into the lymph but only slowly into the rete testis fluid e.g. galactose and the ions  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$  and  $\text{Cl}^-$ ; and thirdly those that passed readily into the lymph but not at all into the rete testis fluid e.g. inulin, glutamic acid and albumen. As all substances measured passed readily into the testicular lymph which bathes the intertubular tissue, whereas there were different concentrations between the rete testis fluid, which originates in the seminiferous tubules, and the lymph and blood plasma, these physiological

studies thus confirm the morphological evidence that there is a barrier in or around the seminiferous tubules.

The presence of a barrier is thought to have significance in relation to testis function. It has been suggested many times that Sertoli cells play a nutritive role for the cells of the germinal epithelium (Vilar, Perez del Cerro and Mancini, 1962; Elftman, 1963; Nicander, 1967) and the demonstration of a secretion of a fluid of unique composition by the Sertoli cells may explain how this nutritive role is carried out (Setchell, 1970). Whereas spermatogonia have access to lymph which has passed through the peritubular cell layer, the other germ cells are only bathed in the fluid which the Sertoli cells secrete into the intercellular spaces. Thus the barrier provides a special fluid environment for spermatogenesis. It is interesting to note that the junctional specialisations of the rat (Vitale et al, 1973) and mouse (Flickinger, 1967) develop just before the appearance of meiotic prophase spermatocytes.

Another role assigned to the barrier is that of immunological isolation of alien antigens of the developing haploid spermatozoa (Johnson and Setchell, 1968). The barrier prevents the leaking of spermatozoal antigen into the circulation and thus reduces autoimmunization. Specific complement fixing and globulin antibody to acrosome of spermatozoa has been found in the normal sera of several species (Johnson, 1968) but only traces of globulin are found in the rete testis fluid (Johnson and Setchell, 1968) or seminiferous tubules (Mancini, et al, 1965) demonstrating the barrier's ability to prevent antibody entering the seminiferous

tubules. Experimental weakening of the barrier requires drastic procedures such as the induction of testicular ischaemia by the administration of cadmium chloride (Johnson, 1969), mechanical trauma, and isoimmunization with testis in adjuvant (Johnson, 1970a). The breakdown of the barrier is demonstrated by the change in the pattern of acriflavine staining of the testis. Isoimmunization causes only partial and temporary damage to the tubule (Johnson, 1970b). Thus the immunological barrier in the seminiferous tubule is highly effective in protecting the tubule contents from an immune response to spermatozoa and also in preventing leakage of spermatozoal antigen.

The presence of a permeability barrier also poses interesting questions about the access of hormones to the developing germ cells. It is thought that the multiplication and growth of pre- and postnatal gonocytes and formation of spermatogonia A are possibly under the control of testosterone. The development of germ cells during meiotic prophase is not thought to require either gonadotrophic or gonadal hormones, although the reduction divisions are under the control of testosterone and maybe also the early steps of spermatid formation. The late stages of spermatid maturation require the presence of follicle stimulating hormone (FSH), (Steinberger, 1971).

From the tracer experiments of this study and those of Fawcett et al. (1970), Dym (1973) and Vital et al. (1973) it seems that peroxidase is unable to reach the later spermatocytes and spermatids via the intercellular spaces. Vitale et al. (1973) think that if a protein enzyme of molecular weight 44,000 is excluded then it is likely that a glycoprotein gonadotropic hormone

of molecular weight 30,000, FSH, would also be excluded. Evidence of this has been provided by morphological studies using immunohistochemical techniques which have demonstrated the diffusion of homologous serum proteins into the seminiferous epithelium and lumen, (Mancini, et al, 1965) and diffusion of ferritin conjugated FSH through the Sertoli cell to the lumen (Mancini, Castro and Seiguer, 1967) at the optical level and at the electron microscopical level, (Castro, Seiguer and Mancini, 1970; Castro, Alonso and Mancini, 1972). Therefore it would seem that the barrier prevents direct access of proteins and glycoproteins to the developing germ cells and any effects of FSH must be mediated by the Sertoli cells.

Vitale et al, (1973) demonstrated that the blood-testis permeability barrier is not present in the immature rat. The establishment of the barrier coincided with the development of the pachytene spermatocytes, the formation of the Sertoli-Sertoli specialised junctions and the opening of the lumen. In the fowl the blood-testis barrier also only becomes operational once pachytene spermatocytes are present at 10-12 weeks, but this is not correlated with the appearance of the subsurface cisternal junctions, because these are present from 1 week of age in the fowl. The development of the barrier is correlated with the formation of the lumen and of Sertoli-Sertoli tight junctions above the spermatogonia.

The hormonal control of the development of the blood-testis barrier was investigated by Vitale et al, (1973) using Clomiphene and oestrogens to suppress circulating gonadotrophins from birth. They found that the barrier developed seven days later than normal, but as the general development of the testis was delayed by a similar time factor, they concluded the development of the barrier

was not under hormonal control. This is in agreement with the results of Johnson (1970b) who found hypophysectomy of mature rats did not result in loss of the barrier.

This study has demonstrated the existence of a blood-testis barrier in the fowl operating at the Sertoli-Sertoli cell junction and correlated its establishment with germ cell differentiation. The barrier has been shown to be the same as that in the rat and monkey in its essential features. This suggests that a blood-testis barrier is of fundamental importance in the proper physiological functioning of not only mammalian testis but also avian testis.

FIXATION STUDIES

1. Good overall fixation of the testis for all ages of bird was obtained by whole body vascular perfusion into the aorta using a controlled pressure/flow perfusion apparatus. 4% GTA/0.1M s-collidine or 2.5% GTA/0.1M Millonig's phosphate with a similarly buffered post-fix gave a good fixation image, but the preferred image was gained by perfusion of 4% GTA/0.075M sodium cacodylate with a phosphate buffered osmium post-fix.

2. Good fixation of cell membranes was obtained by using 4% GTA/0.075M sodium cacodylate followed by a 1% osmium tetroxide post-fix buffered with potassium ferrocyanide.

3. Immersion fixation offered a wider choice of fixative/buffer systems particularly for the prepubertal bird for which 6% GTA/2% acrolein/0.1M phosphate and 1% osmium tetroxide/modified Dalton's buffer were preferred. Modified Karnovsky could give good fixation for pubertal and adult birds but it was very variable in effect.

4. The best fixation image was obtained when the osmolality of glutaraldehyde/buffer fixing solutions was between 550mOsm and 600mOsm.

5. Alterations in the osmolality made by changing the molarity of the buffer had a greater effect on the fixation image than those made by changing the fixative concentration.

ANATOMICAL STUDIES

1. The first and second stages of spermatogenesis present features unique to the fowl, whereas the second stage of involving meiotic division has similar morphological characteristics to that of mammalian species.

2. /



2. An A- and B-type spermatogonium have been identified and differentiate at the start of spermatogenesis.

3. The acrosome is the only part of the spermatid that protrudes into the Sertoli cell cytoplasm and this leads to a different mechanism of spermiation in the fowl compared to that of mammals. The spermatid is released from the cytoplasm by a swelling and coalescence of ER vesicles and released into the lumen by the obliteration of the Sertoli cell recess holding the acrosome.

4. The lack of widespread germ cell synchronisation and presence of atypical cell-associations during the spermatogenic cycle in the fowl is considered to be due to (a) a smaller number of cytoplasmic bridges between spermatogonia and spermatocytes than the number observed in mammals, because of the completion of cytokinesis; (b) a lack of the gap junctions which are thought to aid cell communication between adjoining Sertoli cells.

5. The pattern of organisation of the intertubular tissue is similar to that of primates and man. This correlates with the boundary tissue organisation of the fowl which also has a similar pattern to primates and man.

6. The Sertoli-Sertoli tight junctions were not as extensive as those observed in primates and were not observed in conjunction with gap junctions.

#### TRACER STUDIES

1. A blood-testis permeability barrier has been demonstrated by the use of lanthanum and peroxidase. The morphological basis of the barrier was found to lie at the tight junction between adjoining Sertoli cells above the spermatogonial cell layer. No barrier was found in the peritubular boundary tissue. This correlates with /

with the position of the barrier in the primates.

2. The barrier was found to develop once pachytene spermatocytes were present in the tubules.

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ULTRASTRUCTURAL STUDIES ON THE  
TESTIS OF THE DOMESTIC FOWL,  
GALLUS DOMESTICUS

A Thesis Submitted By

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a Candidate for the Degree of

Doctor of Philosophy

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Key to Figures

- A - acrosome
- ax - axonemal complex
- B - bridge
- Bl - basal lamina
- BT - boundary tissue
- C - capillary
- cc - chromosomal core
- ce - centriole
- cf - collagen fibres
- cj - cisternal junction
- D - dense amorphous material
- dc - distal centriole
- E - endothelial cell
- ER - endoplasmic reticulum
- f - fibrils
- G - Golgi complex
- I - interdigitating plasma membrane
- Is - interstitial or intercellular space
- IT - intertubular tissue
- J - junction
- L - Leydig cell
- La - lanthanum nitrate
- Li - lipid droplet
- LSc - leptotene spermatocyte
- Lu - lumen
- Lv - lymphatic vessel
- Ly - lysosome

m - mitochondrion  
ma - macula adherens  
mgb - multigranular body  
mt - microtubule  
mvb - multivesicular body  
N - nucleus  
P - peroxidase  
pA - proacrosome  
pc - proximal centriole  
pf - perforatorium  
pm - peritubular myofibroblast cell  
PSc - pachytene spermatocyte  
pv - pinocytotic vesicle  
R - ribosomes  
RB - residual body  
Rbc - red blood cell  
RER - rough endoplasmic reticulum  
RSc - resting spermatocyte  
s - synaptnemal complex  
Sc - spermatocyte  
Sc1 - primary spermatocyte  
Sc2 - secondary spermatocyte  
Se - Sertoli cell  
SER - Smooth endoplasmic reticulum  
sf - 'sunflower' organelle  
Sg - spermatogonium  
Sp - spermatid  
ST - seminiferous tubule



sv - sex vesicle  
tj - tight junction  
v - vacuole  
Ve - vesicle  
za - zonula adherens  
zo - zonula occludens



Figure 2.1

1% OsO<sub>4</sub>/0.1M Millonig's PO<sub>4</sub>

Shows good preservation of intertubular and boundary tissue, and seminiferous tubules. Note well preserved fine grained precipitate in intercellular spaces.

X 4,000

Figure 2.2

1% OsO<sub>4</sub>/0.1M s-collidine

Shows leached appearance of cytoplasm and nucleoplasm.

Note preservation of intercellular material in the interstitial space,

X 3,400

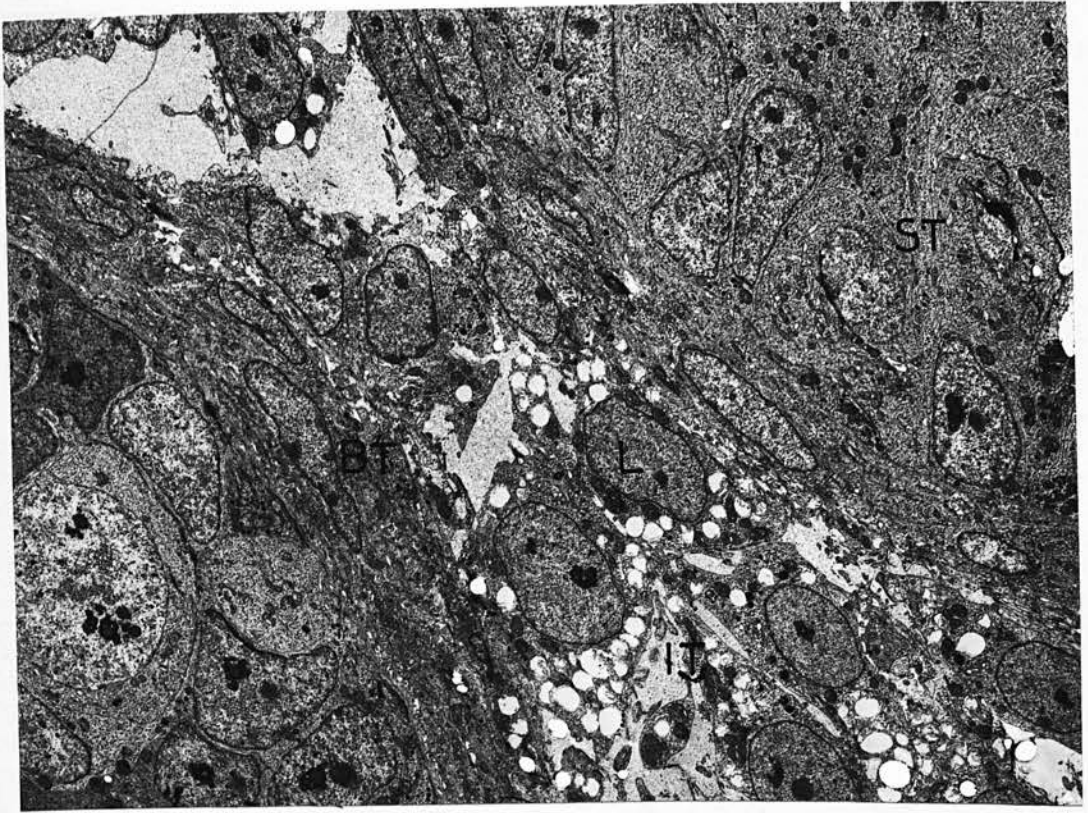


Figure 2.3

1% OsO<sub>4</sub>/modified Dalton's buffer

Intertubular tissue has separated from the boundary tissue and seminiferous tubules. Sertoli cell mitochondria have a very dense matrix.

X 3,800

Figure 2.4

1% OsO<sub>4</sub>/Palade's buffer

Intertubular tissue has separated from the boundary tissue.

X 3,400

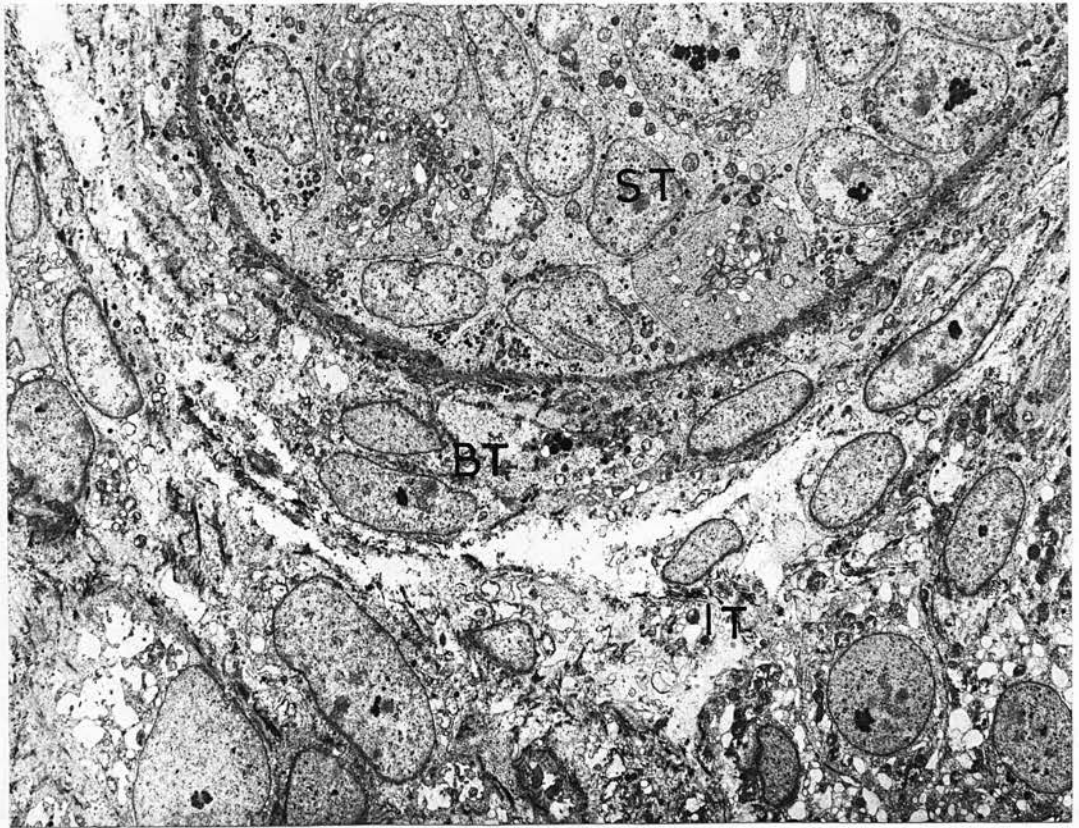
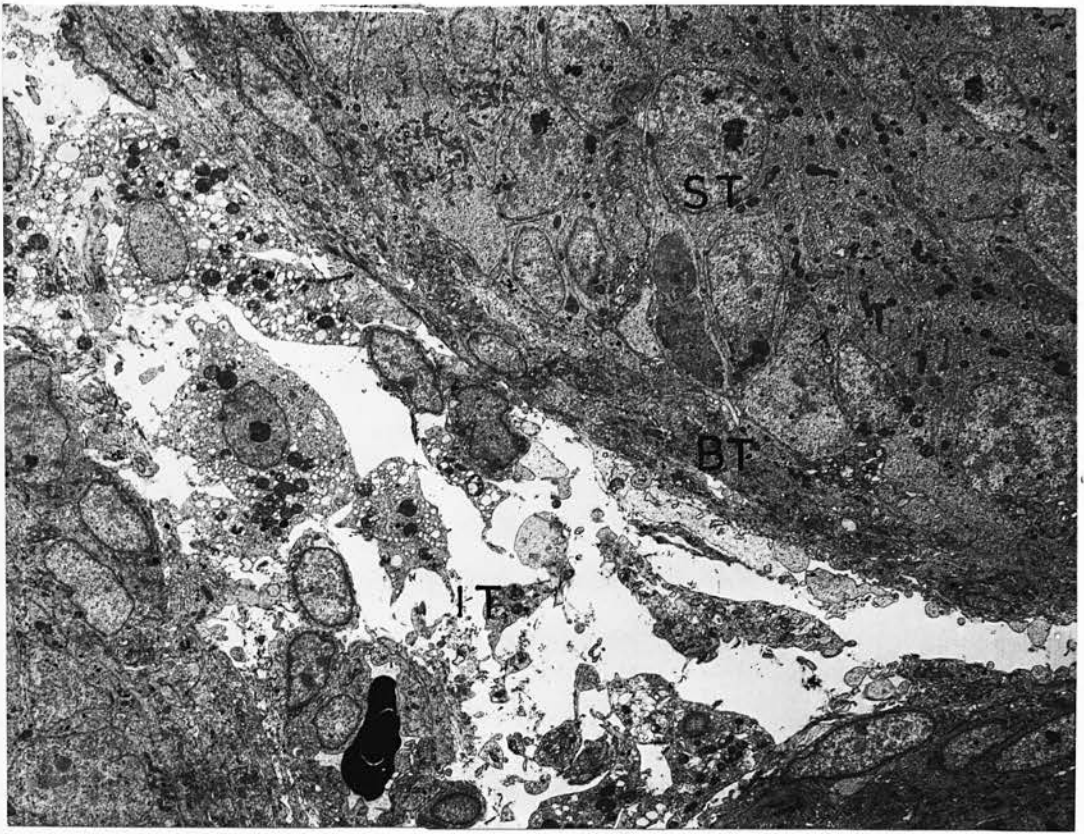


Figure 2.5

1% OsO<sub>4</sub>/modified Dalton's buffer

Leydig cells exhibit a hypotonic image of swollen mitochondria and vesiculated ER.

X 9,500

Figure 2.6

1% OsO<sub>4</sub>/Palade's buffer

Leydig cells exhibit a hypotonic image of swollen mitochondria and vesiculated ER. Note prominent interchromatin granules of nucleus.

X 9,500

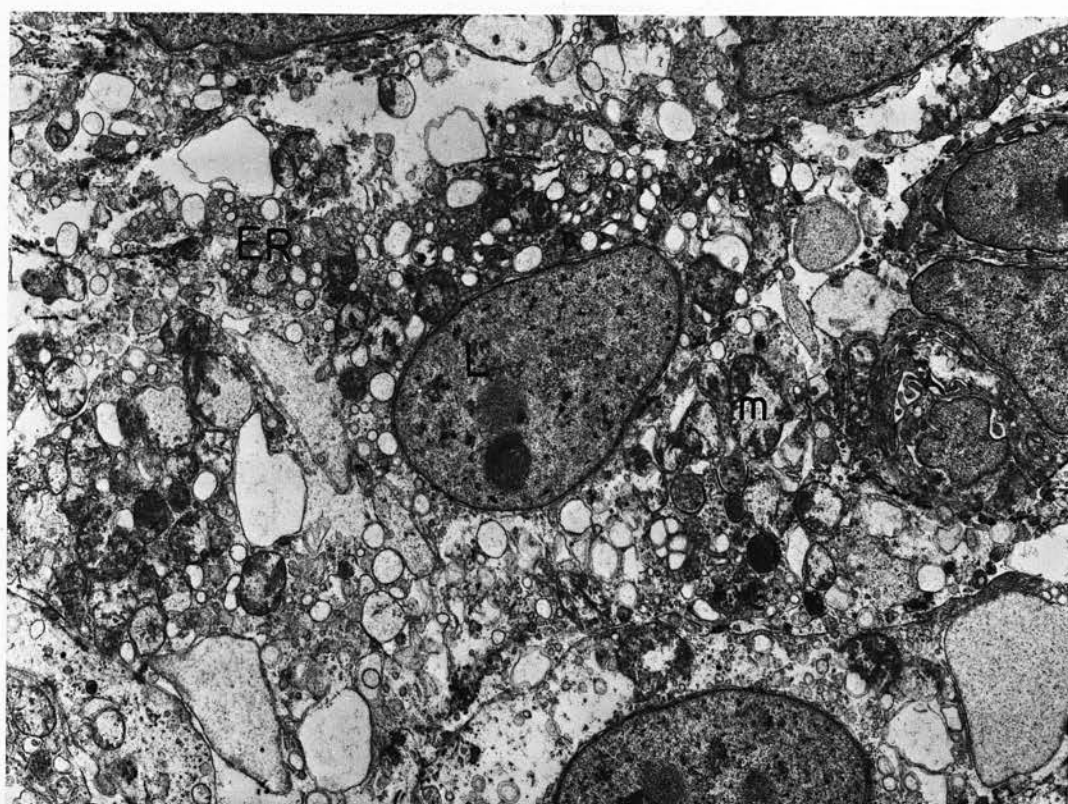
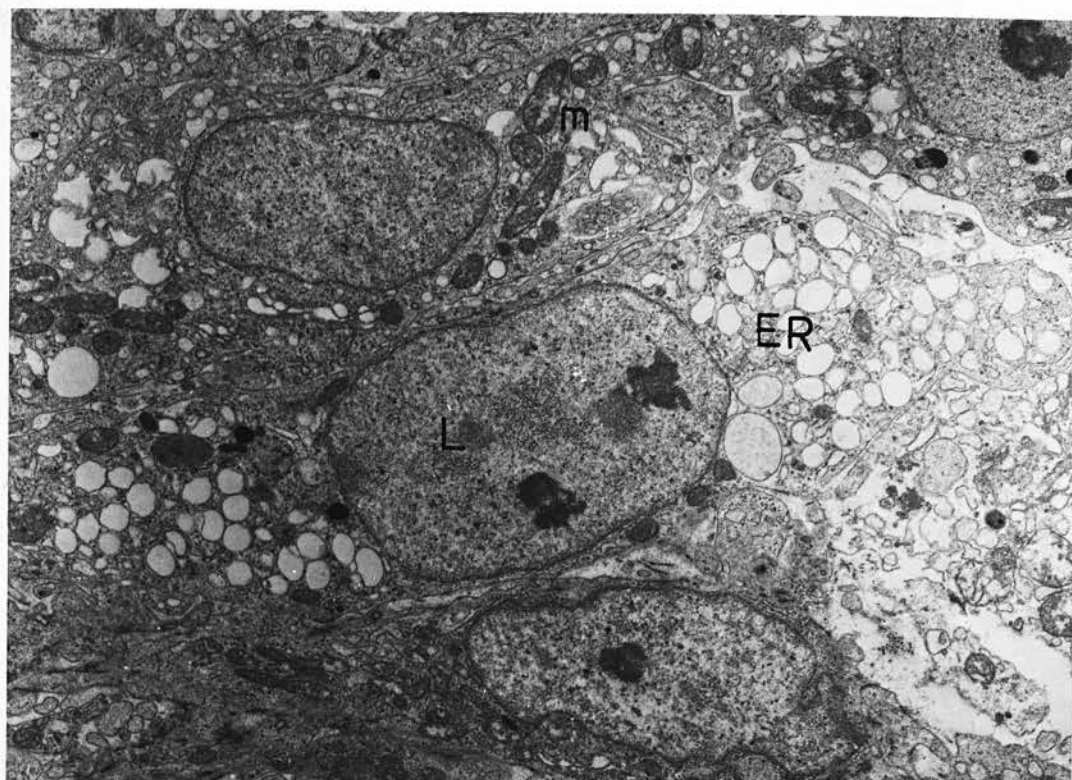




Figure 2.7

1% OsO<sub>4</sub>/0.1M s-collidine

Mitochondria have a pale matrix and free ribosomes are seen aggregated in clumps. Note perinuclear clumping of chromatin.

X 26,000

Figure 2.8

1% OsO<sub>4</sub>/0.1M Millonig's PO<sub>4</sub>

Note good preservation of cell membranes and profiles of ER. Lysosomes have a dark matrix.

X 26,000

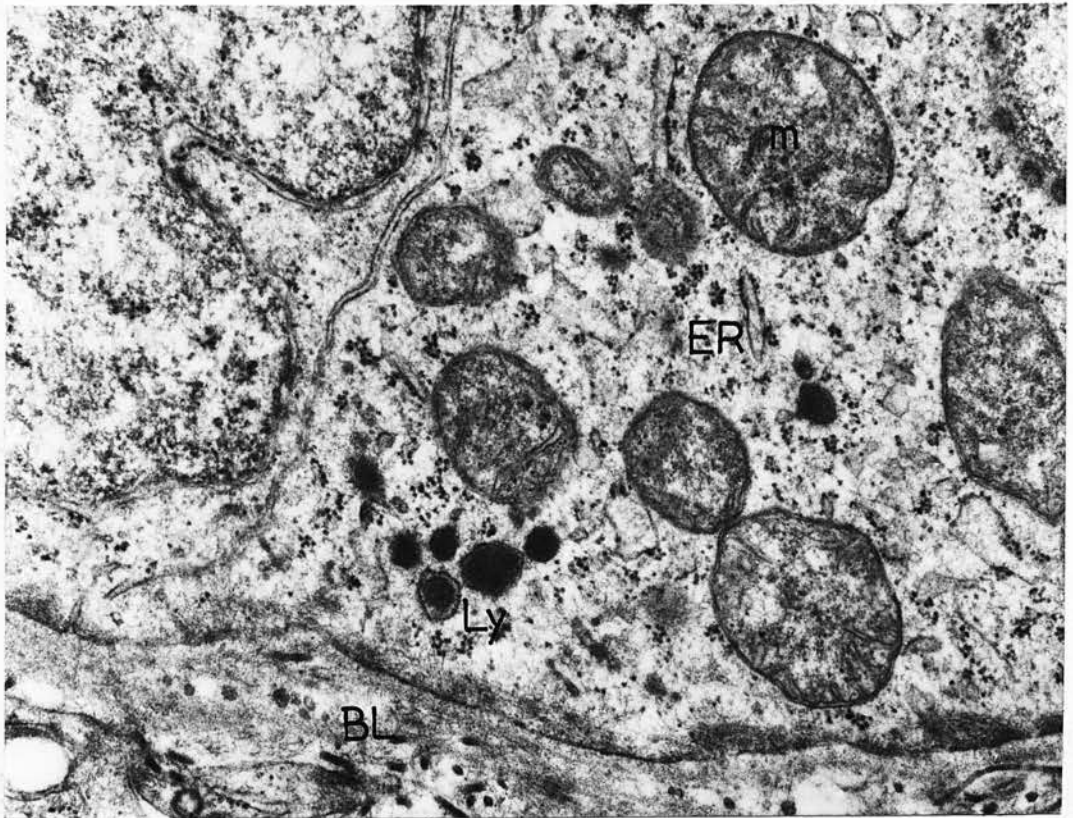
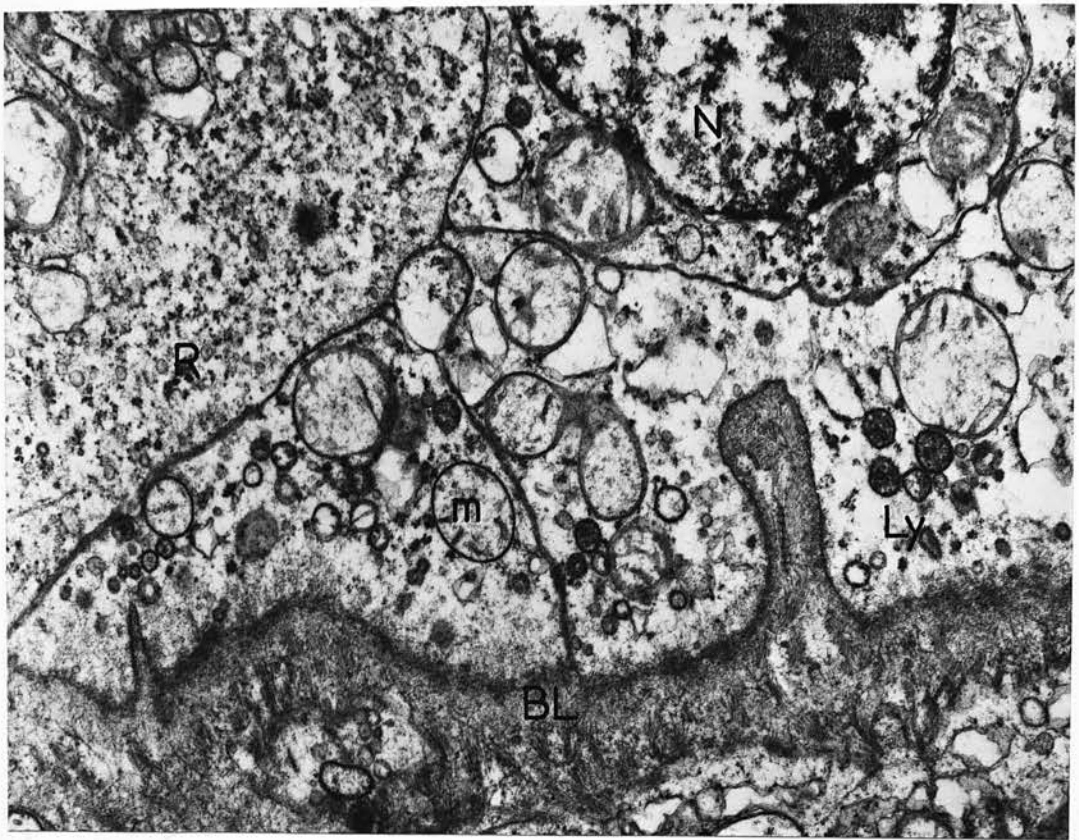


Figure 2.9

1% OsO<sub>4</sub>/modified Dalton's buffer

Shows good preservation of all cell membranes and a vesicular form of rough ER.

X 26,000

Figure 2.10

1% OsO<sub>4</sub>/Palade's buffer

Shows indistinct broken plasma membranes and lysosomes with a dark matrix but distinct limiting membrane.

X 26,000

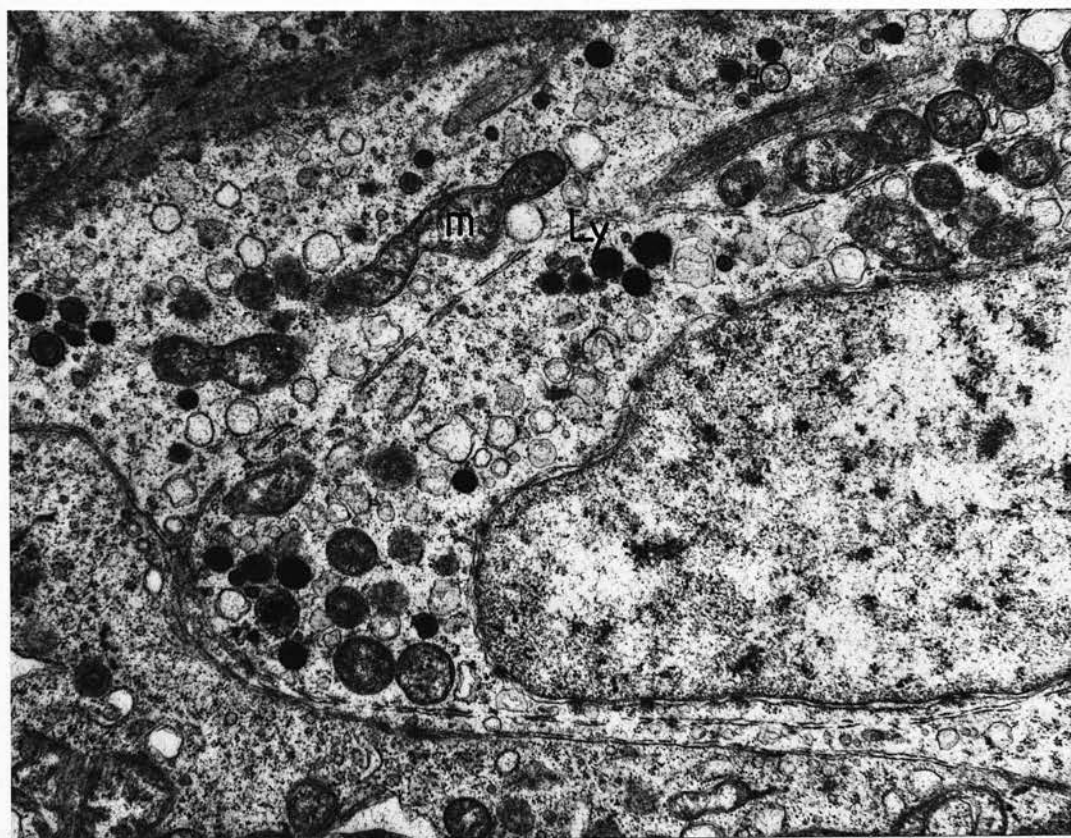
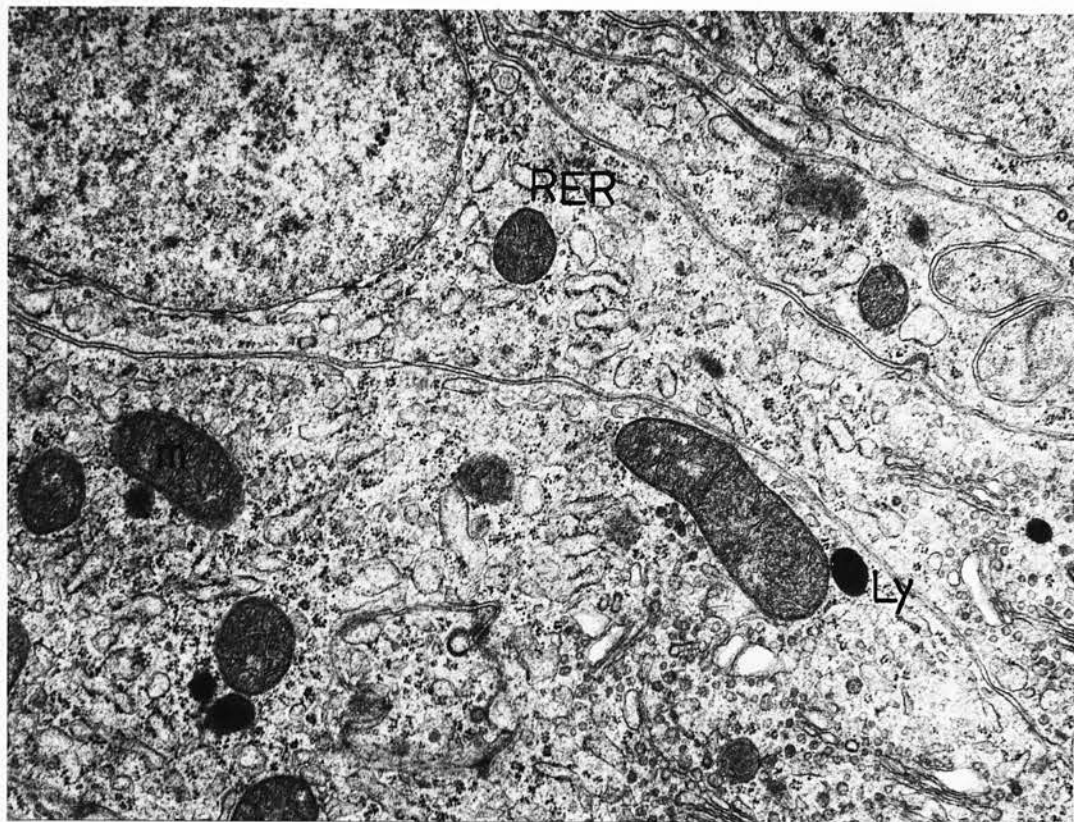


Figure 2.11

2.5% GTA/modified Dalton's buffer

Tissue exhibits a hyper-tonic image with many dark  
and contracted looking cells.

X 3,000

Figure 2.12

4% GTA/0.1M s-collidine

Tissue exhibits a pale leached appearance

X 3,500

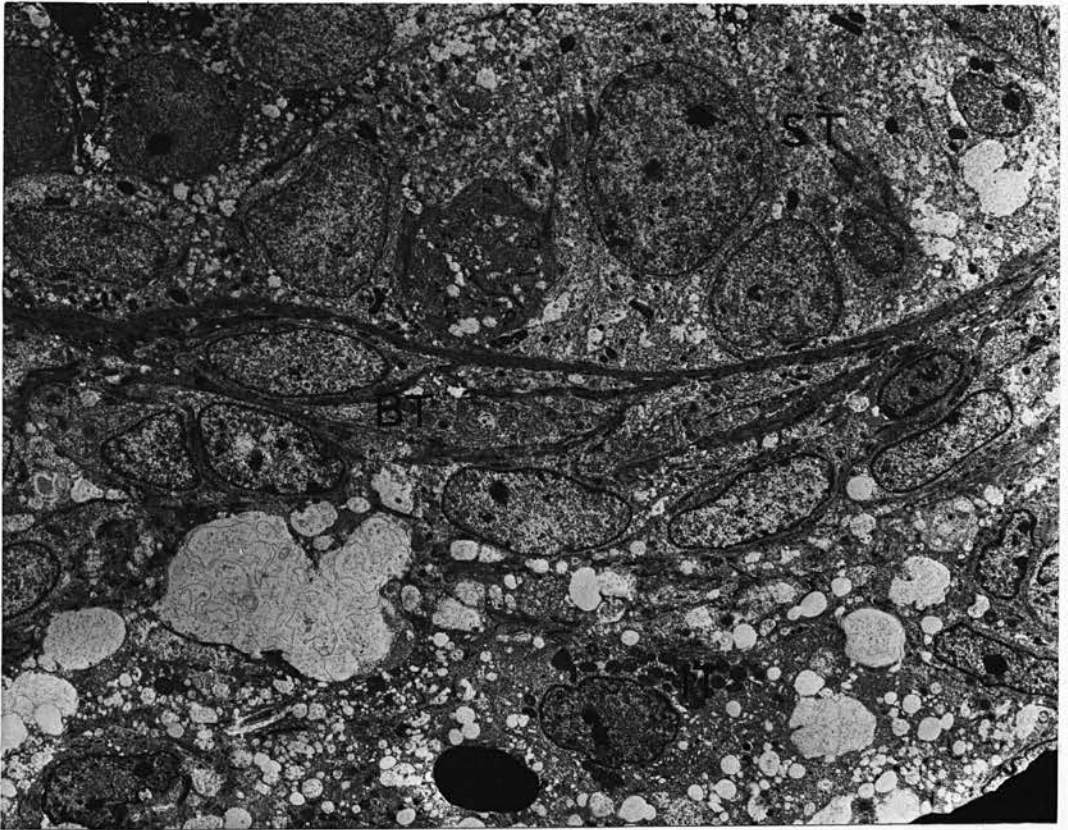
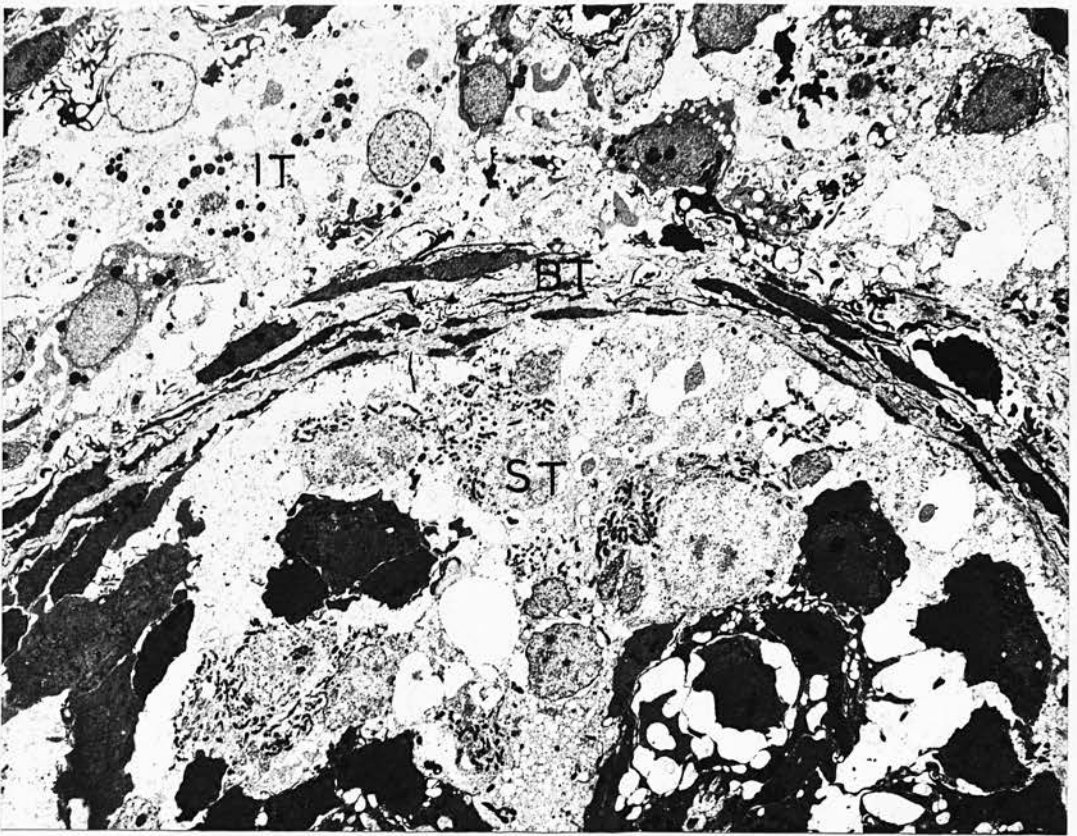


Figure 2.13

4% GTA/0.1M s-collidine

Patches of cell membrane and cytoplasm are lost and the tissue has a leached appearance.

X 26,000

Figure 2.14

2.5% GTA/0.1M Millonig's  $PO_4$

Tissue is well preserved apart from Leydig cells in which patches of leached cytoplasm are observed.

X 3,800

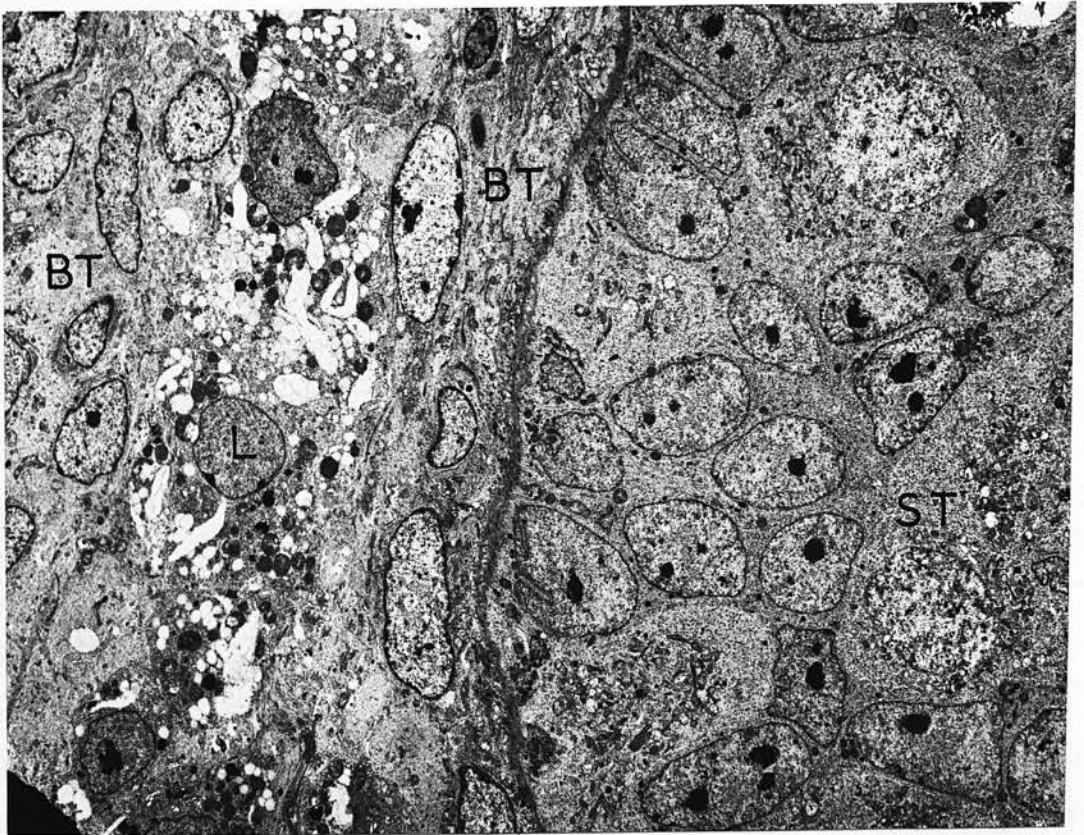
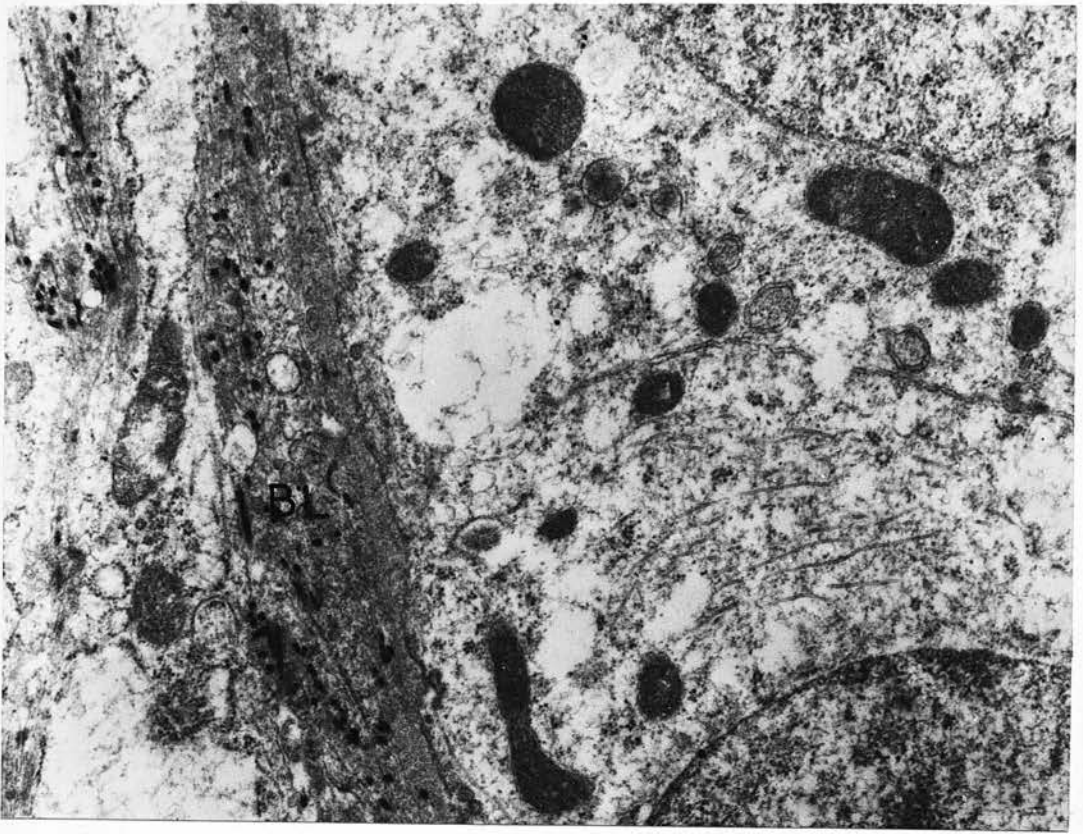




Figure 2.15

2.5% GTA/0.1M Millonig's PO<sub>4</sub>

Leydig cell exhibits patches of leached cytoplasm  
and swollen mitochondria.

X 12,000

Figure 2.16

2.5% GTA/0.1M Millonig's PO<sub>4</sub>

Shows preservation of the ground substance of the  
cytoplasm of a Sertoli cell. Smooth ER and micro-  
tubules are seen.

X 34,000

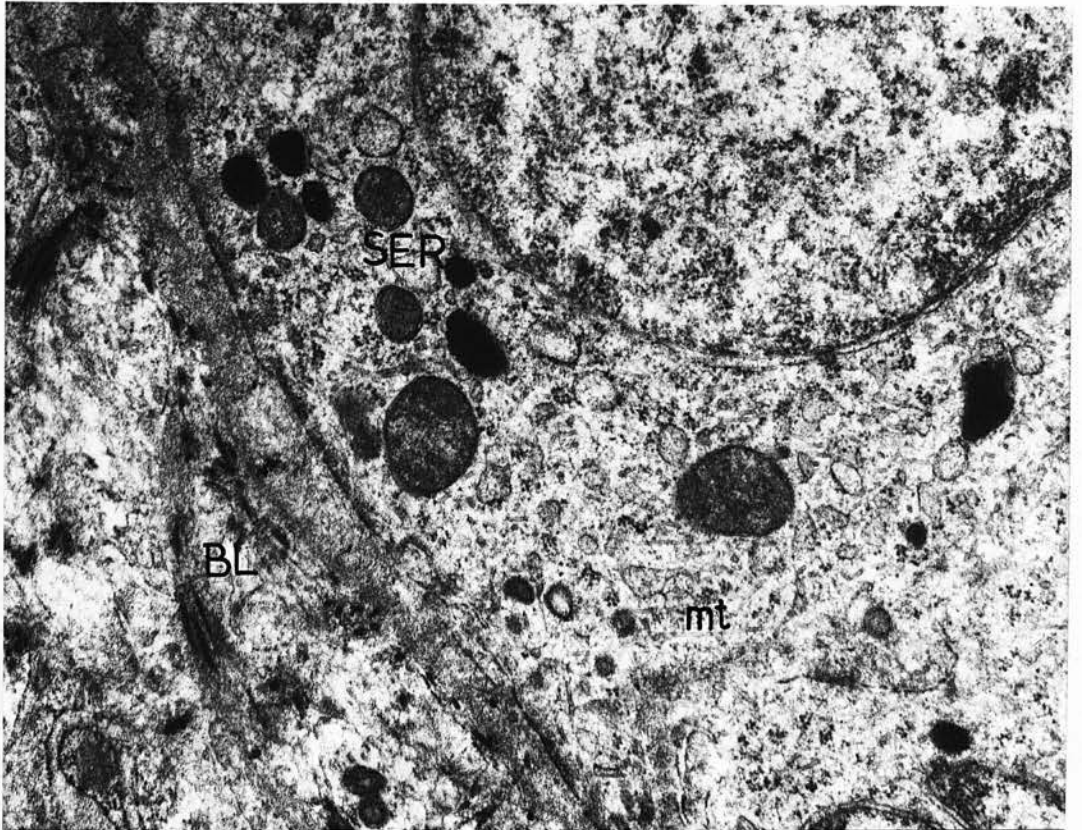
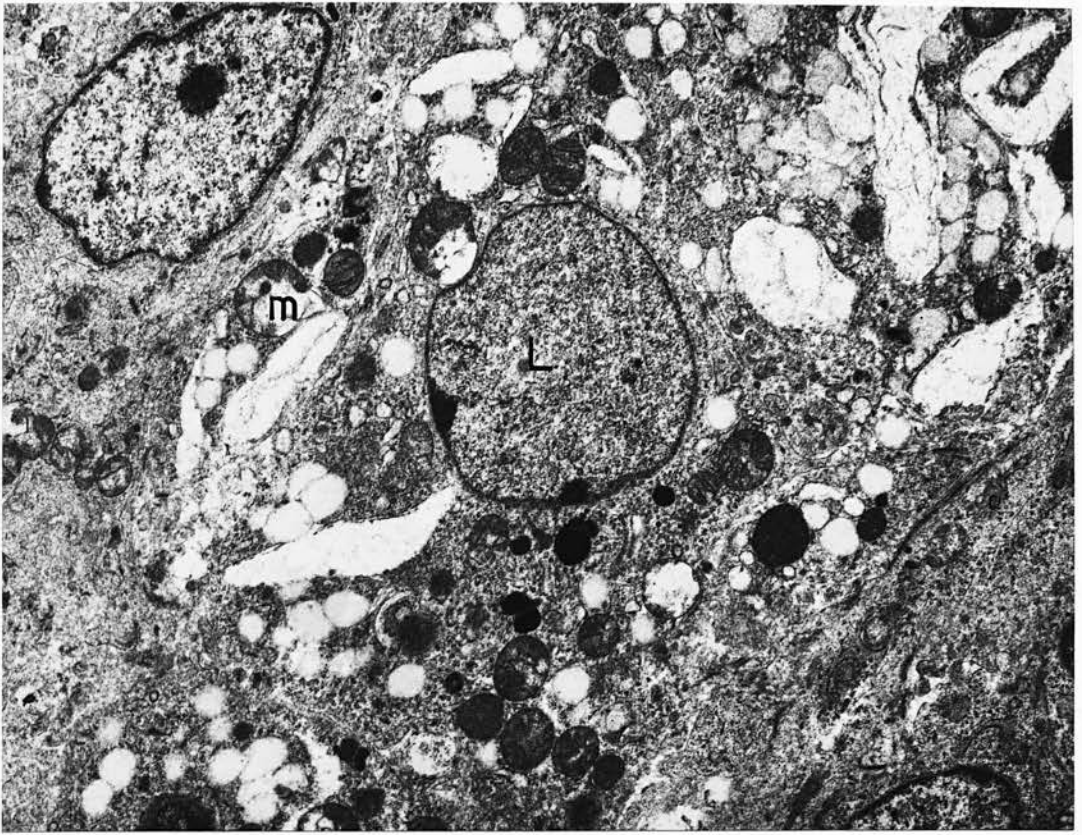


Figure 2.17

4% GTA/0.075M Na cacodylate and 1% OsO<sub>4</sub>/0.175M Na cacodylate  
Shows good preservation of intertubular tissue, boundary  
tissue and seminiferous tubules.

X 6,000

Figure 2.18

4% GTA/0.075M Na cacodylate and 1% ~~6~~ OsO<sub>4</sub>/0.175M Na  
cacodylate.

Plasma membranes and microtubules are well preserved  
but ER membranes are indistinct.

X 26,000

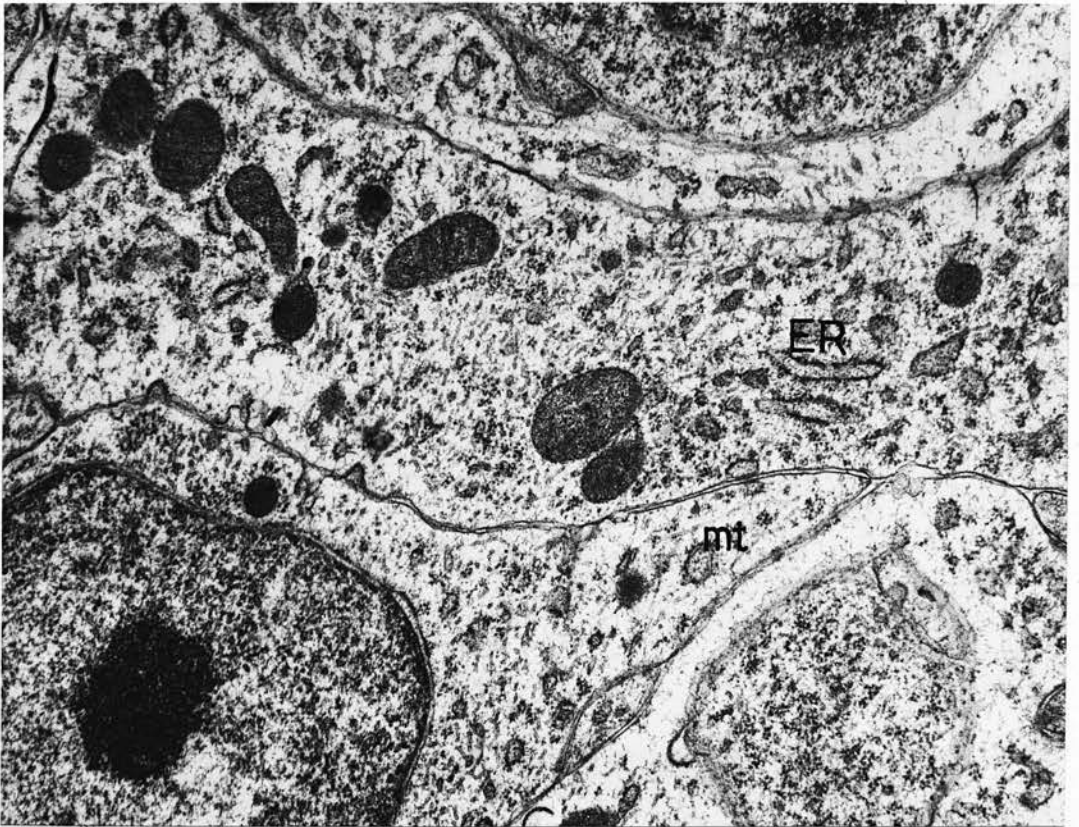


Figure 2.19

1% OsO<sub>4</sub>/0.1M Millonig's PO<sub>4</sub>

Shows good preservation of the intertubular and boundary tissue and seminiferous tubule.

X 3,800

Figure 2.20

2.5% GTA/0.1M Millonig's PO<sub>4</sub>

Shows good preservation of the intertubular and boundary tissue, and seminiferous tubule.

X 3,600

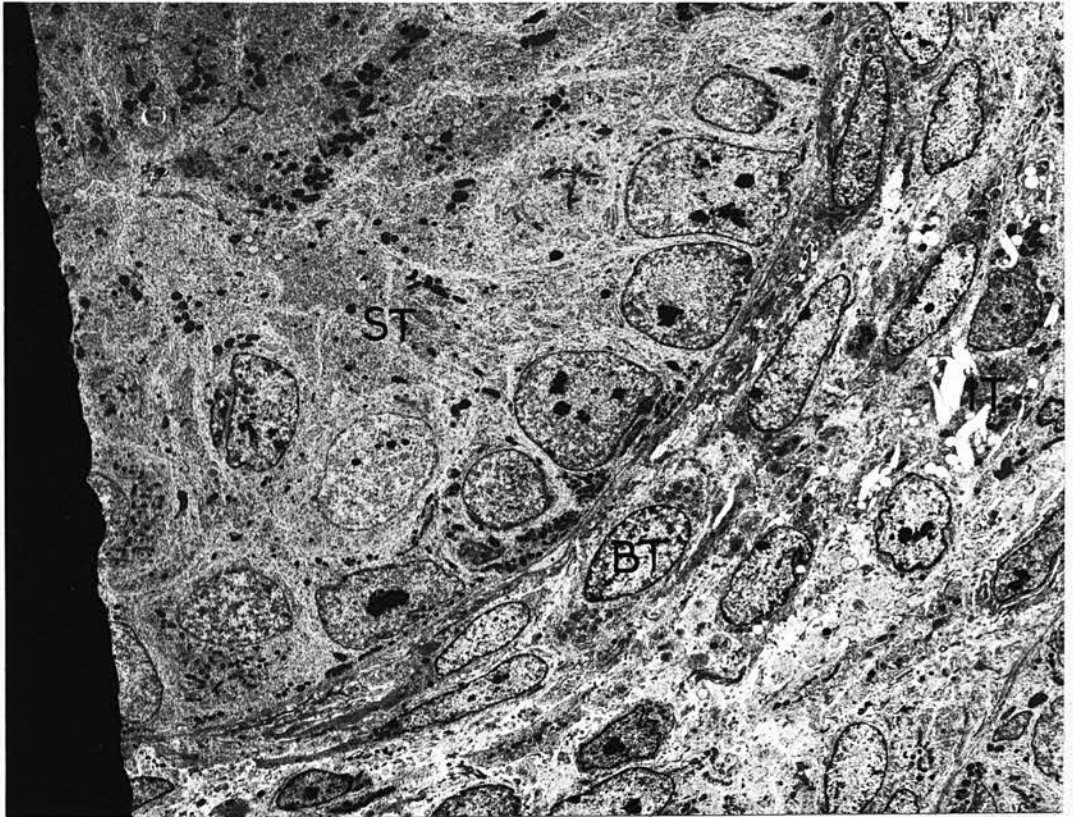
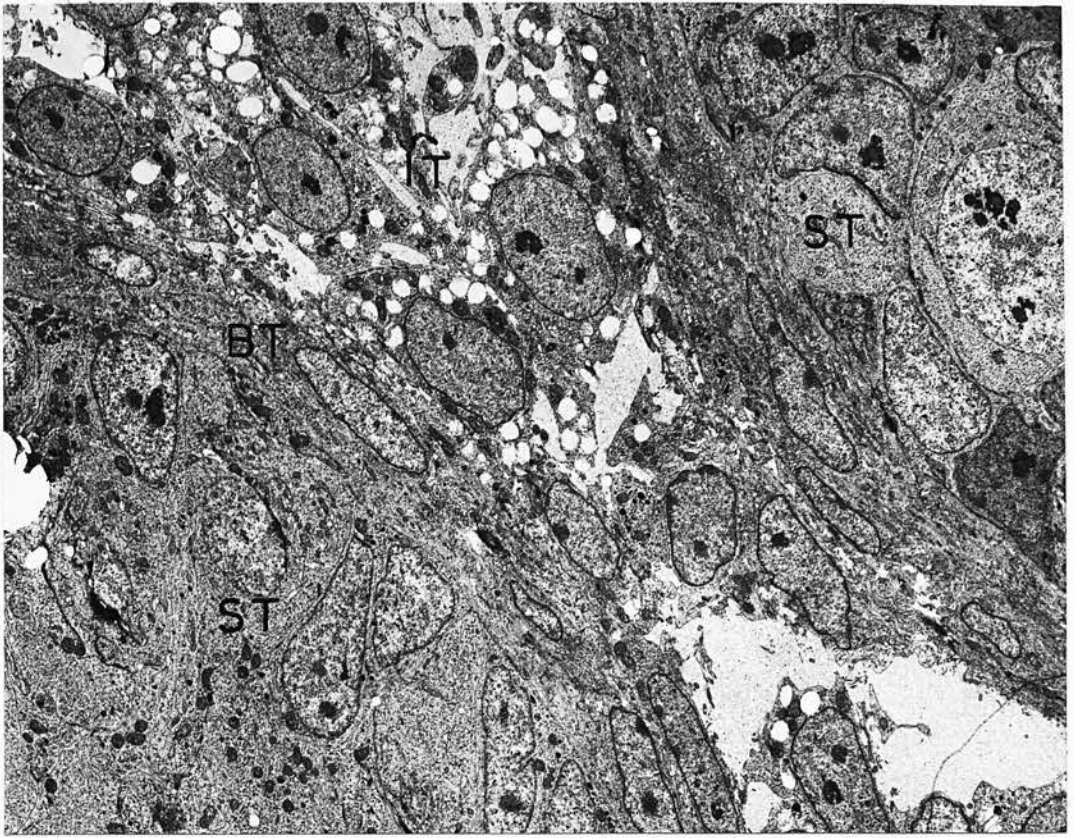


Figure 2.21

1% OsO<sub>4</sub>/0.1M Millonig's PO<sub>4</sub>

Short lengths of ER and many free ribosomes are observed.

Note even distribution of chromatin in nucleus.

X 26,000

Figure 2.22

2.5% GTA/0.1M Millonig's PO<sub>4</sub>

Long lengths of ER, polyribosomes and microtubules are observed in the cytoplasm. Note the perinuclear clumping of the chromatin.

X 26,000

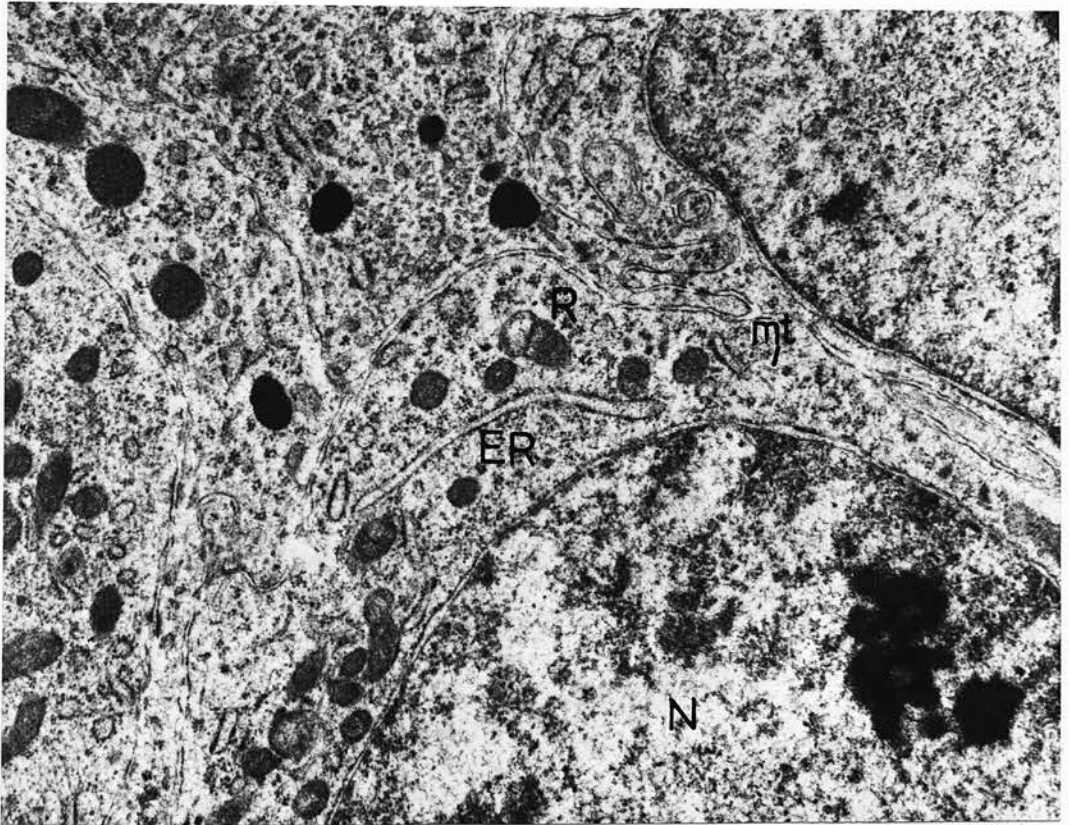
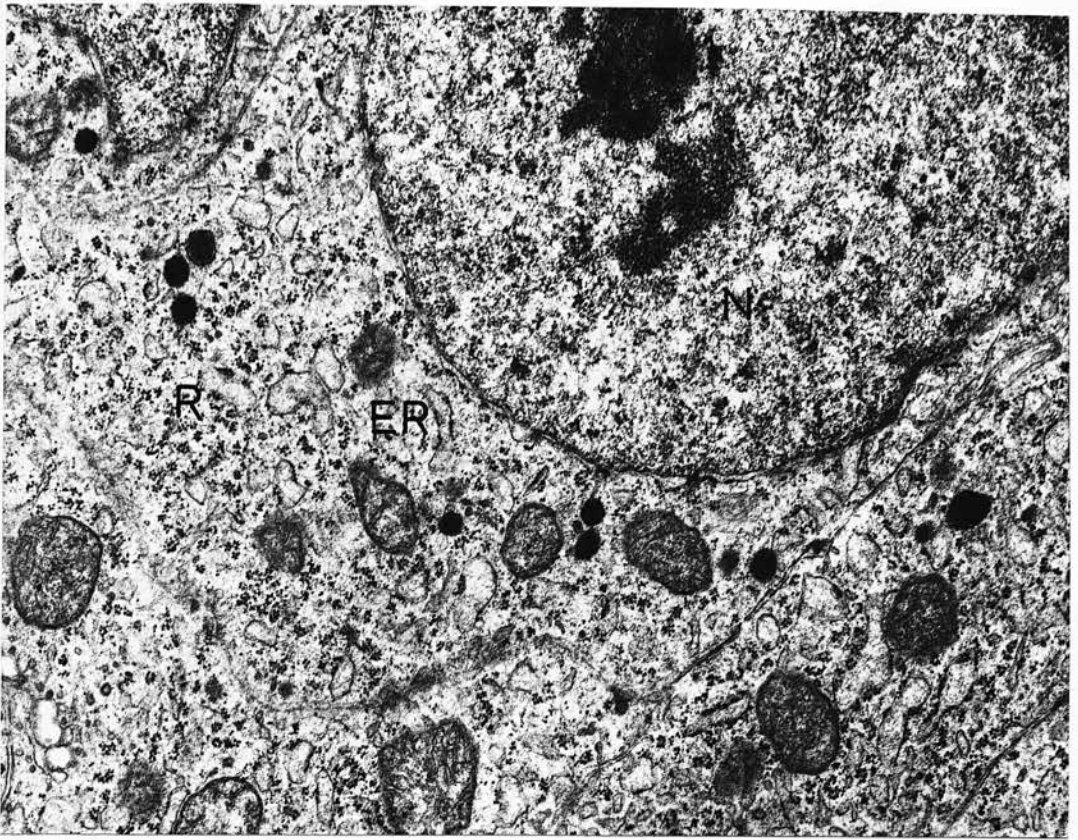




Figure 2.23

5% acrolein/0.1M PO<sub>4</sub> shows very leached intertubular cytoplasm with loss of cell outline.

X 2,800

Figure 2.24

5% acrolein/0.1M PO<sub>4</sub>

Parts of the cytoplasm of the tubule cells are lost.

Single ribosomes and vesicular ER are observed.

X 26,000

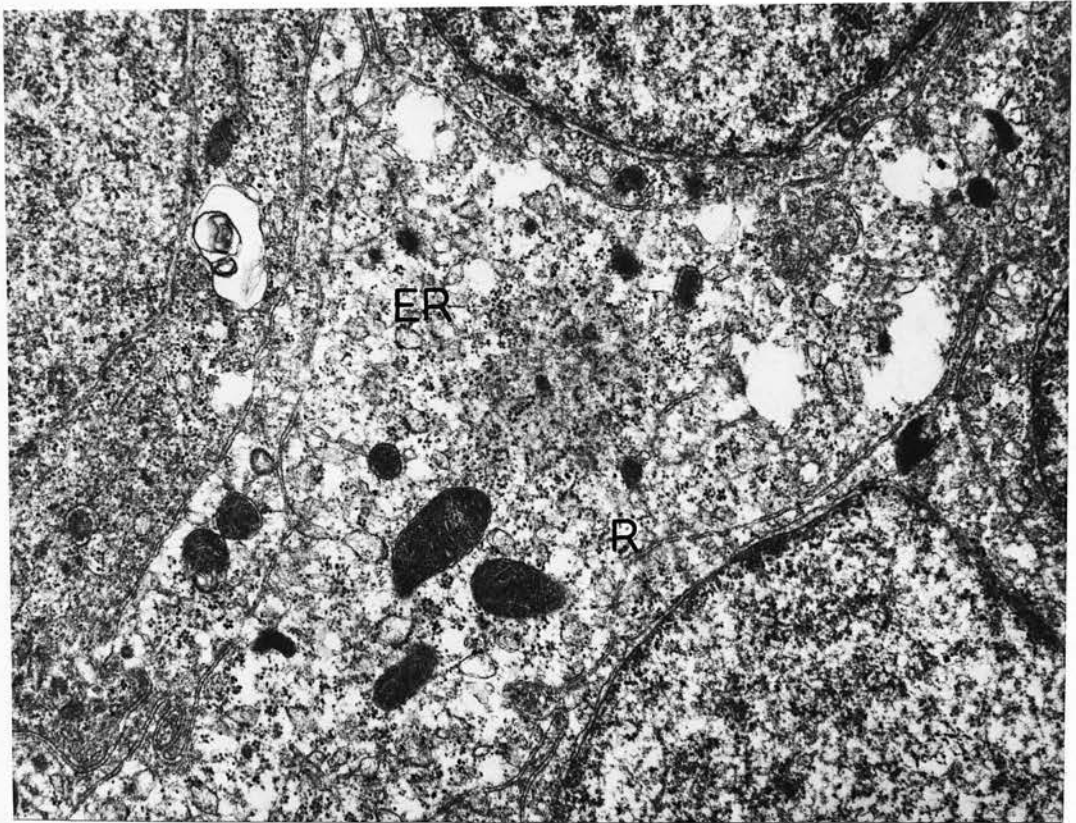
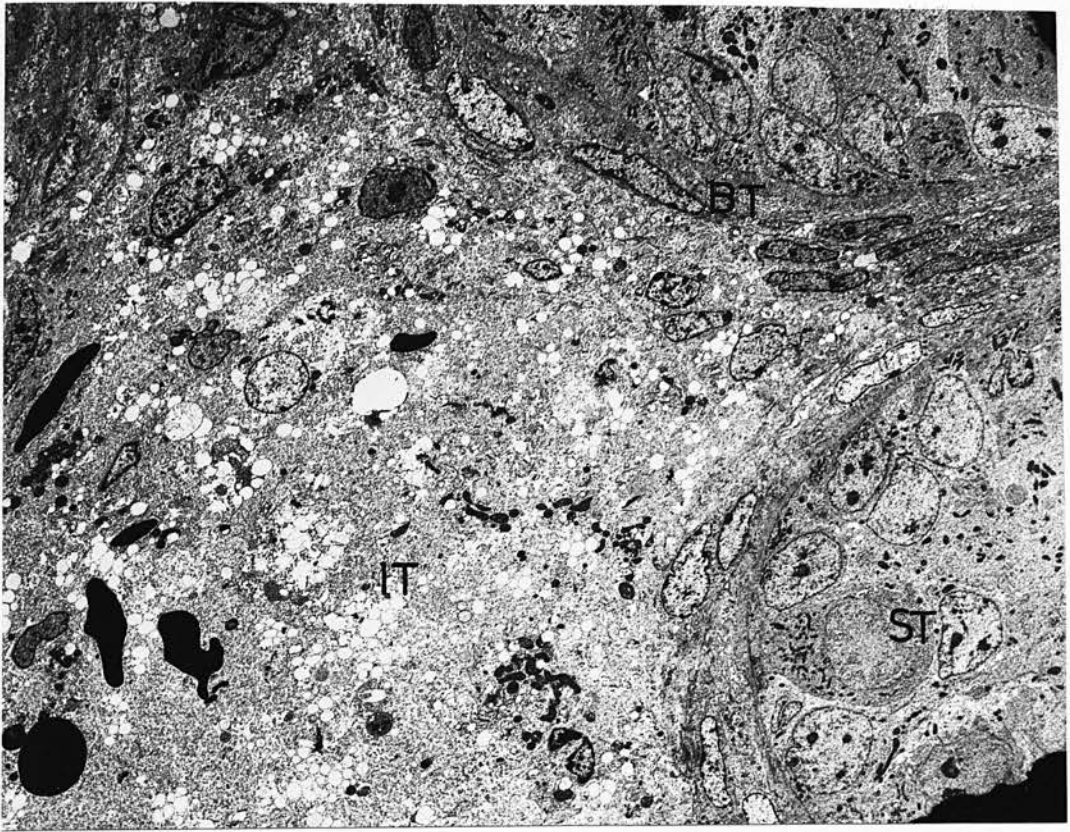


Figure 2.25

6% GTA/2% acrolein/0.1M  $\text{PO}_4$

The intertubular tissue has a leached appearance but preservation of the tubules and boundary tissue is good.

X 3,800

Figure 2.26

6% GTA/2% acrolein/0.1M  $\text{PO}_4$

Shows well preserved mitochondria and vesicular ER.

Note the preservation of microtubules.

X 26,000

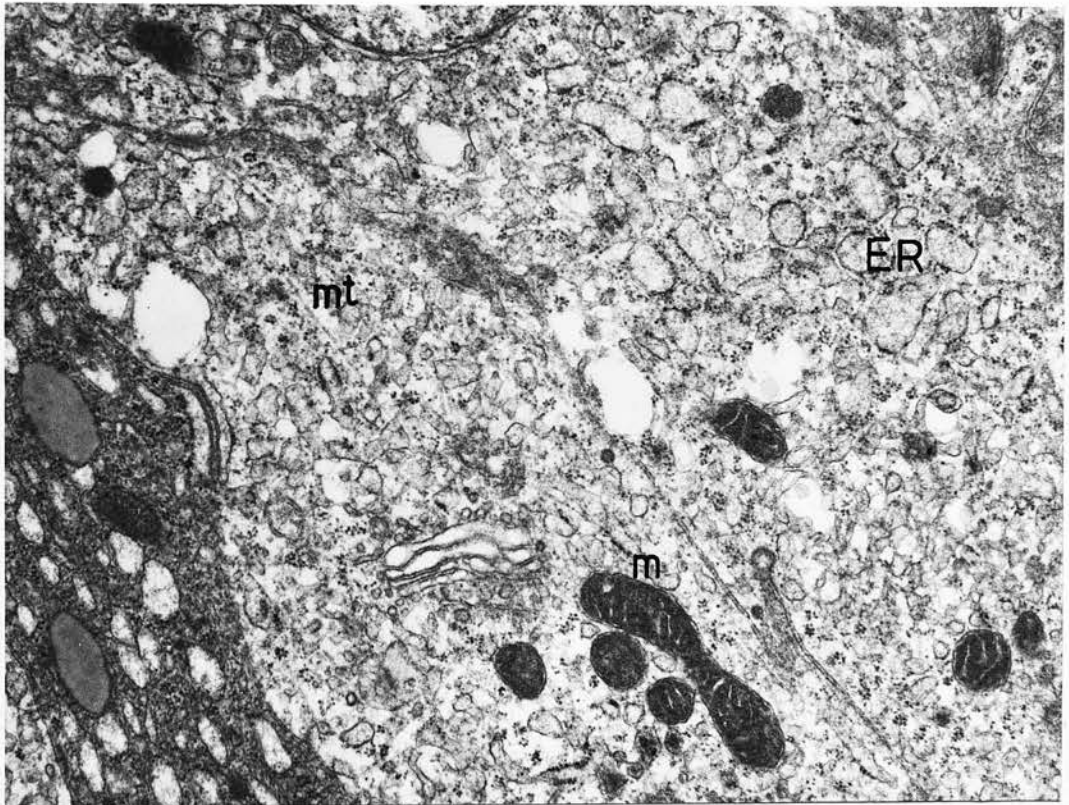
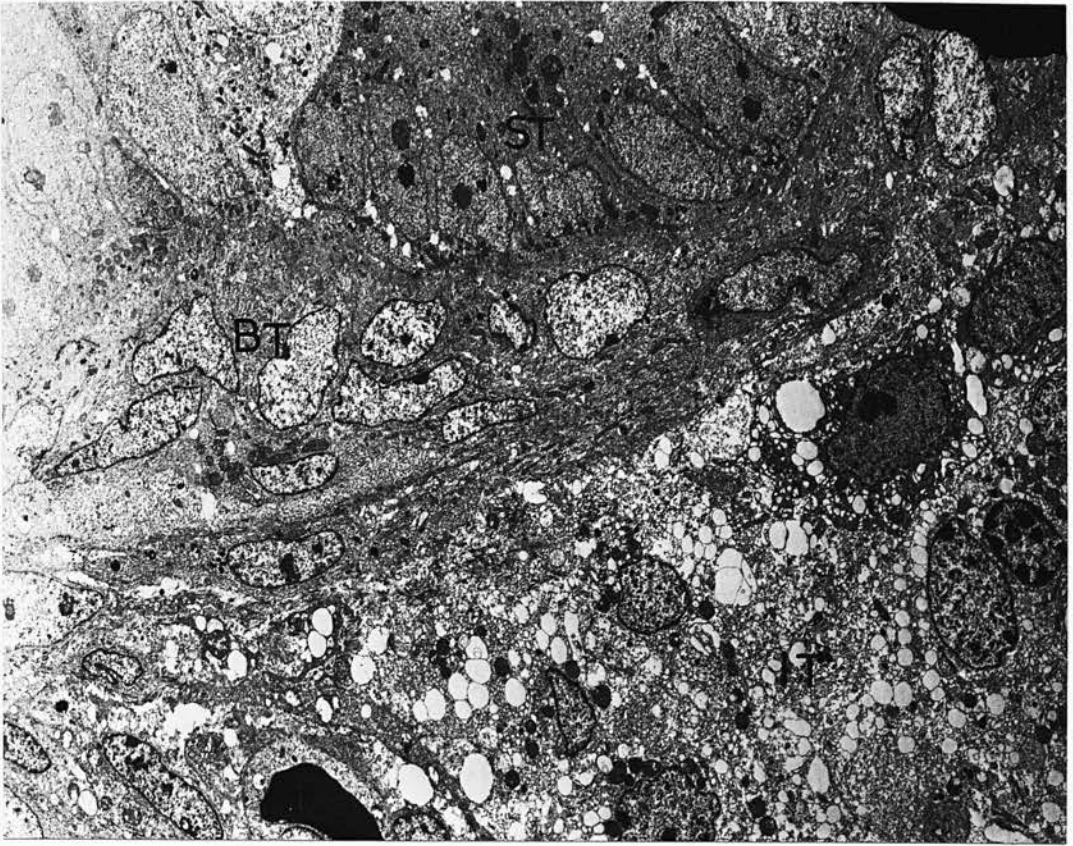


Figure 2.27

4% GTA/1% pHCHO/0.1M Millonig's  $\text{PO}_4$

Shows clear distinct cell membranes. Perinuclear vacuoles formed by the widening of the perinuclear cisterna are observed (arrow) in the germ cells.

X 5,000

Figure 2.28

4% GTA/1% pHCHO/0.1M Millonig's  $\text{PO}_4$

Shows clear distinct Golgi complex membranes and mitochondrial membranes.

X 26,000

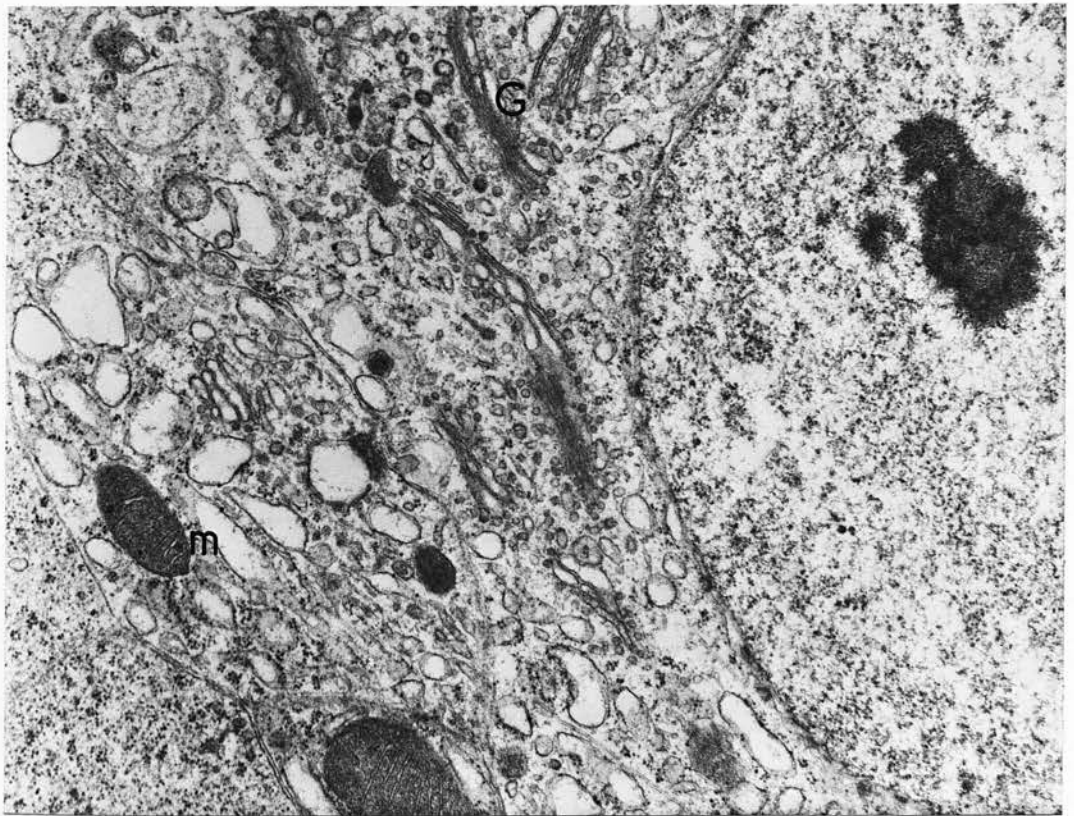
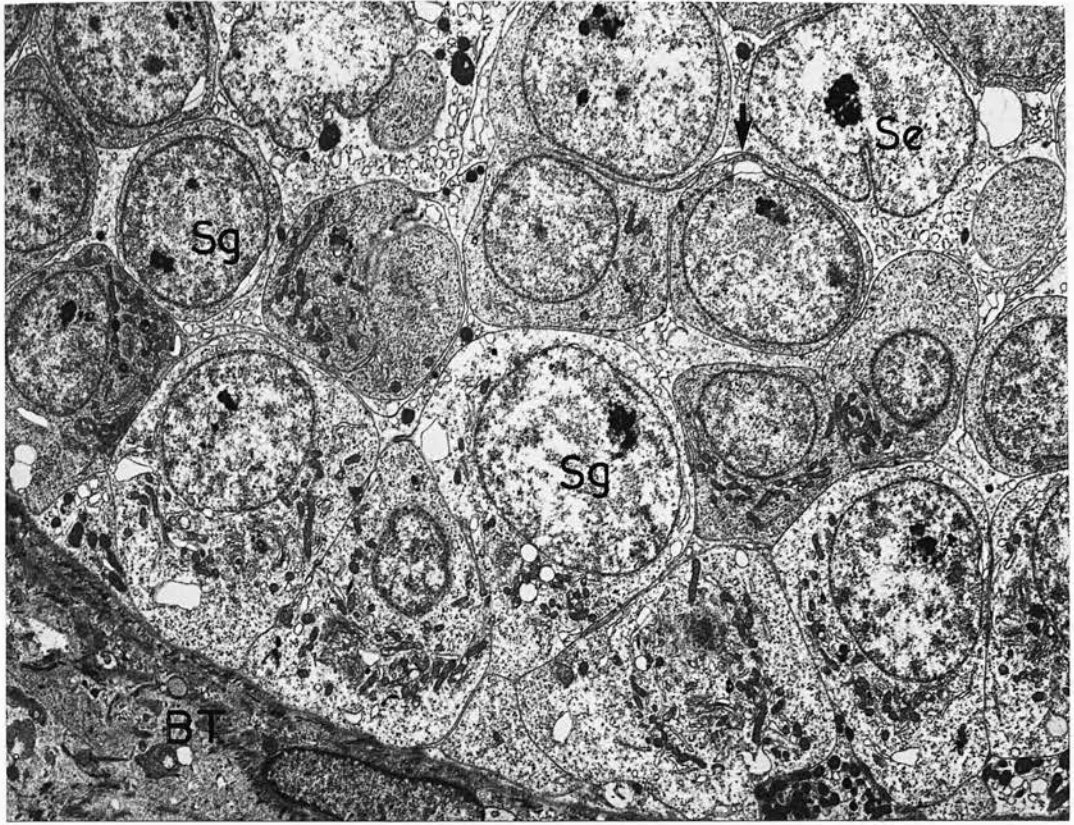


Figure 2.29

4% GTA/1% pHCHO/0.1M Millonig's  $\text{PO}_4$

Leydig cells exhibit hypertonic image. Seminiferous tubule cells are well preserved. Note widening of perinuclear cisternae.

X 26,000

Figure 2.30

Picric acid/pHCHO/ $\text{PO}_4$

All tissues show the formation of cytoplasmic vacuoles.

X 3,500

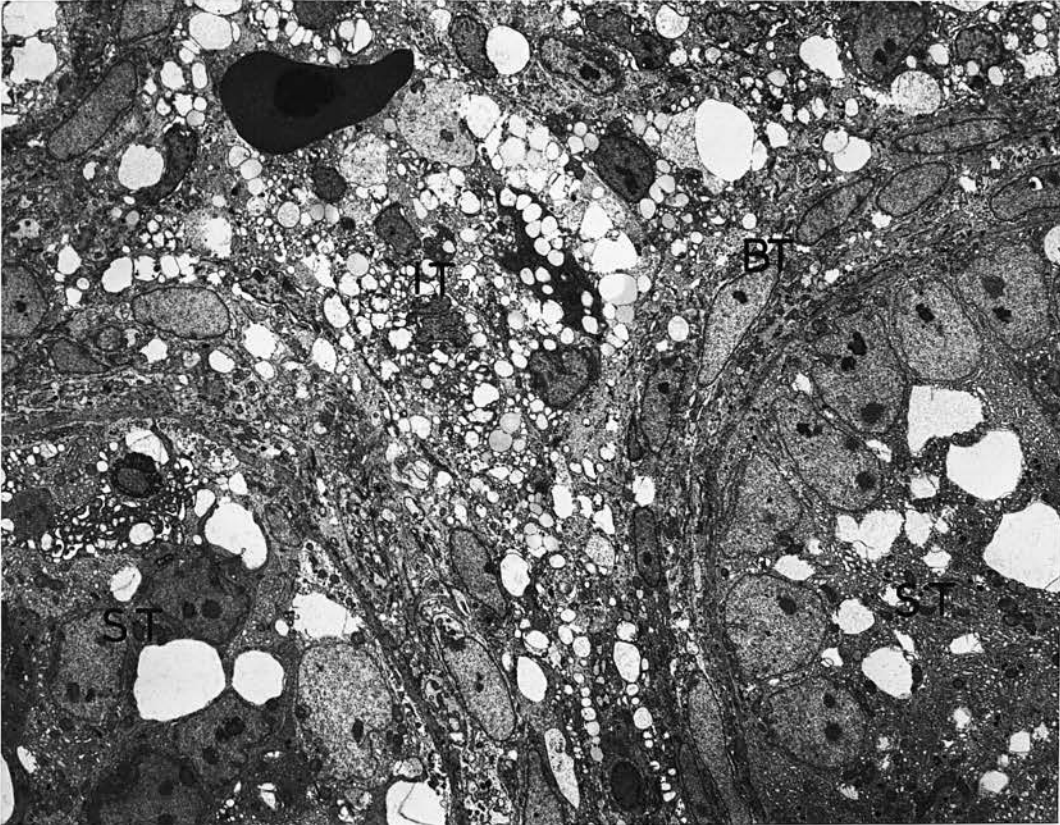
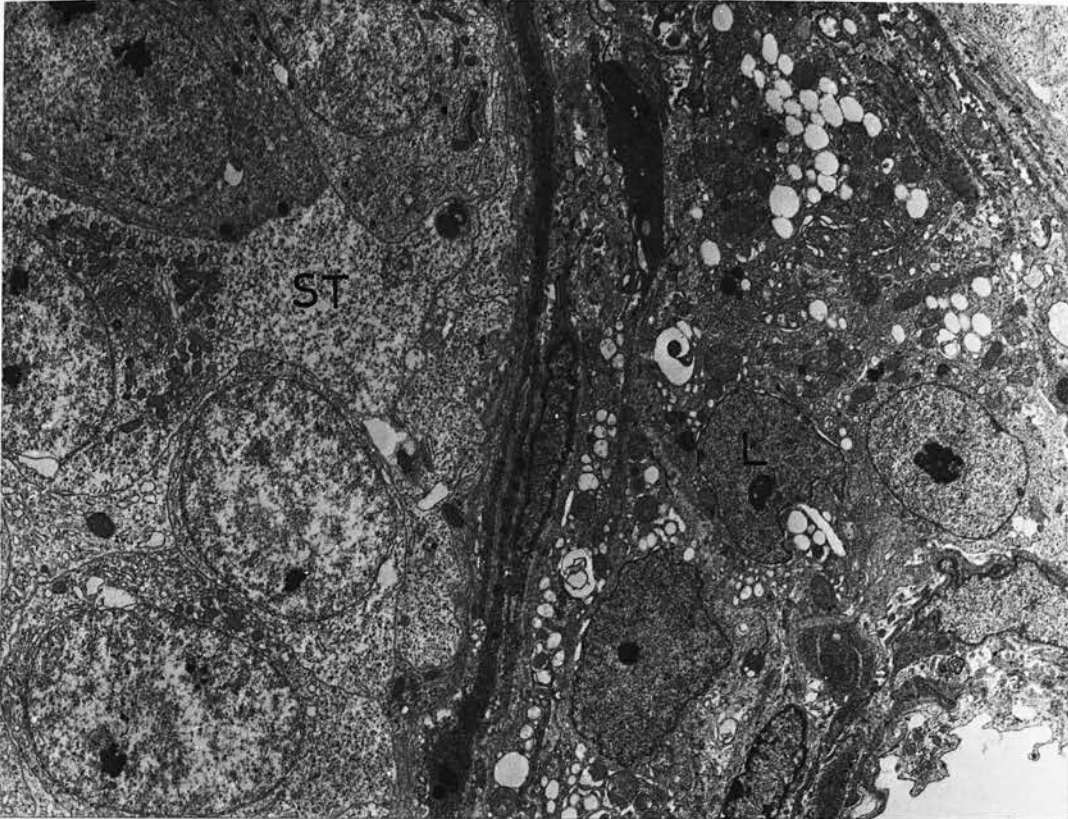




Figure 2.31

Picric acid/pHCHO/PO<sub>4</sub>

Shows formation of cytoplasmic vacuoles in tubule cells  
and swollen mitochondria.

X 9,500

Figure 2.32

Picric acid/pHCHO/PO<sub>4</sub>

Leydig cells exhibit swollen mitochondria and  
vesicular ER

X 12,000

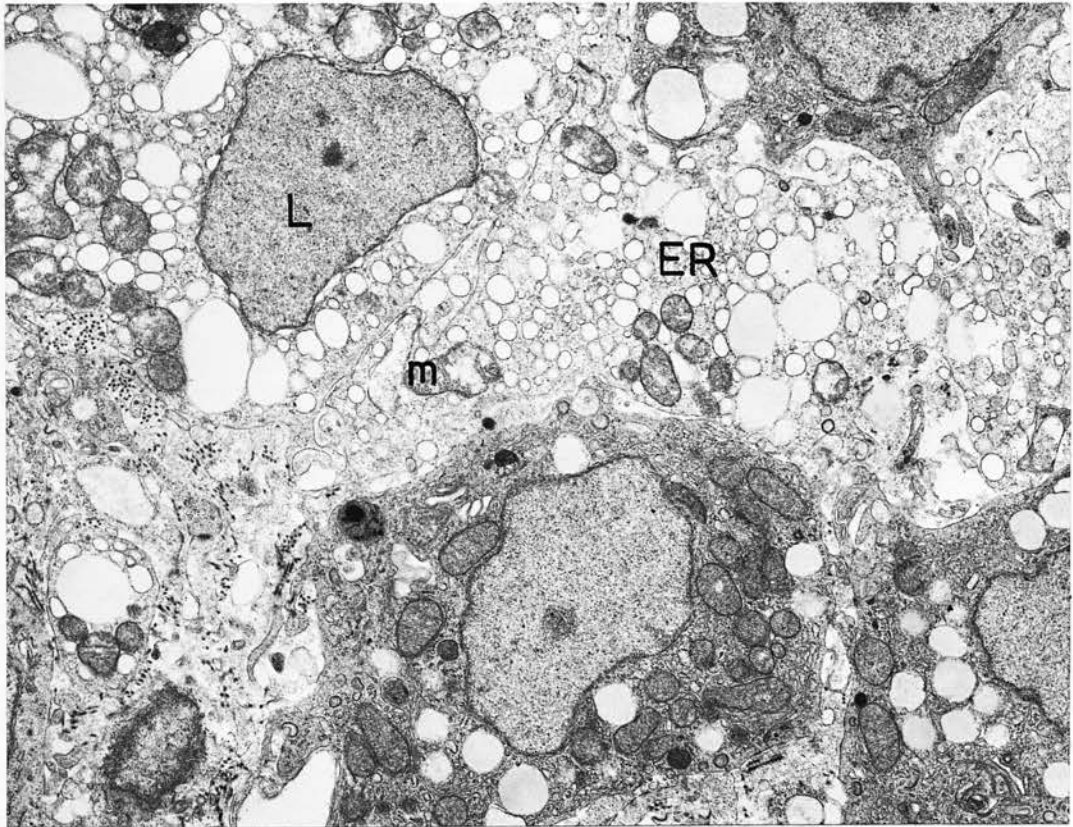
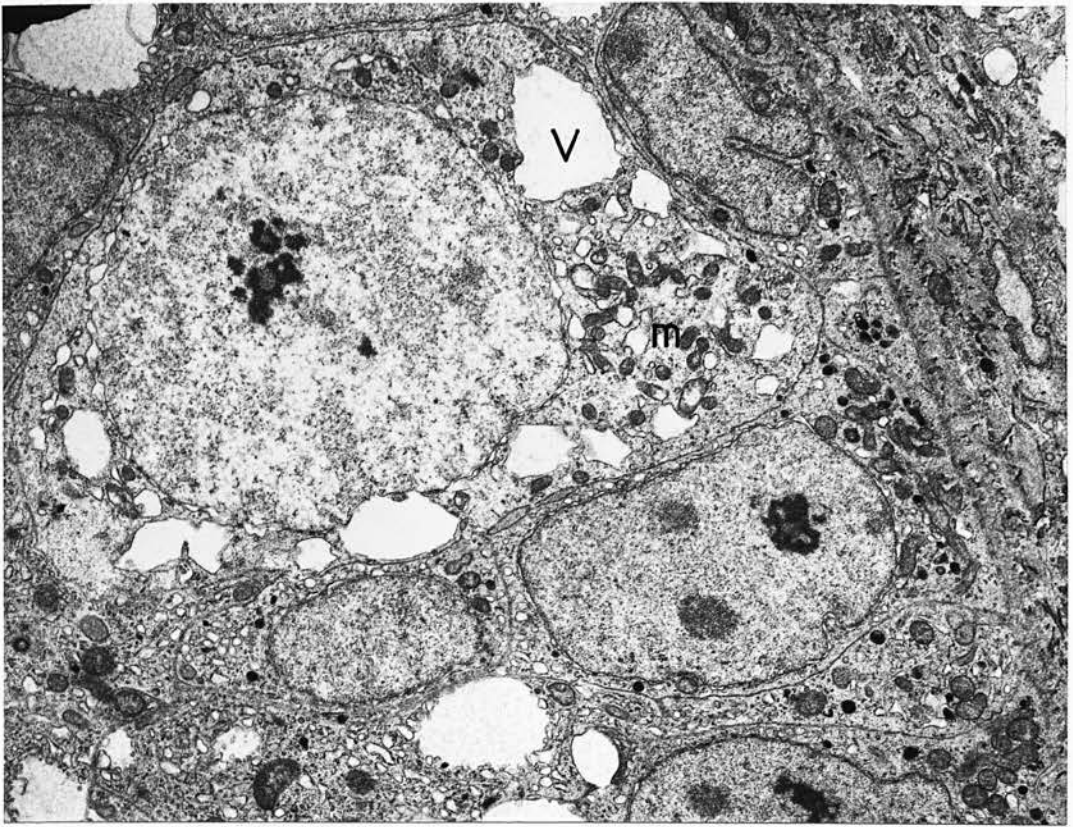


Figure 2.33

Picric acid/pHCHO/PO<sub>4</sub>

Shows lysosomes with matrices of varying density.

X 43,000

Figure 2.34

3% GTA/0.1M Millonig's PO<sub>4</sub>

Demonstrates well preserved boundary tissue and  
seminiferous tubules.

X 6,700

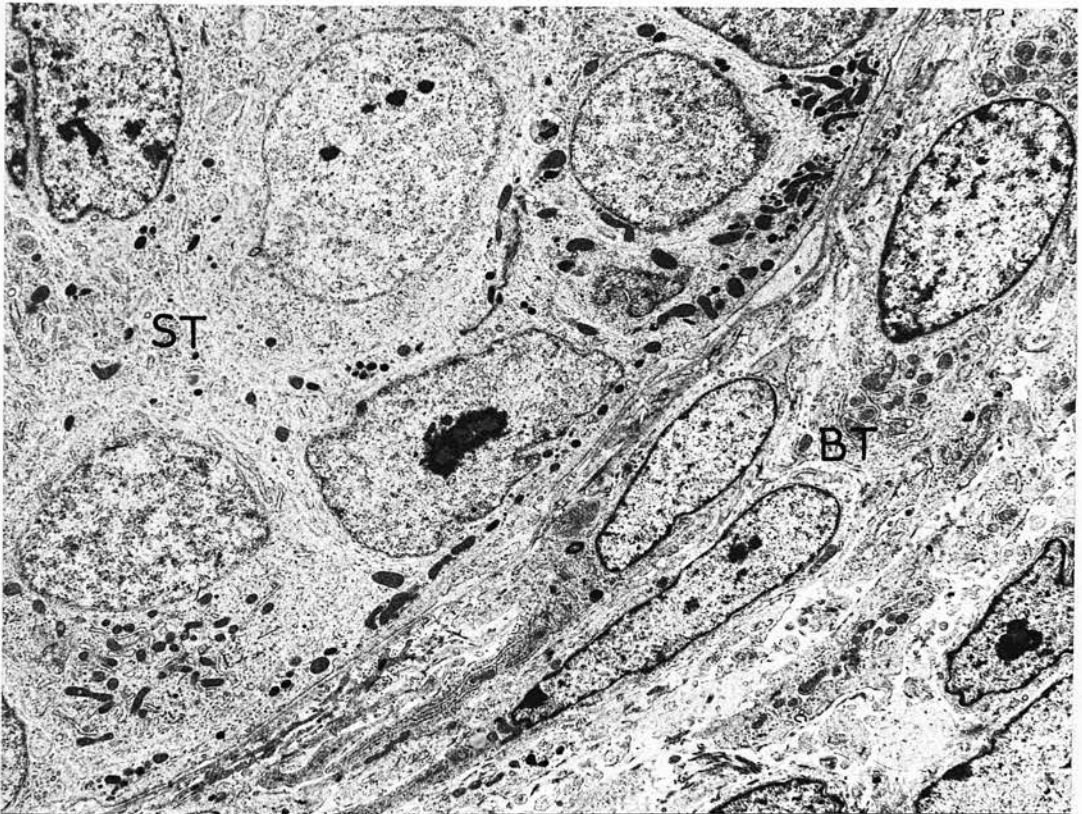
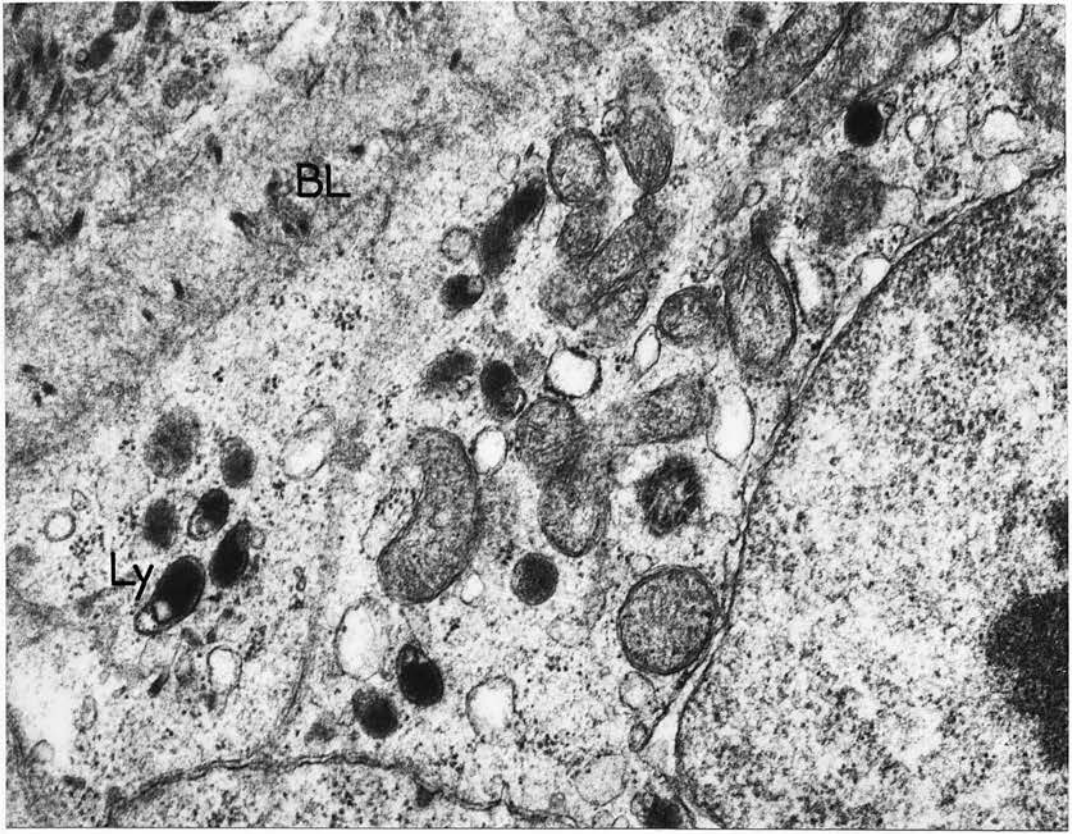


Figure 2.35

1% GTA/0.2M Millonig's  $\text{PO}_4$

Demonstrates a hypertonic image. Cells have dense mitochondria, perinuclear blisters and the cytoplasm has a dark contracted appearance.

X 3,500

Figure 2.36

5% GTA/0.05M Millonig's  $\text{PO}_4$

Demonstrate a hypotonic image. Cells have a leached appearance.

X 2,800

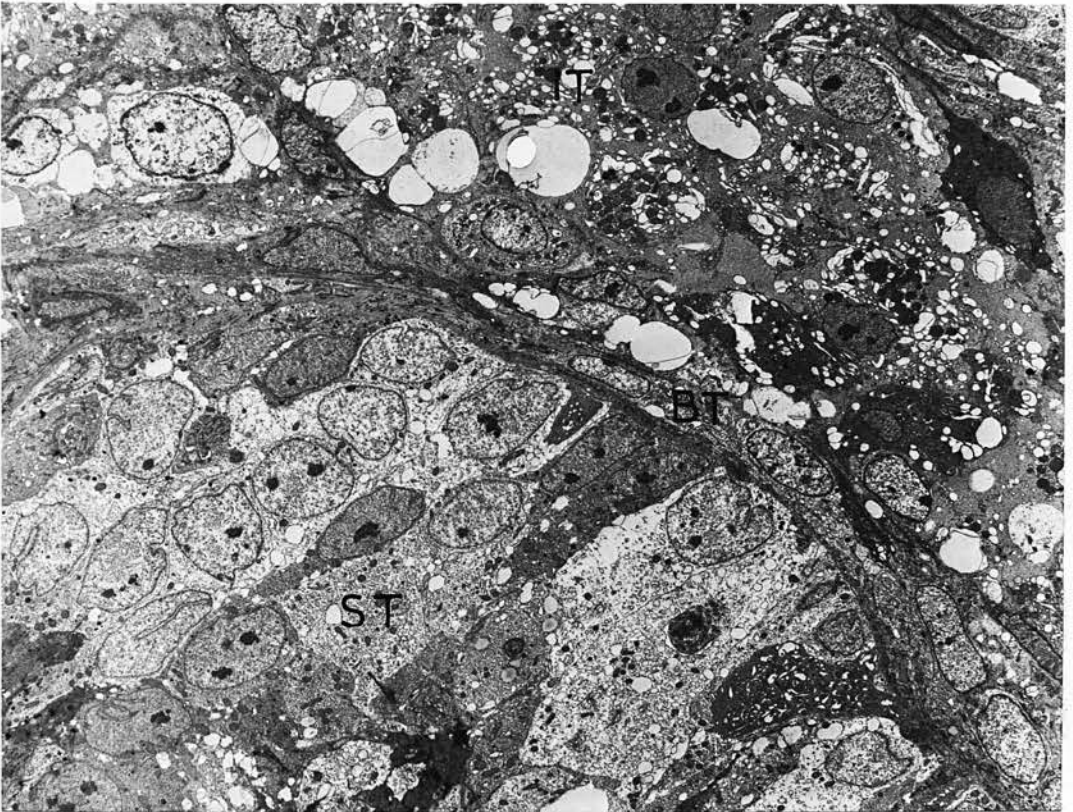
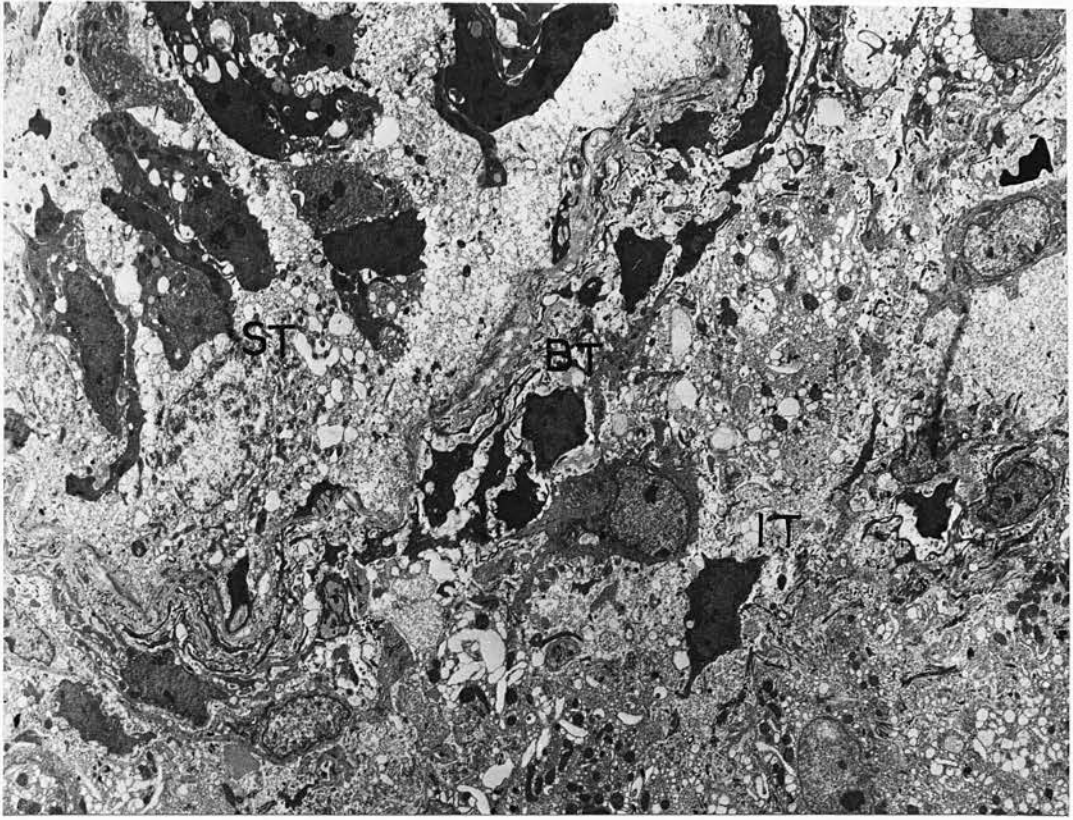


Figure 2.37

1% GTA/0.02M Millonig's  $\text{PO}_4$

Demonstrates a hypertonic image. Dense cytoplasm and mitochondria, and perinuclear blisters are seen.

X 6,700

Figure 2.38

5% GTA/0.05M Millonig's  $\text{PO}_4$

Demonstrate a hypotonic image. Distended mitochondria and swollen ER are seen.

X 6,700

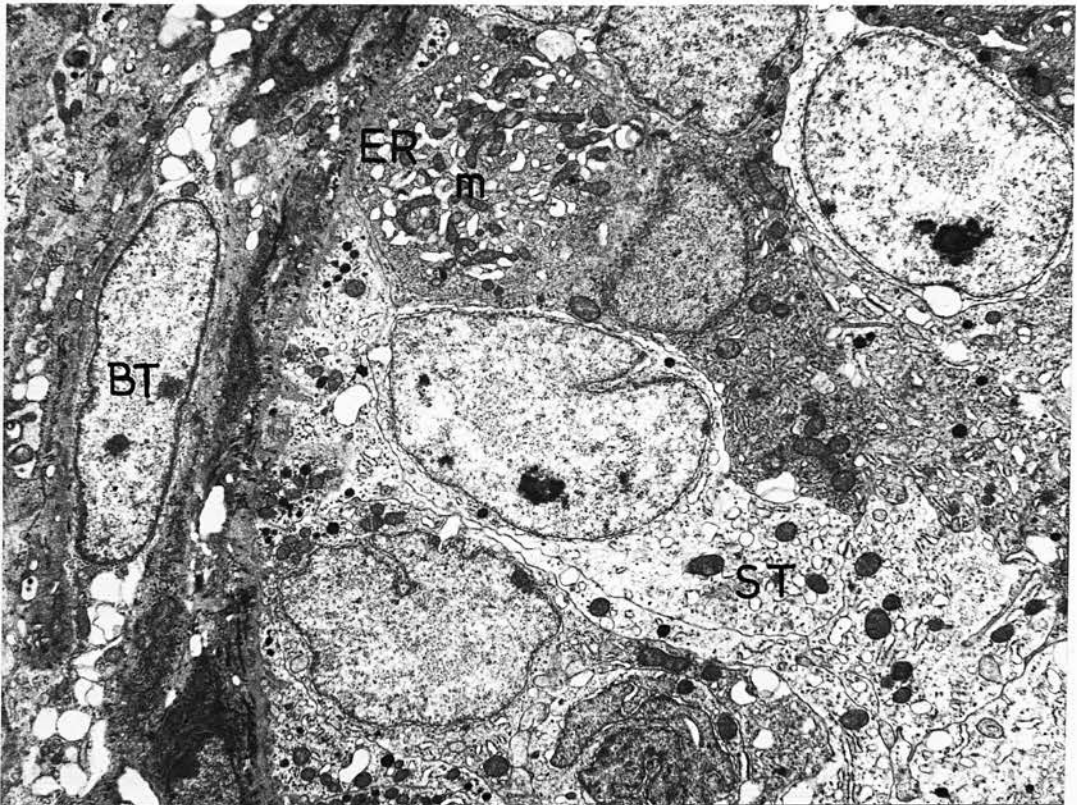
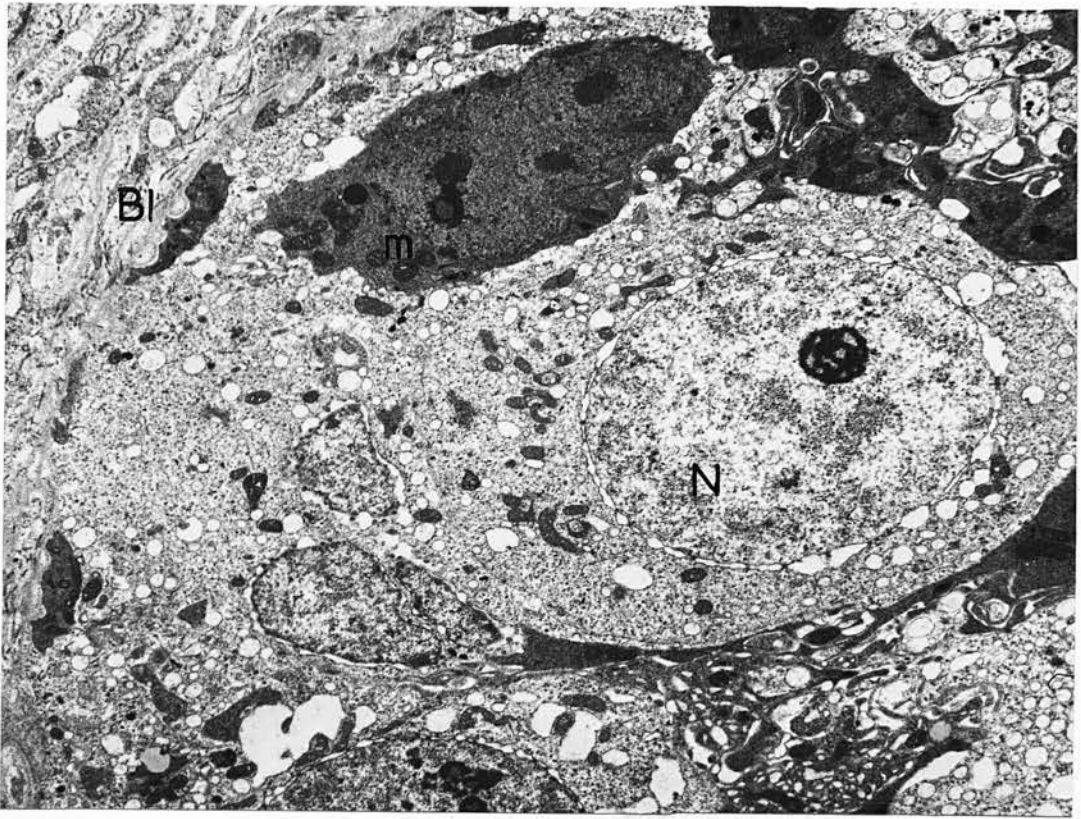




Figure 2.39

5% GTA/0.1M Millonig's  $\text{PO}_4$

Demonstrates a slightly hypertonic image. The cytoplasm is dense but no contracted appearance of the cells is evident.

X 9,500

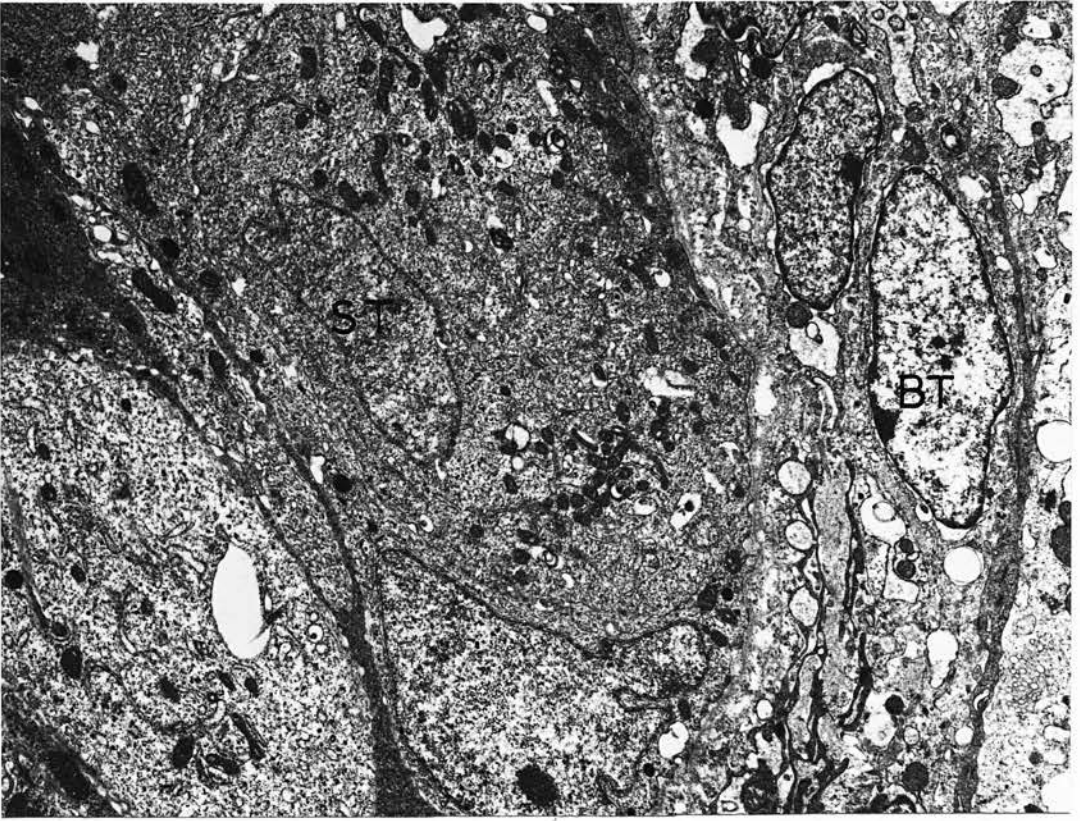


Figure 2.40

4% GTA/0.1M s-collidine

Demonstrates well preserved intertubular tissue,  
boundary tissue and seminiferous tubule.

X 3,600

Figure 2.41

4% GTA/0.1M s-collidine

Demonstrates well preserved seminiferous tubule and  
boundary tissue.

X 6,700

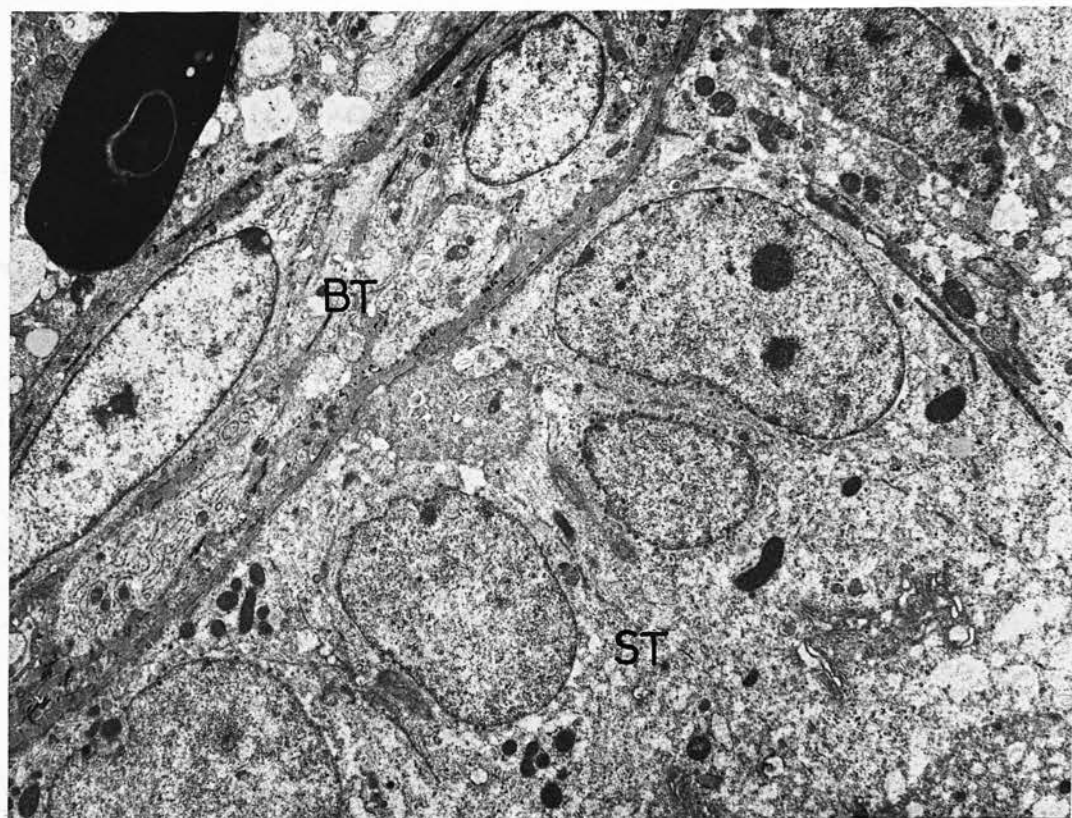
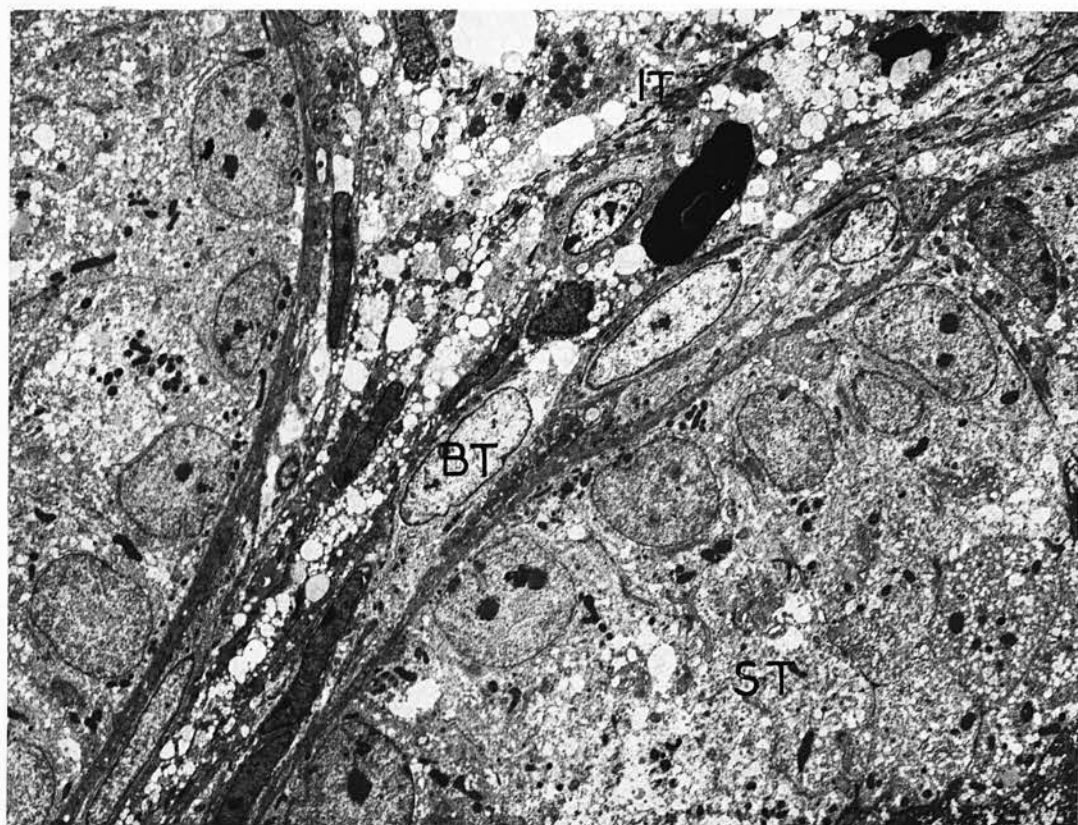


Figure 2.42

4% GTA/0.075M Na cacodylate

Demonstrates well preserved intertubular and boundary tissue and seminiferous tubule.

X 2,800

Figure 2.43

4% GTA/0.075M Na cacodylate.

Demonstrates well preserved mitochondria and Golgi complex.

X 26,000

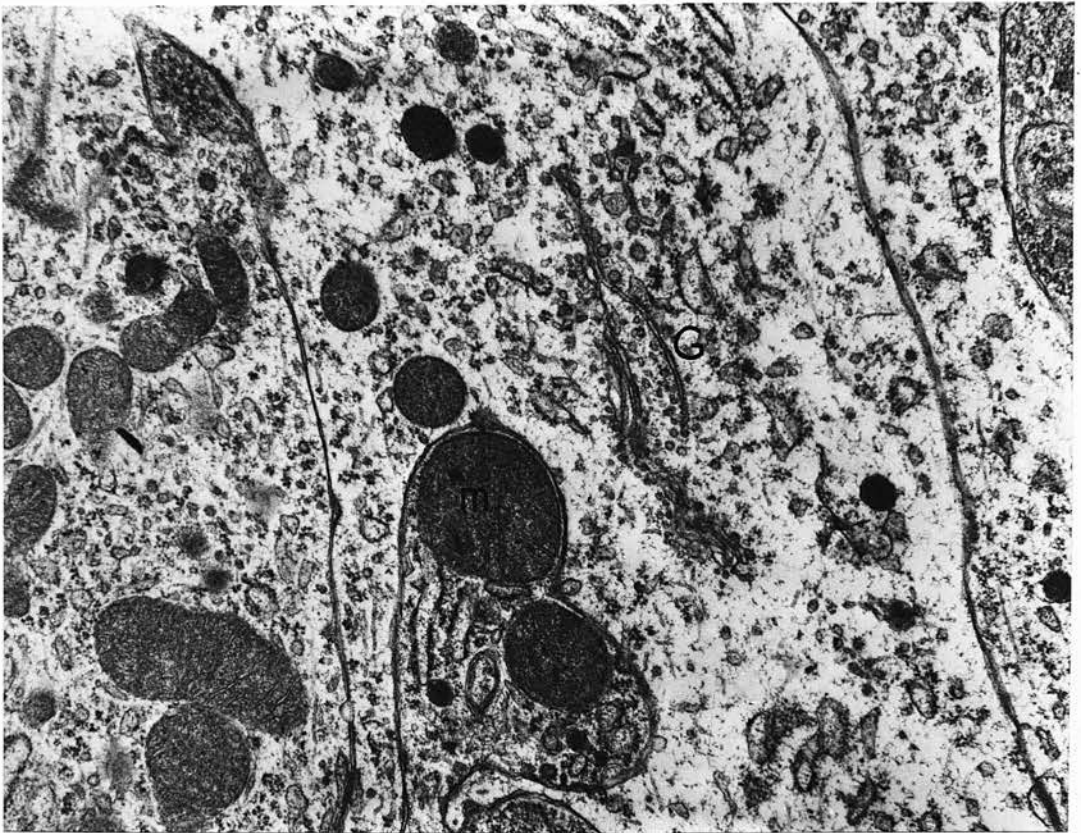
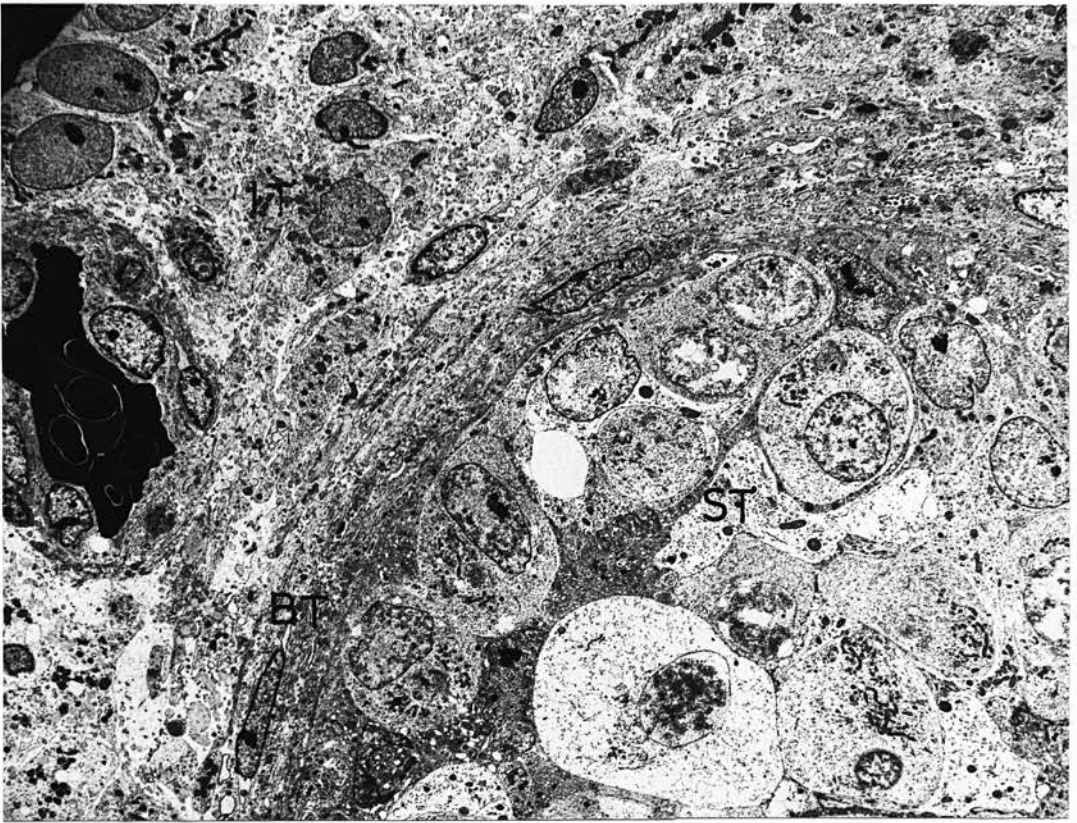


Figure 2.44

5% GTA/0.2M s-collidine

Demonstrates a hypertonic image. Note tubule cells  
have dark contracted nuclei.

X 3,600

Figure 2.45

5% GTA/0.2M s-collidine

Demonstrates hypertonic image. Note dark contracted  
appearance of tubule cells.

X 6,700

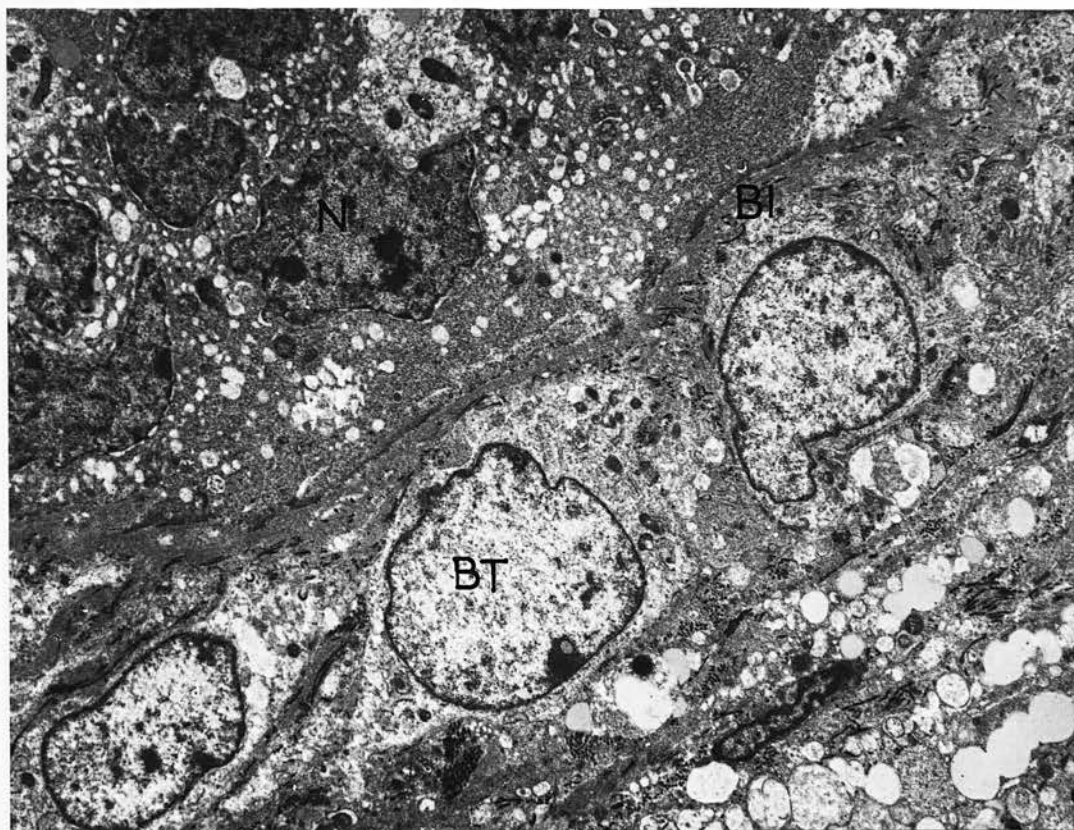
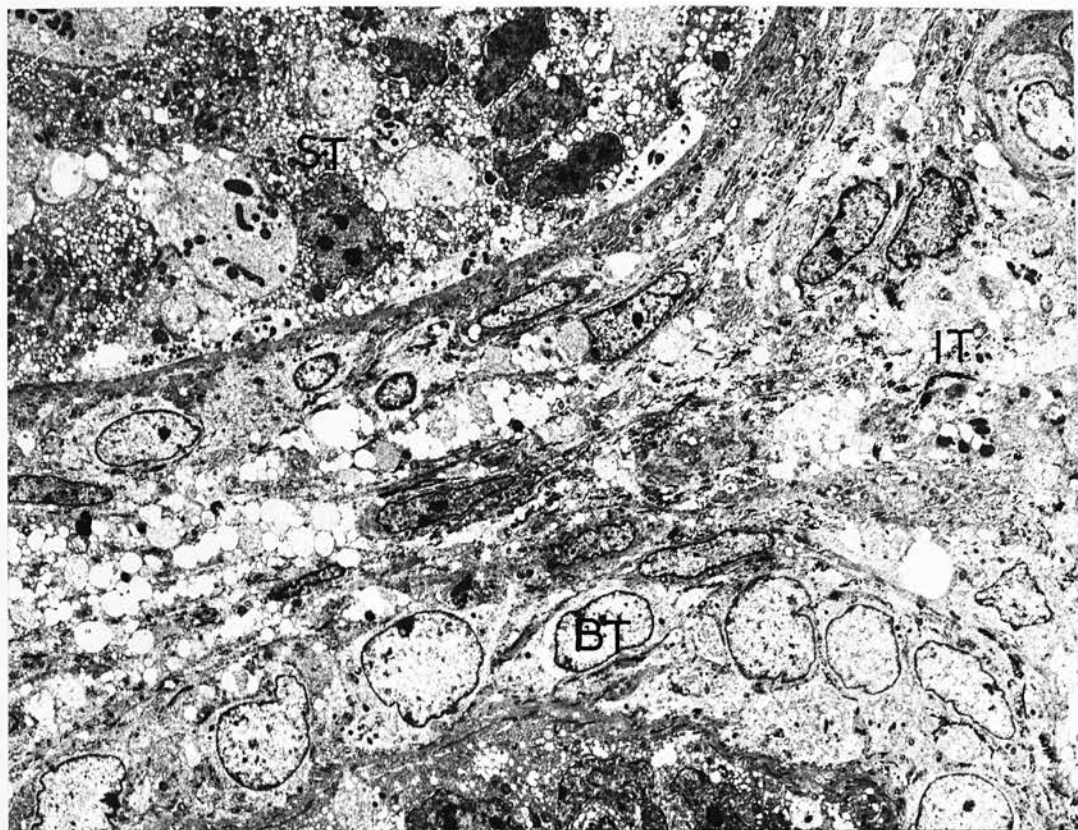




Figure 2.46

3% GTA/0.1M Na cacodylate.

Demonstrates hypertonic image. Note dark contracted  
~~nuclei~~ nuclei with perinuclear blisters and cytoplasmic  
vacuole formation.

X 3,800

Figure 2.47

3% GTA/0.1M Na cacodylate

Demonstrates hypertonic image. Note formation of  
perinuclear vacuoles and dense cytoplasm.

X 15,000

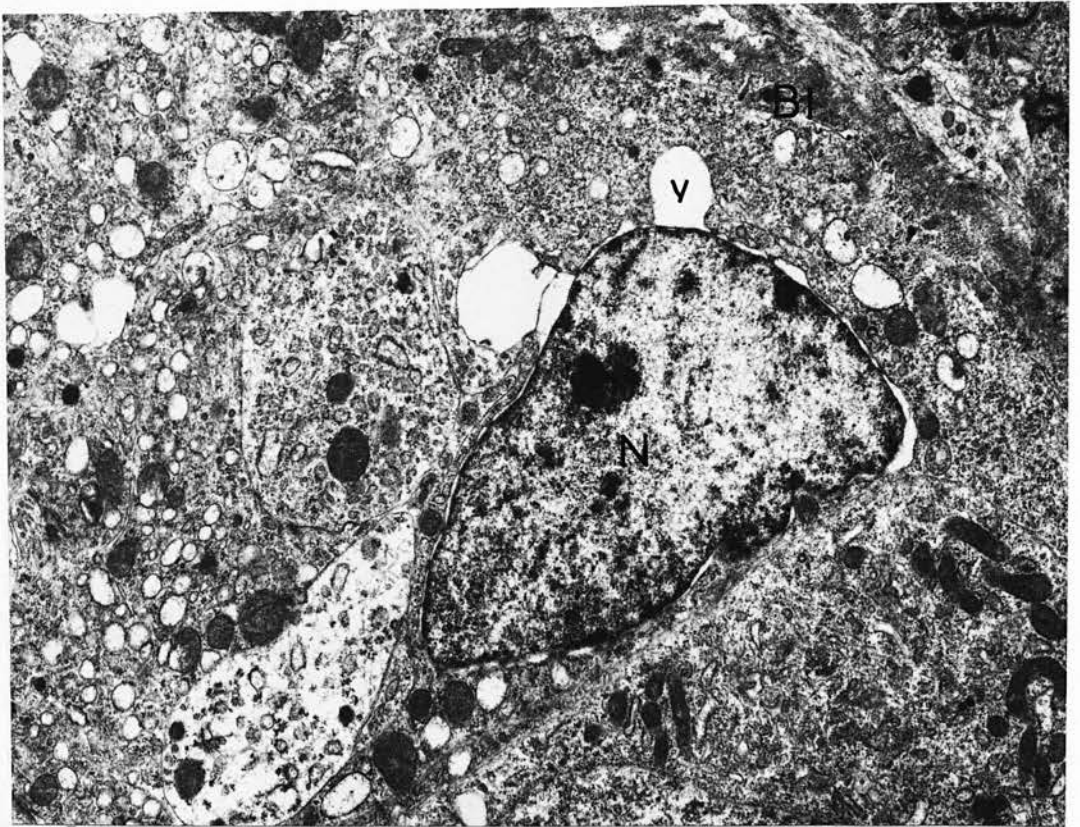
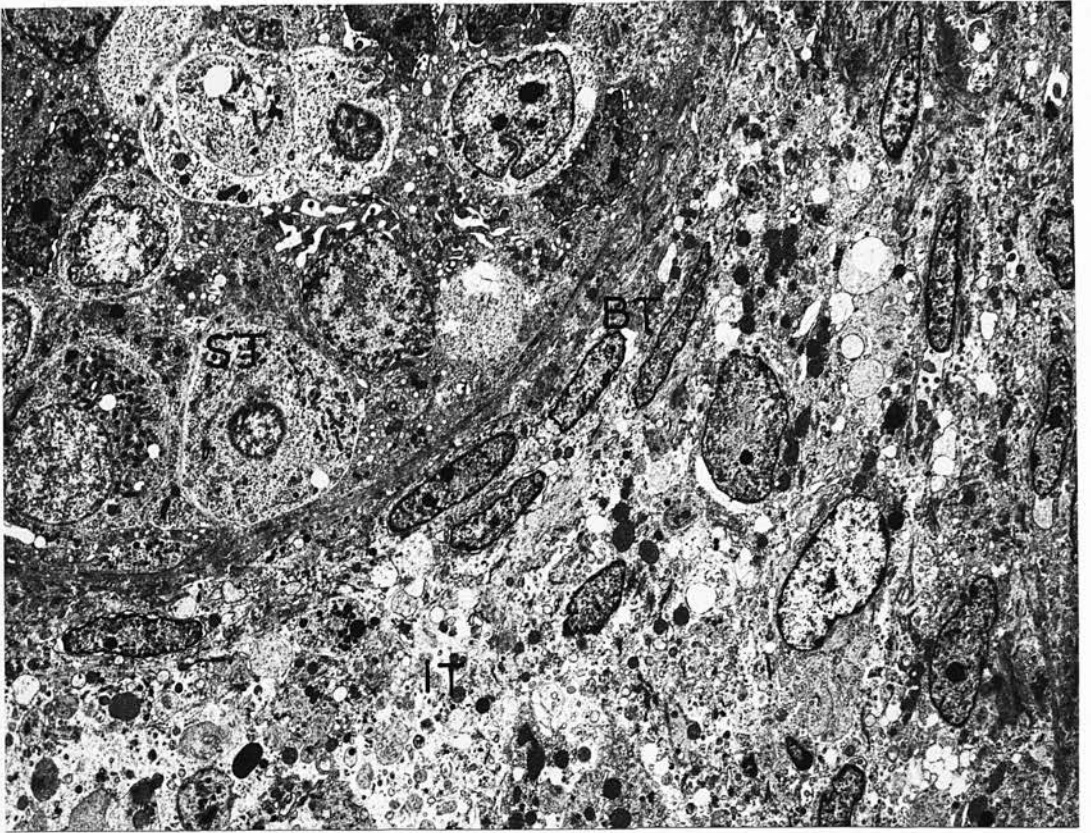


Figure 2.48

2.5% GTA/0.15M Na cacodylate

Demonstrates a very hypertonic image. Note very dense tubule cells and loss of cell outline.

X 3,500

Figure 2.49

4% GTA/0.075M Na cacodylate, 1% OsO<sub>4</sub>/0.175M Na cacodylate

Shows loss of cytoplasmic ground substance in some cells.

X 25,000

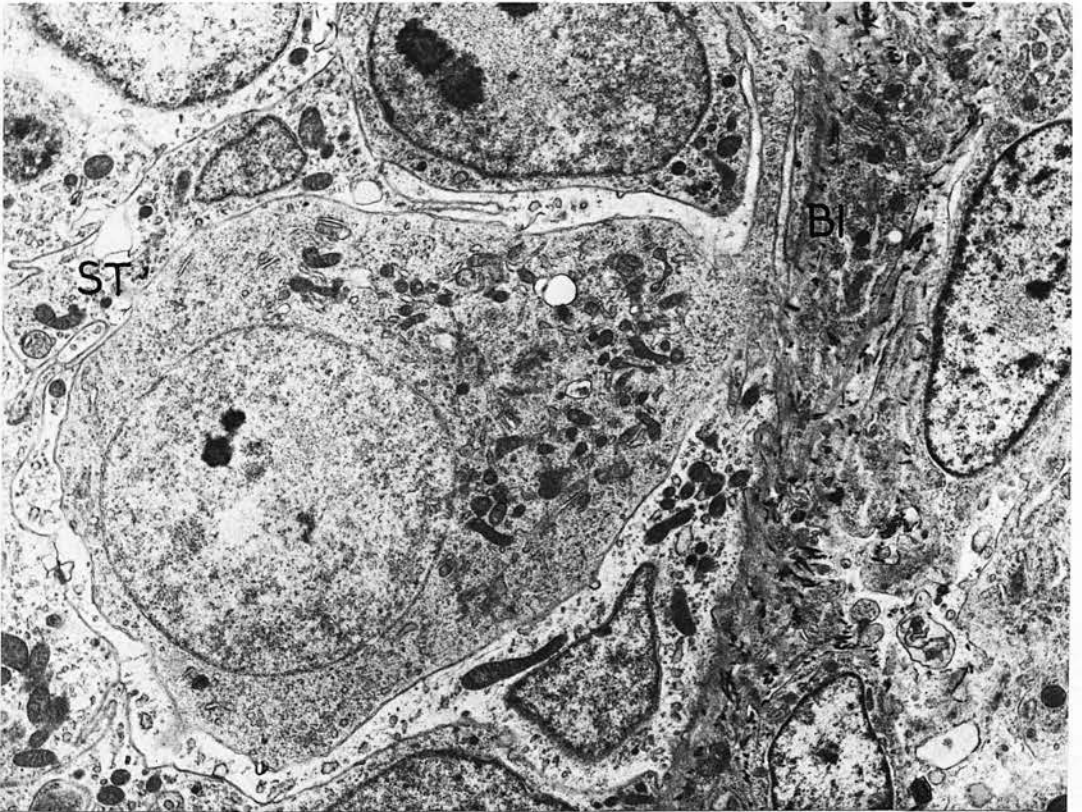
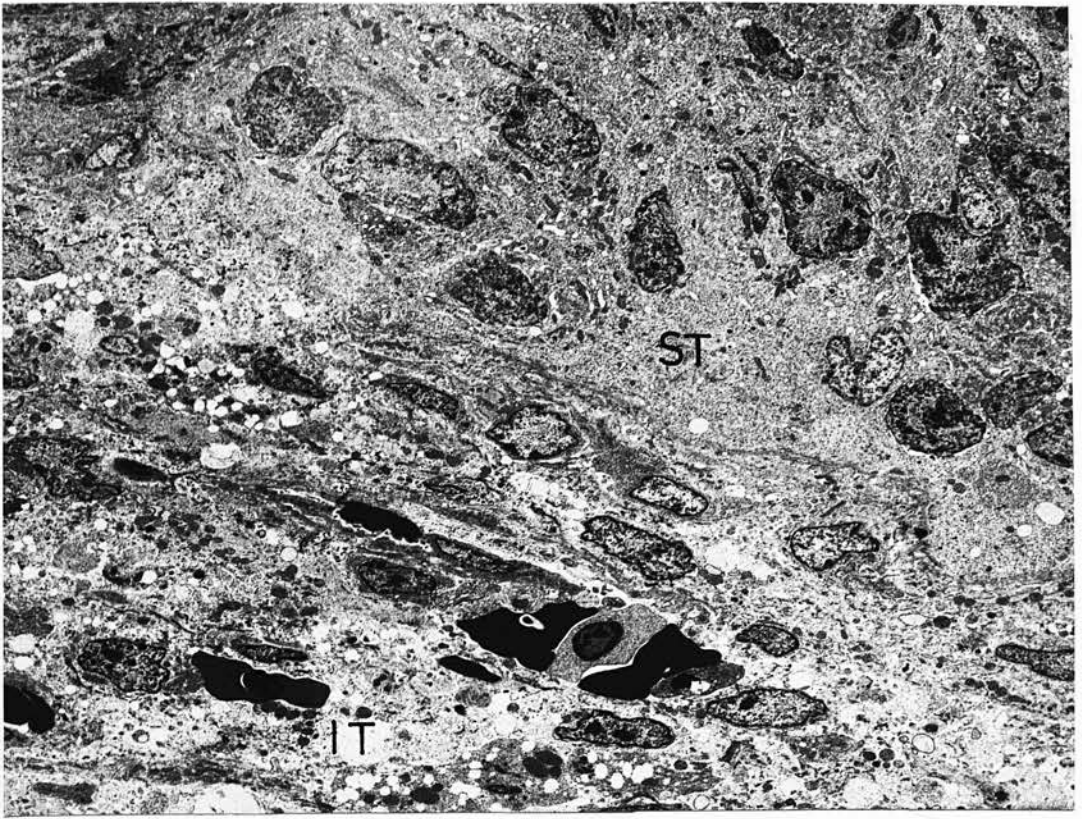


Figure 2.50

4% GTA/0.075M Na cacodylate, 1% OsO<sub>4</sub>/0.1M PO<sub>4</sub>

The cytoplasmic ground substance is well preserved.

X 7,500

Figure 2.51

4% GTA/0.075M Na cacodylate, 1% OsO<sub>4</sub>/modified

Dalton's buffer.

The cytoplasmic ground substance is leached and  
large cytoplasmic vacuoles are observed.

X 7,500

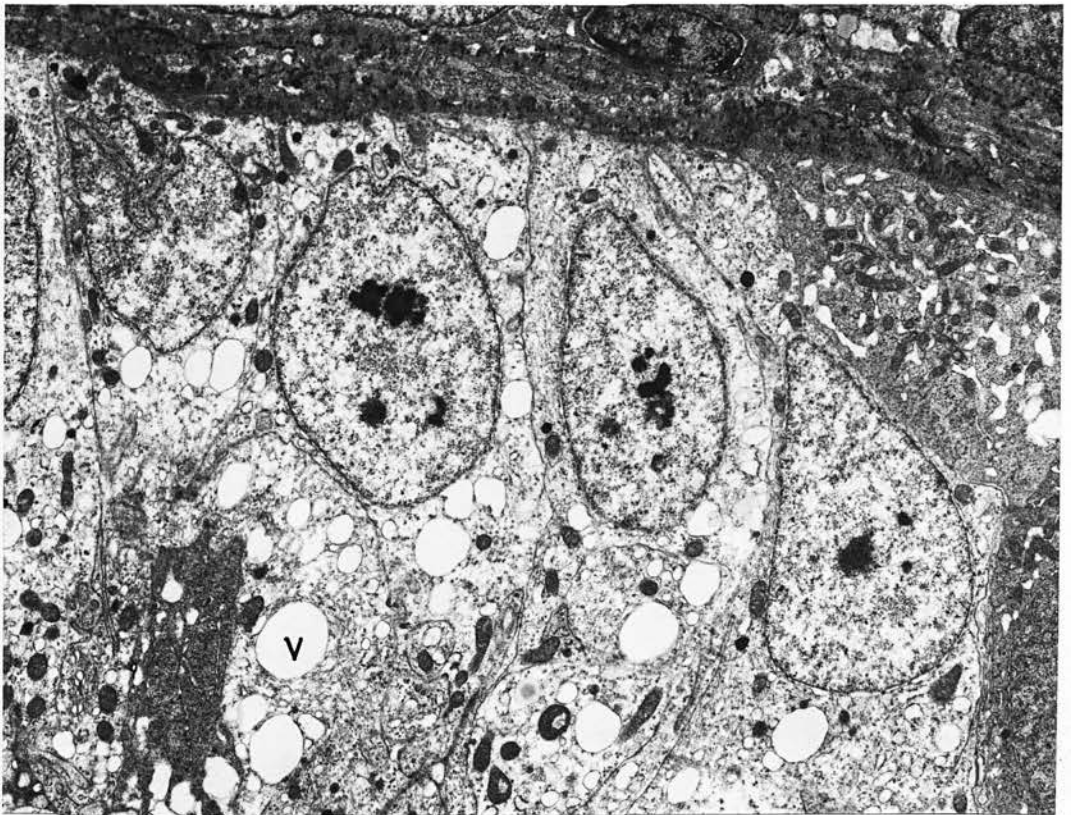
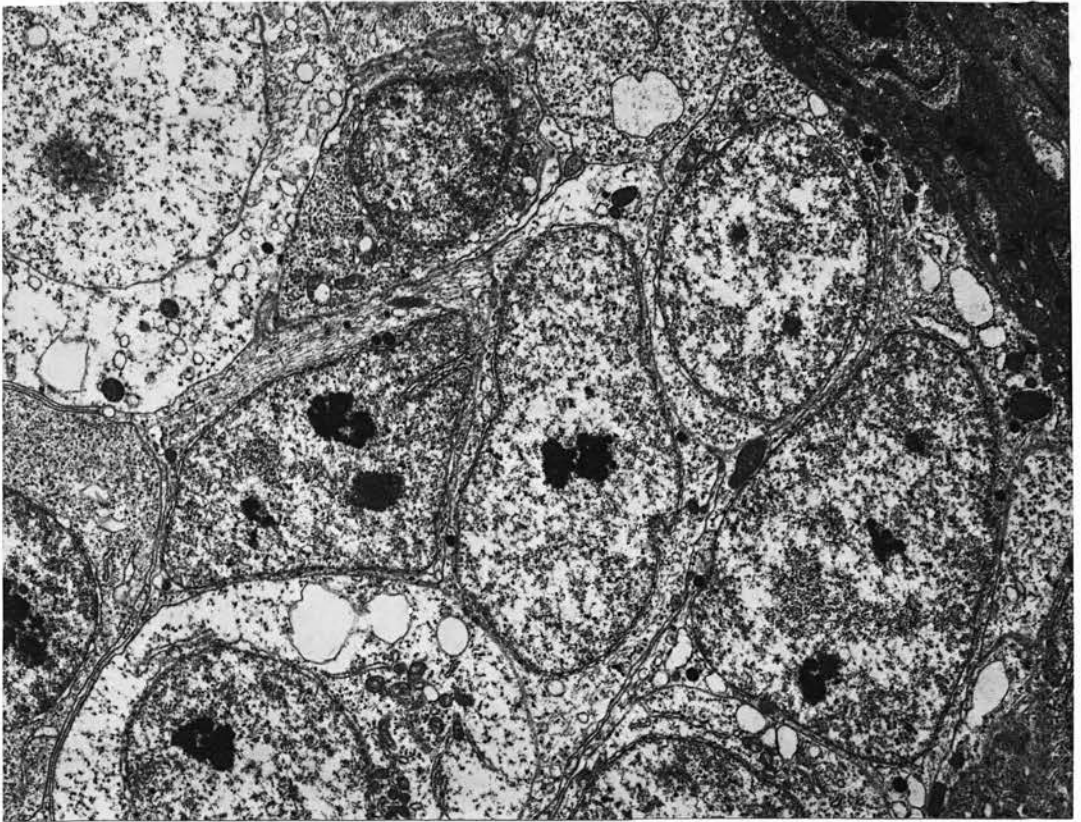


Figure 2.52

4% GTA/0.075M Na cacodylate, 1% OsO<sub>4</sub>/1.5% K<sub>4</sub>Fe(CN)<sub>6</sub>  
A pale cytoplasmic ground substance of even density  
is observed.

X 11,172

Figure 2.53

4% GTA/0.075M Na cacodylate, 1% OsO<sub>4</sub>/1.5% K<sub>4</sub>Fe(CN)<sub>6</sub>  
The trilaminar structure of the unit membrane is observed  
within a cisternal junction.

X 137,000

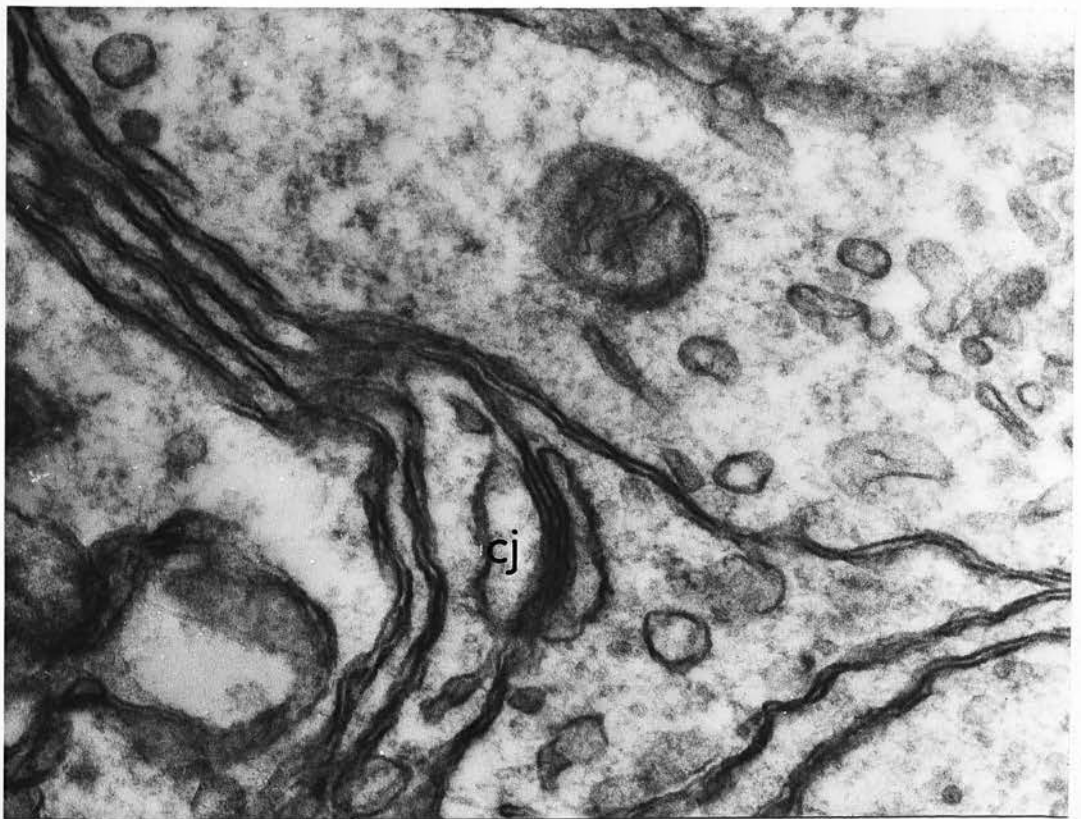
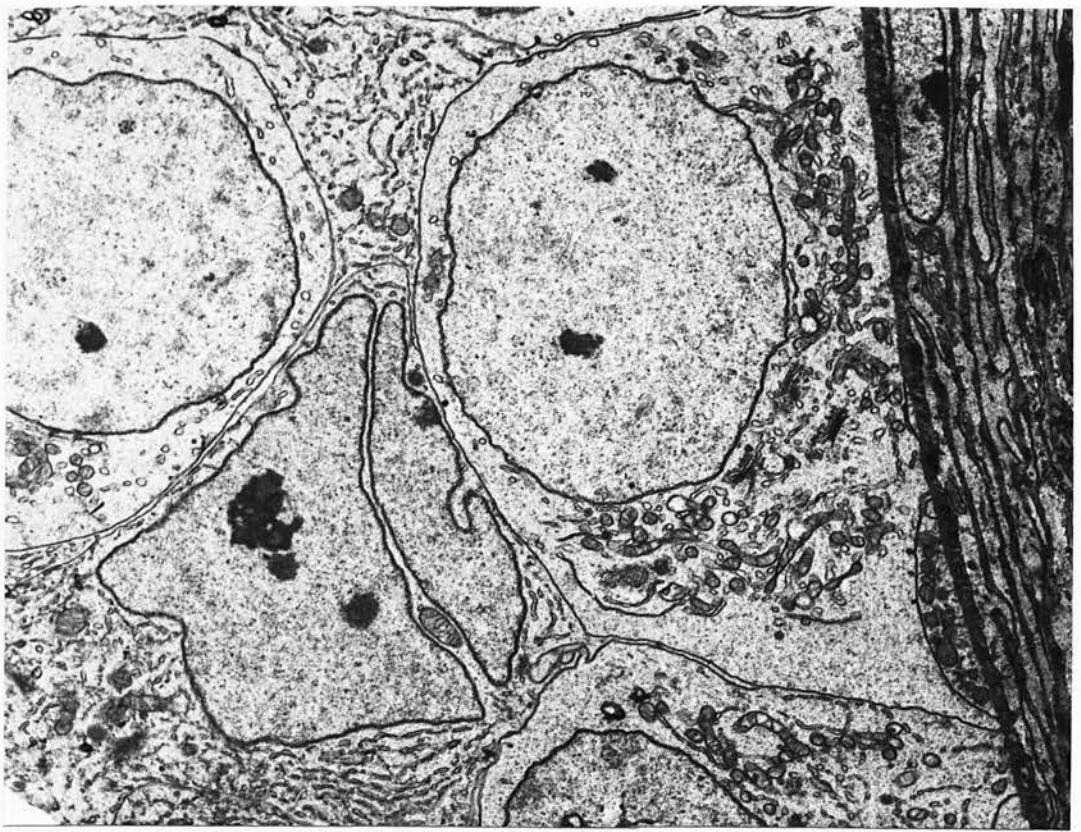




Figure 2.54

1% OsO<sub>4</sub>/modified Dalton's buffer.

21 week old bird. The cells of the seminiferous tubule have separated and many Sertoli cells are lost. The intercellular spaces of the boundary tissue are widened causing cell layer separation.

X 2,800

Figure 2.55

5% acrolein/0.1M PO<sub>4</sub>

21 week old bird. The intertubular tissue is destroyed and the boundary tissue is observed separated into cell layers.

X 2,800

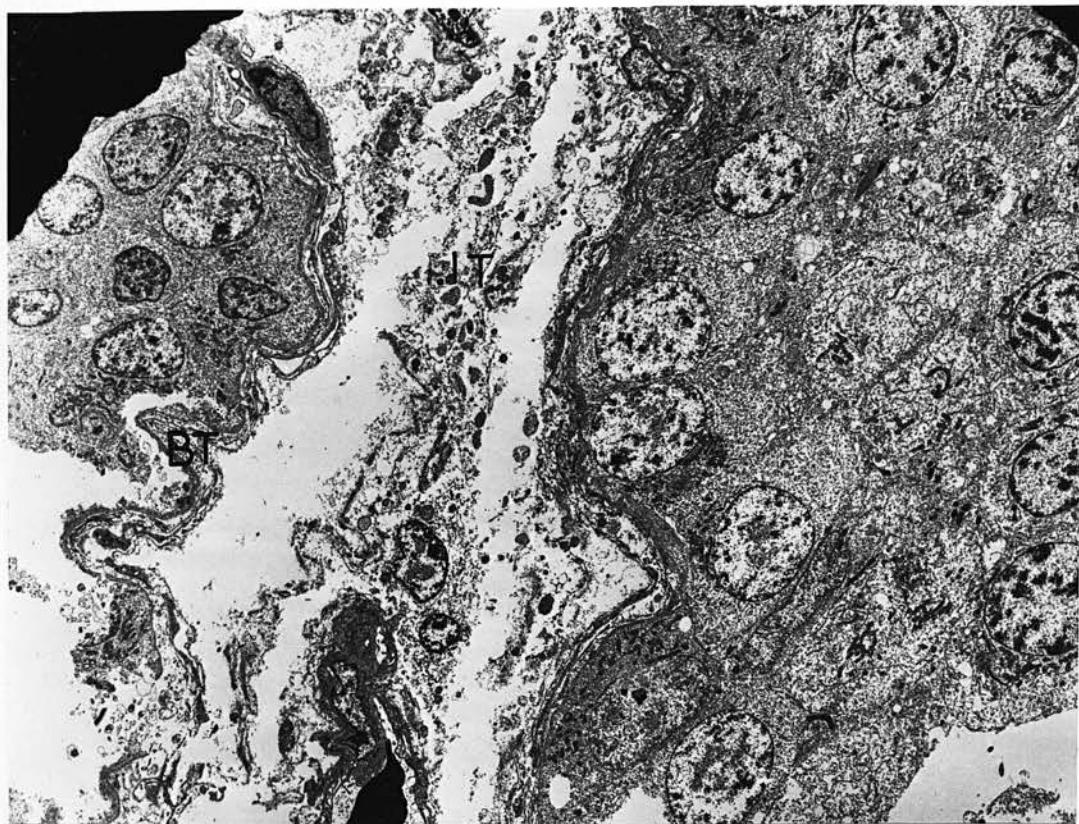
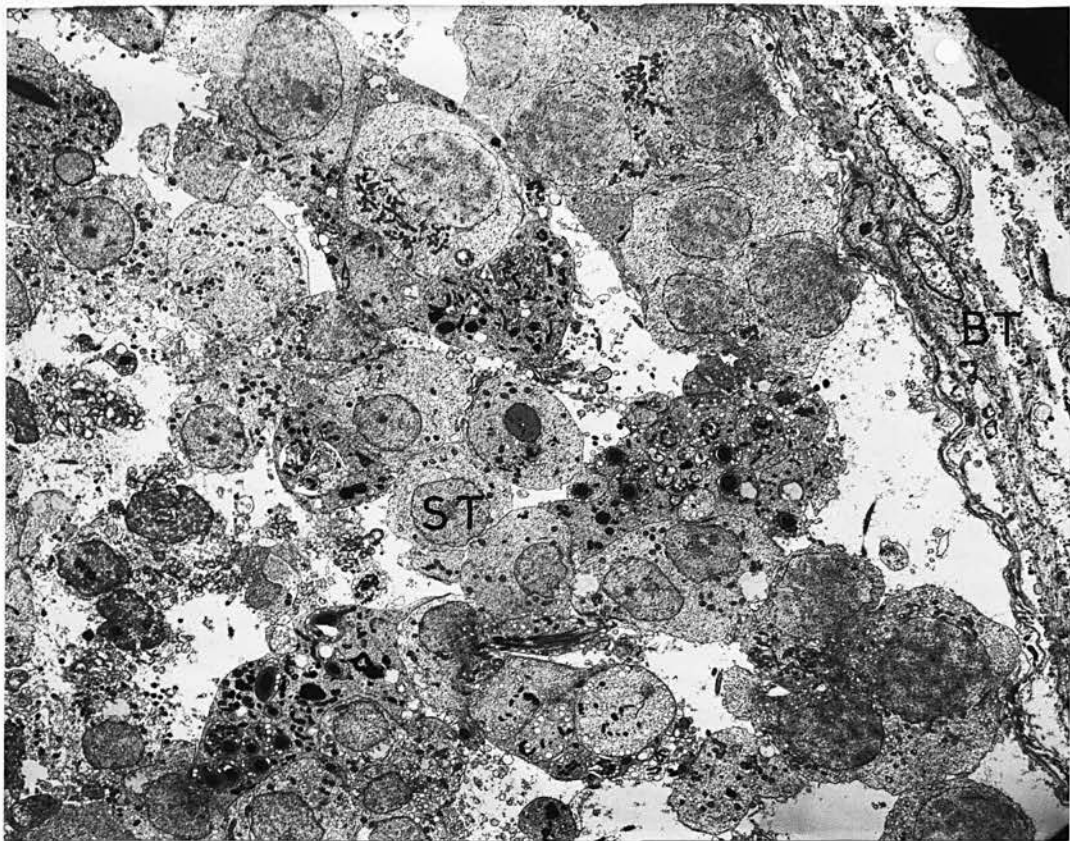


Figure 2.56

Picric acid/pHCHO/PO<sub>4</sub>

21 week old bird. Intertubular cell membranes are destroyed and the cytoplasm lost. Large vacuoles are observed in the tubule cell cytoplasm and nucleoplasm.

X 3,800

Figure 2.57

4% GTA/1% pHCHO/0.1M Millonig's PO<sub>4</sub>

21 week old bird. Large vacuoles formed by the protrusion of cell or nuclear membranes are observed in the basal cells of the tubule. The cytoplasm of the spermatogonial cells is more dense than the adluminal cells.

X 2,800

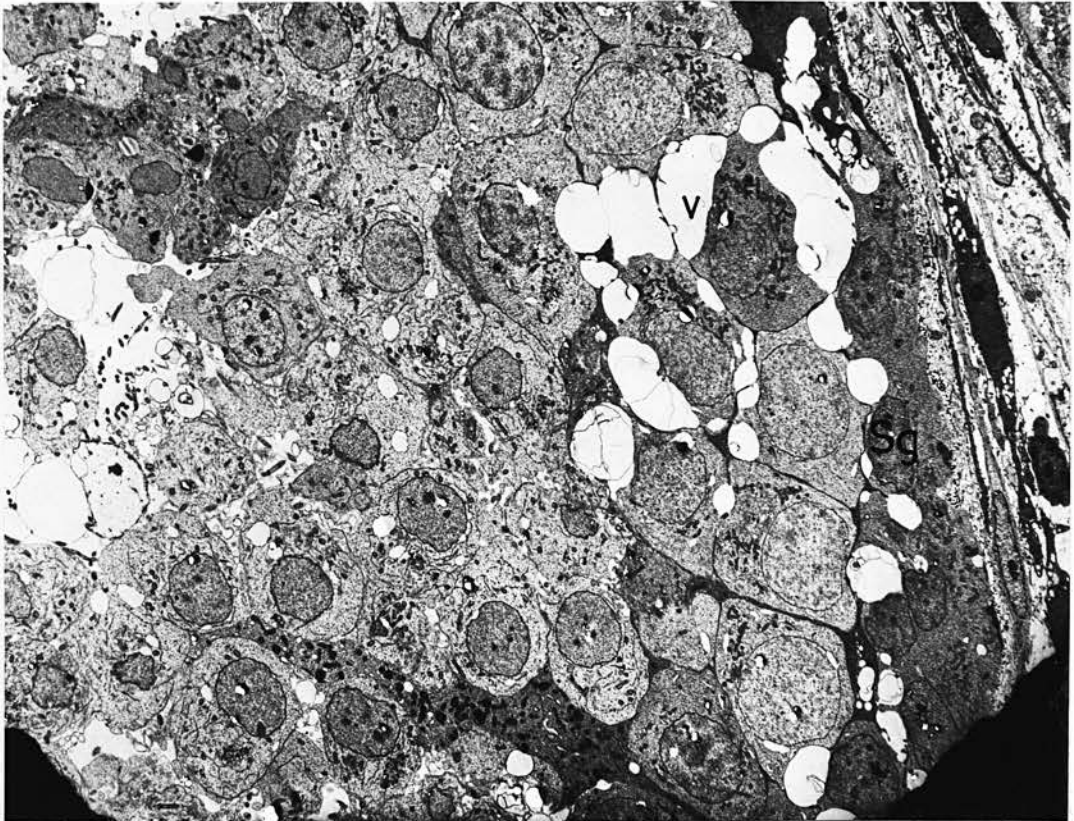
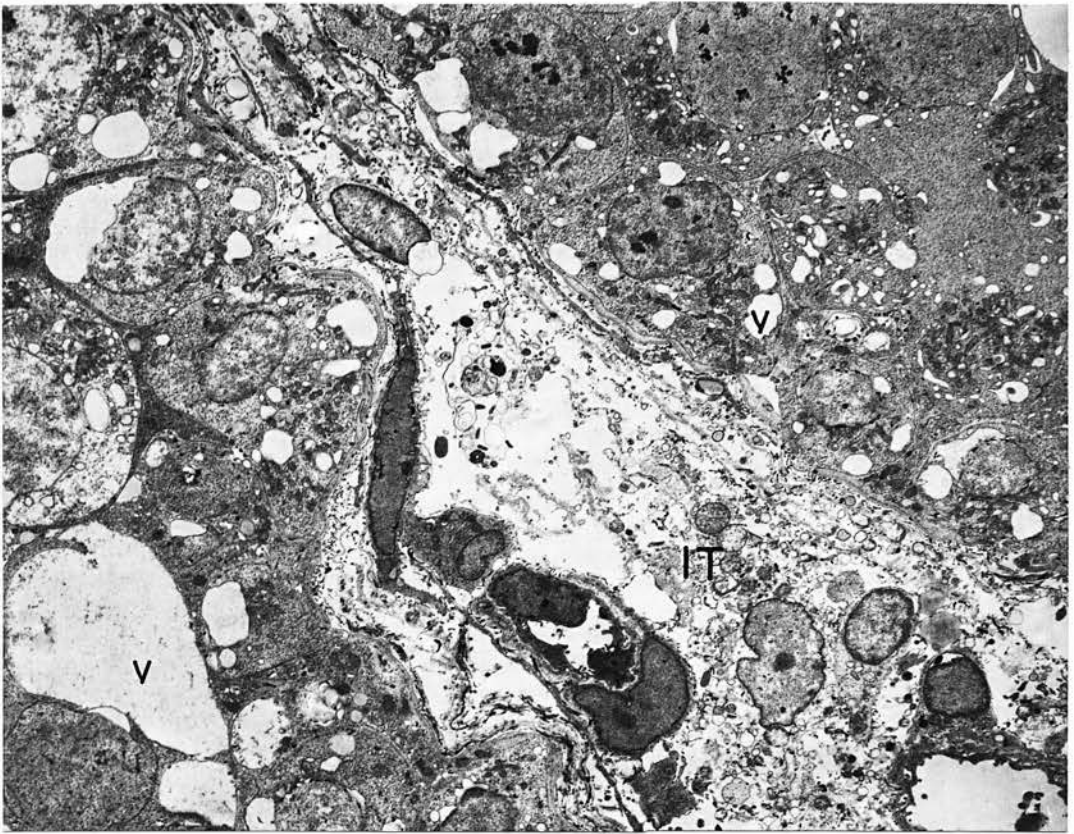


Figure 2.58

3% GTA/0.1M Millonig's  $\text{PO}_4$

21 week old bird. Demonstrates good preservation  
of the seminiferous tubule.

X 7,500

Figure 2.59

4% GTA/0.075M Na cacodylate, 1%  $\text{OsO}_4$ /0.175M Na cacodylate

21 week old bird. Demonstrates good preservation of the  
seminiferous tubule.

X 2,800

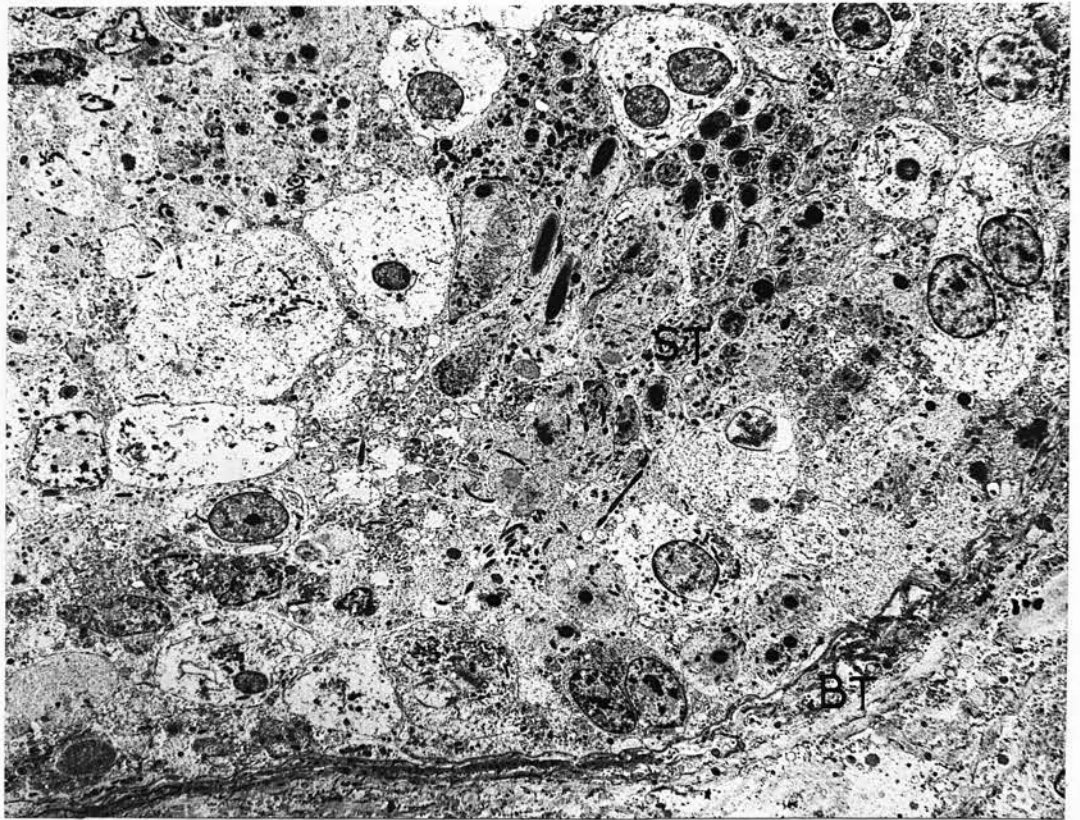
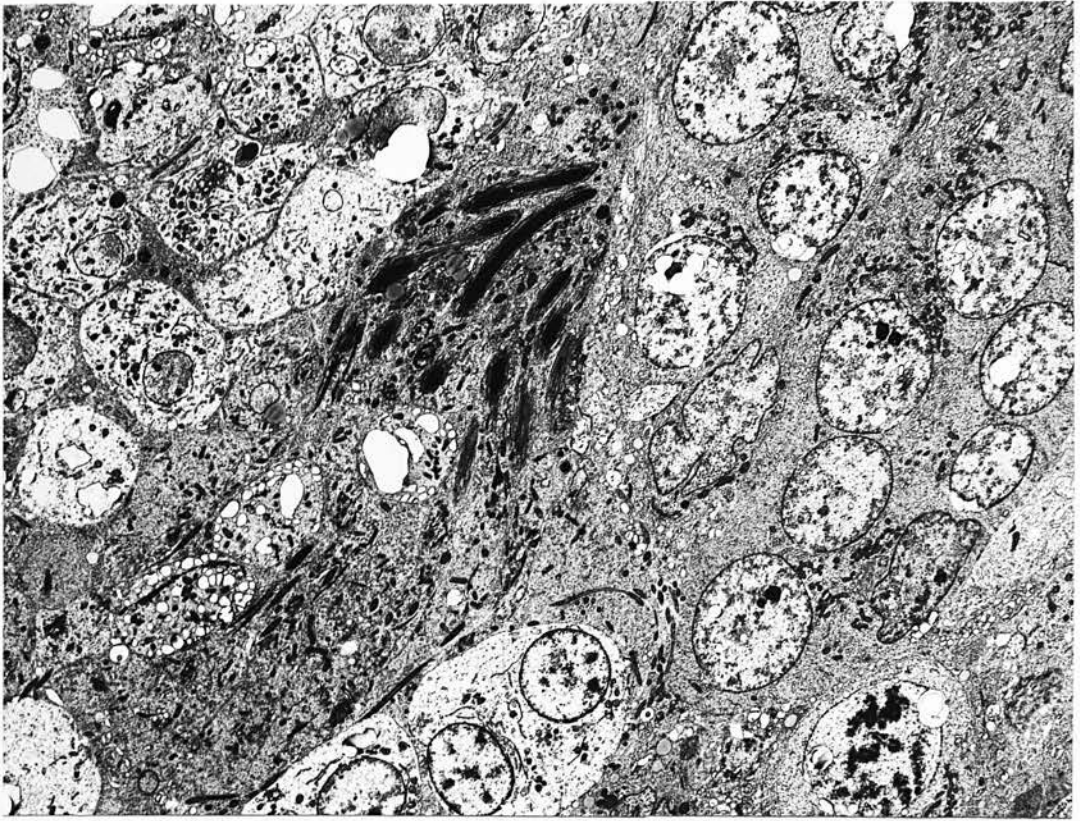


Figure 2.60

4% GTA/0.1M s-collidine

21 week old bird. Demonstrates good preservation of  
intertubular and boundary tissue, and seminiferous tubule.

X 3,800

Figure 2.61

4% GTA/1% pHCHO/0.1M Millonig's  $PO_4$

1 week old bird. Shows a very hypertonic image with  
dark contracted-looking cells for all tissues.

X 3,800

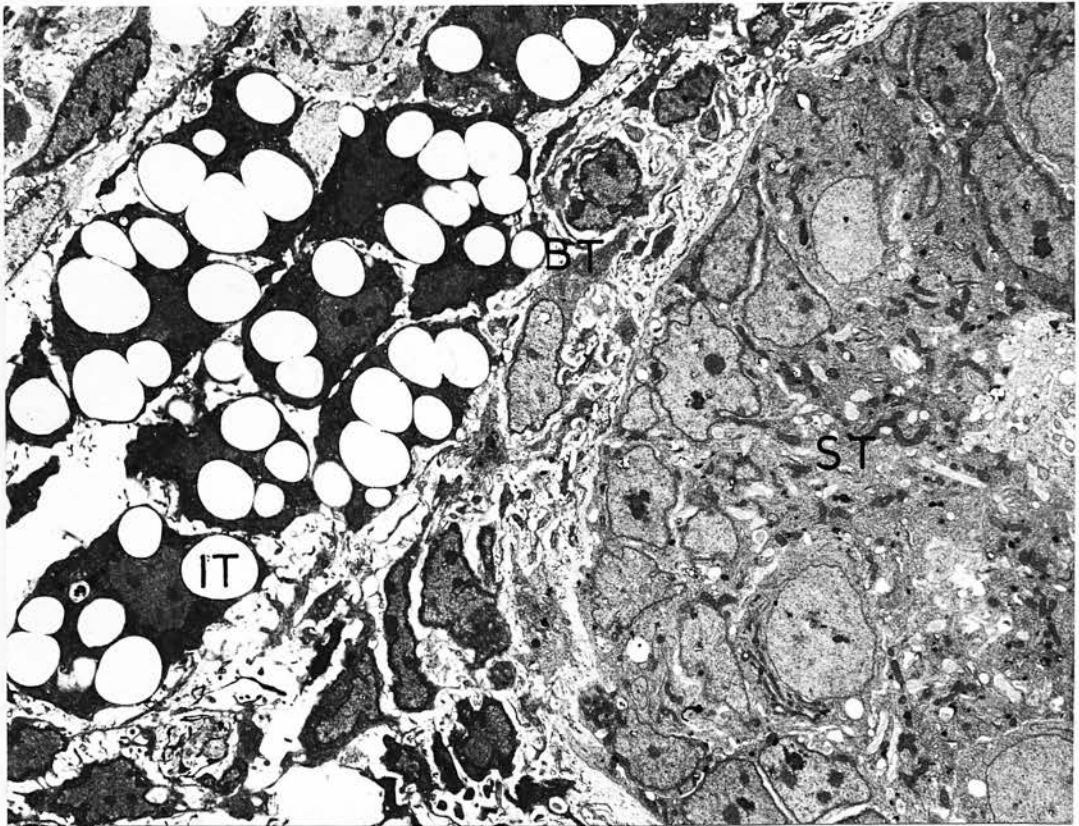
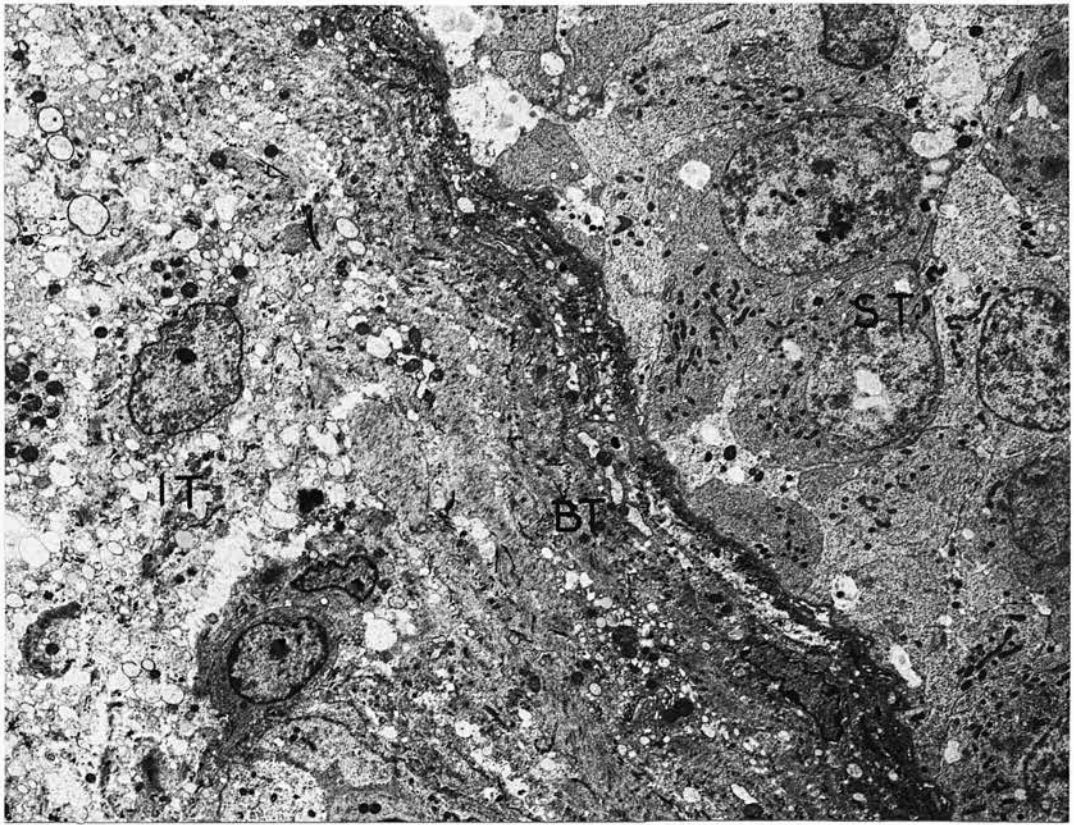




Figure 2.62

5% acrolein/0.1M  $\text{PO}_4$

1 week old bird. Shows a hypotonic image with swollen mitochondria, vesiculated ER and loss of plasma membranes.

X 4,000

Figure 2.63

4% GTA/0.1M s-collidine

1 week old bird. Shows a hypotonic image with vesiculated ER and swollen perinuclear cisternae. Loss of plasma membranes results in a lack of cell integrity.

X 5,100

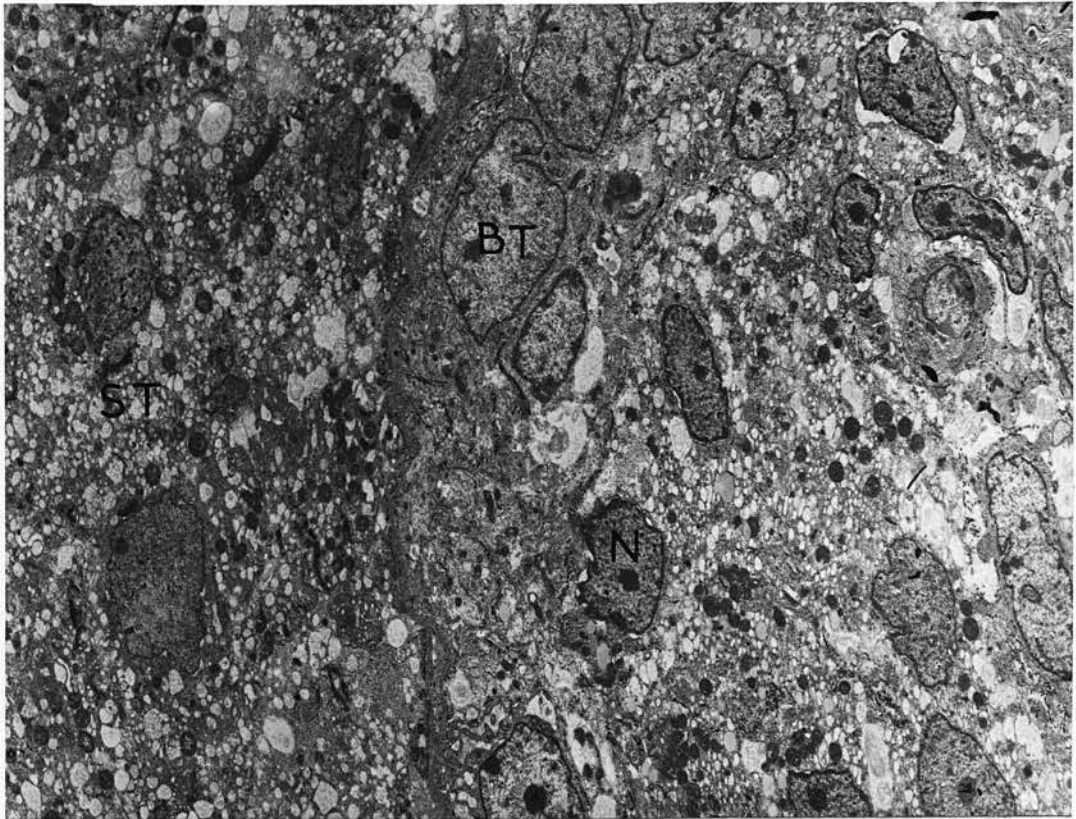
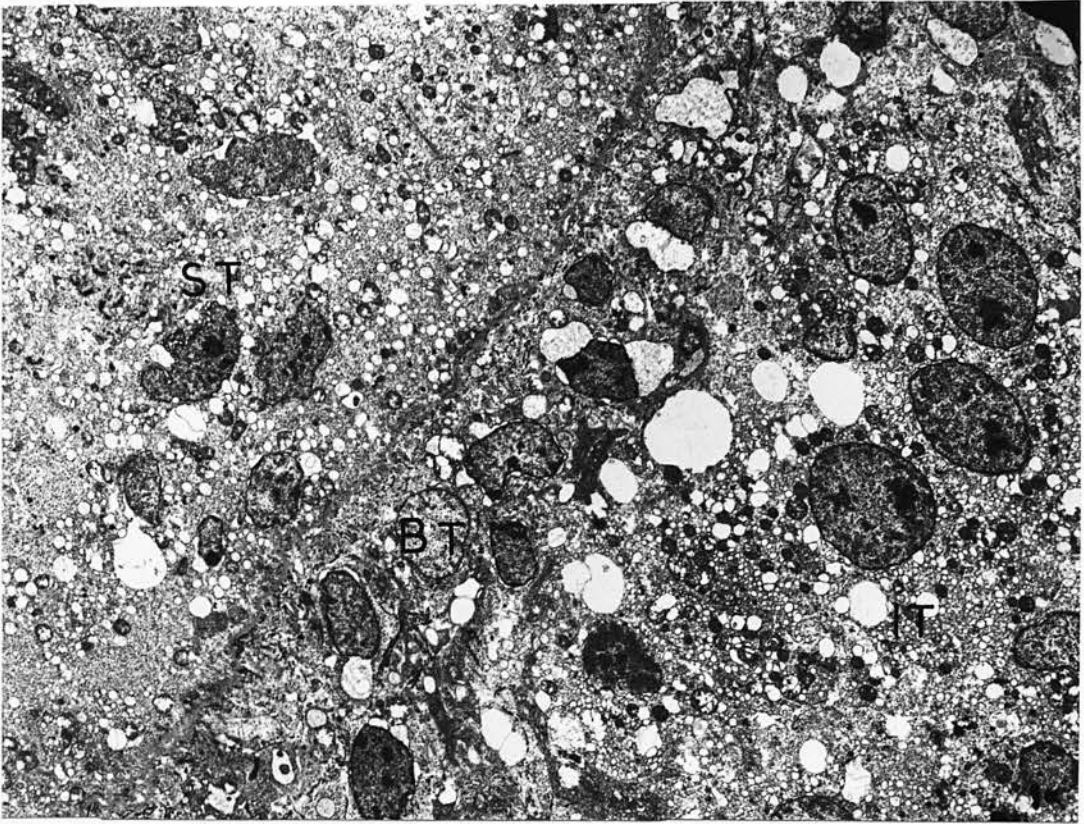


Figure 2.64

3% GTA/0.1M Millonig's  $\text{PO}_4$

1 week old bird. Demonstrates good preservation  
of seminiferous tubule.

X 9,500

Figure 2.65

3% GTA/0.1M Millonig's  $\text{PO}_4$

1 week old bird. Demonstrates Leydig cell  
separation but good preservation of boundary tissue  
and tubule.

X 3,400

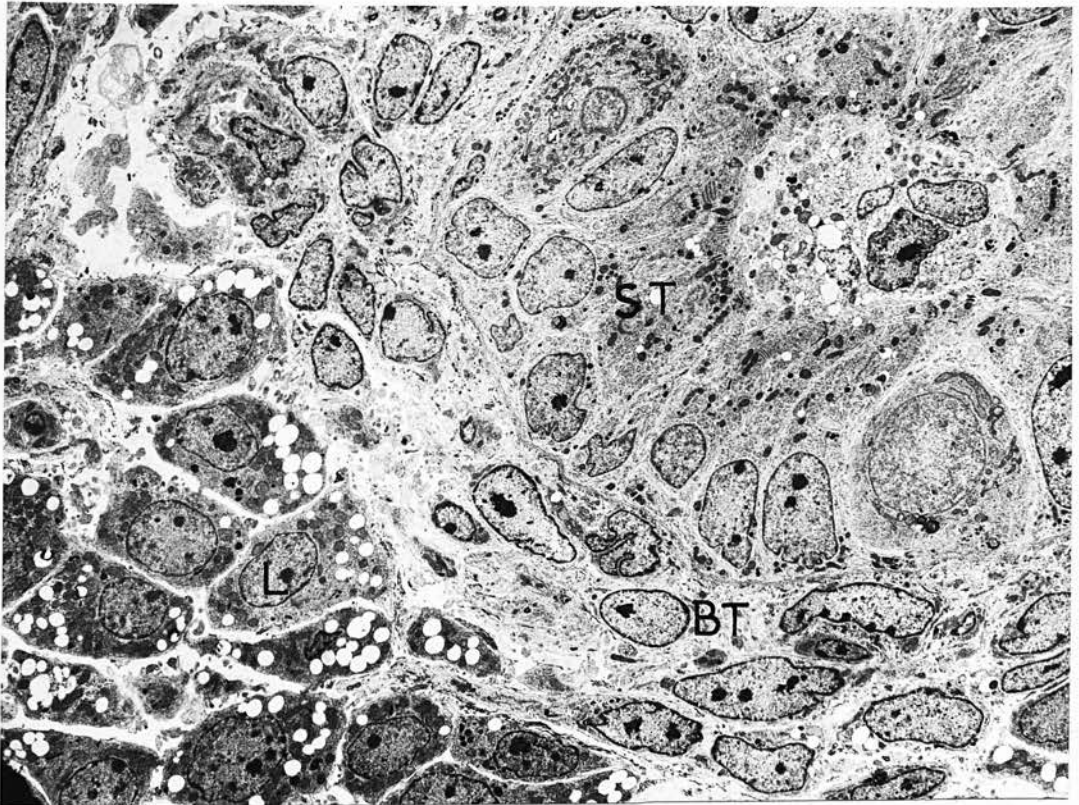
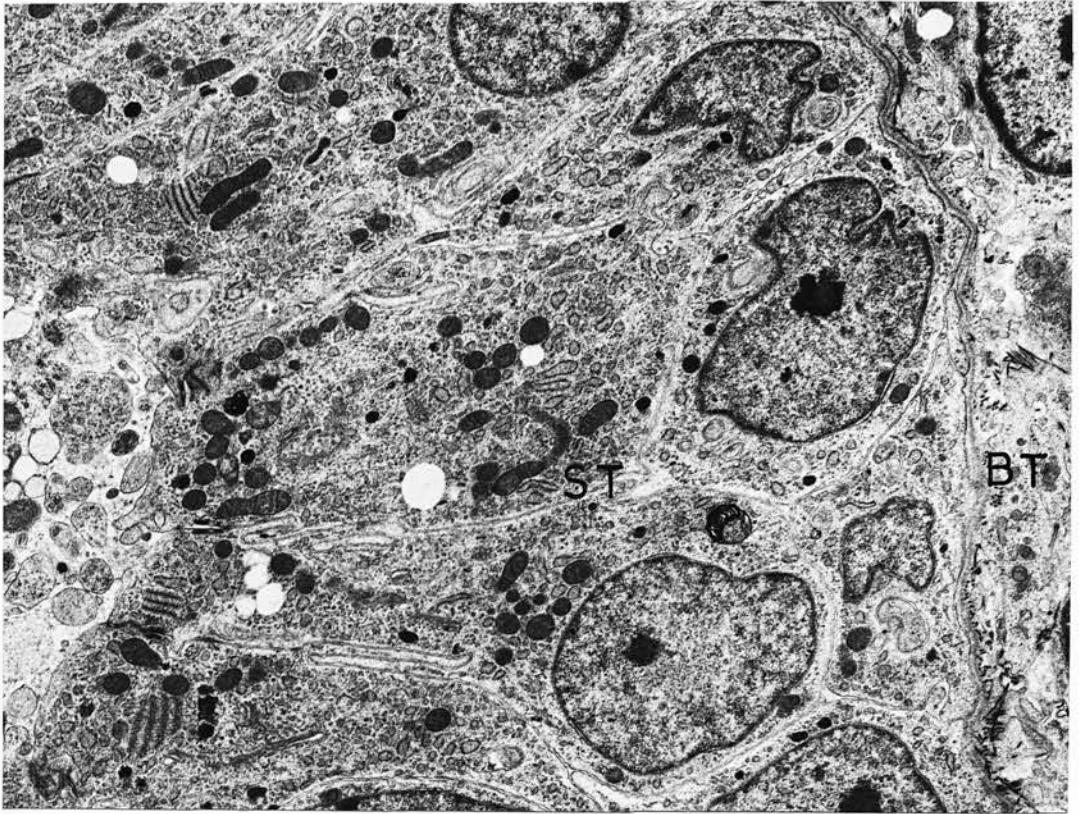


Figure 2.66

1% OsO<sub>4</sub>/modified Dalton's buffer

1 week old bird. Demonstrates preservation of the relationship of the intertubular tissue, boundary tissue and seminiferous tubule.

X 3,500

Figure 2.67

1% OsO<sub>4</sub>/modified Dalton's buffer

1 week old bird. Sertoli cell exhibits vesiculated ER and swollen mitochondria

X 15,000

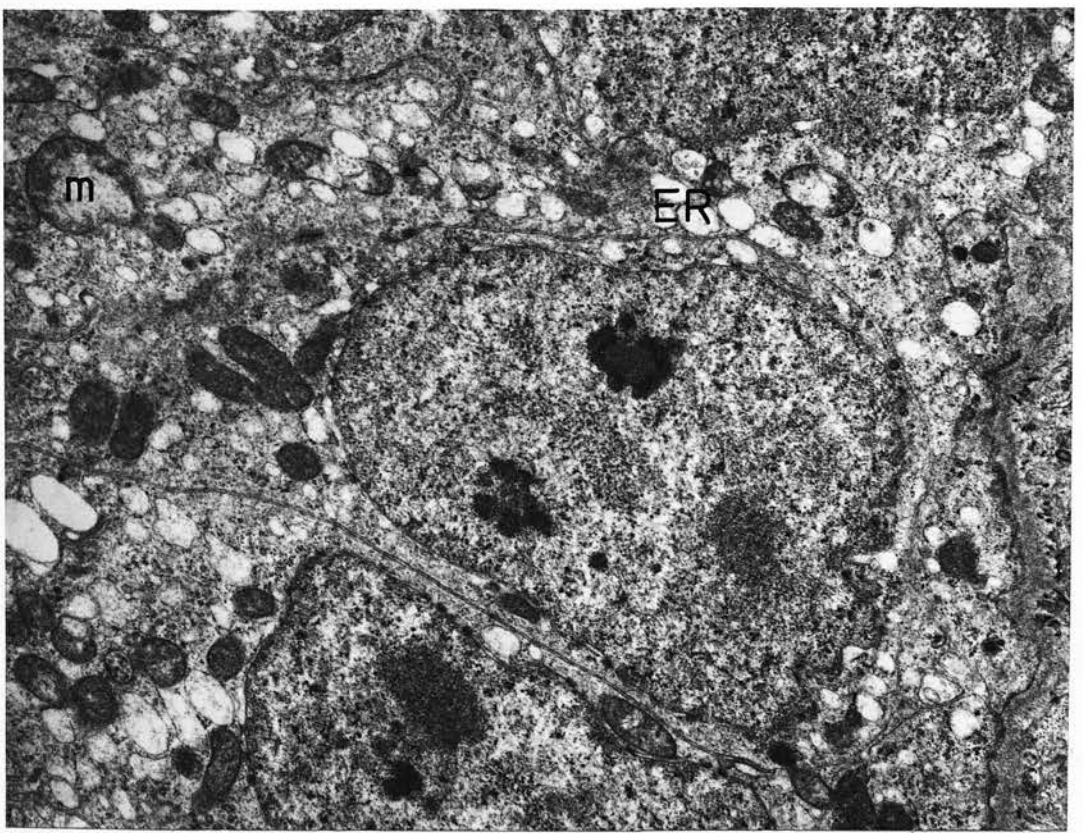


Figure 2.68

6% GTA/2% acrolein/0.1M  $\text{PO}_4$

1 week old bird. Demonstrates good preservation of intertubular tissue, boundary tissue and seminiferous tubule. Note the well preserved cytoplasm of the Leydig cells.

X 3,500

Figure 2.69

4% GTA/0.075M Na cacodylate, 1%  $\text{OsO}_4$ /0.175M Na cacodylate

1 week old bird. Demonstrates good preservation of Leydig cells. Note the mitochondria with tubular cristae, smooth ER and preservation of lipid droplets.

X 12,000

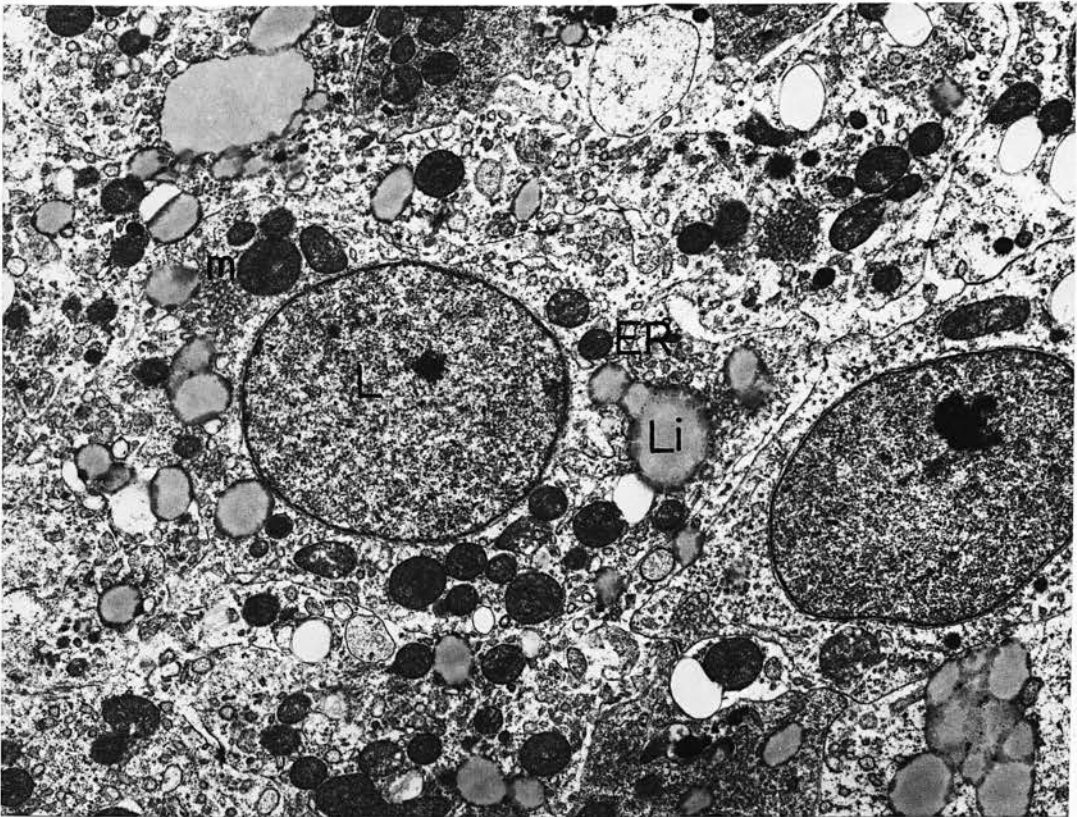
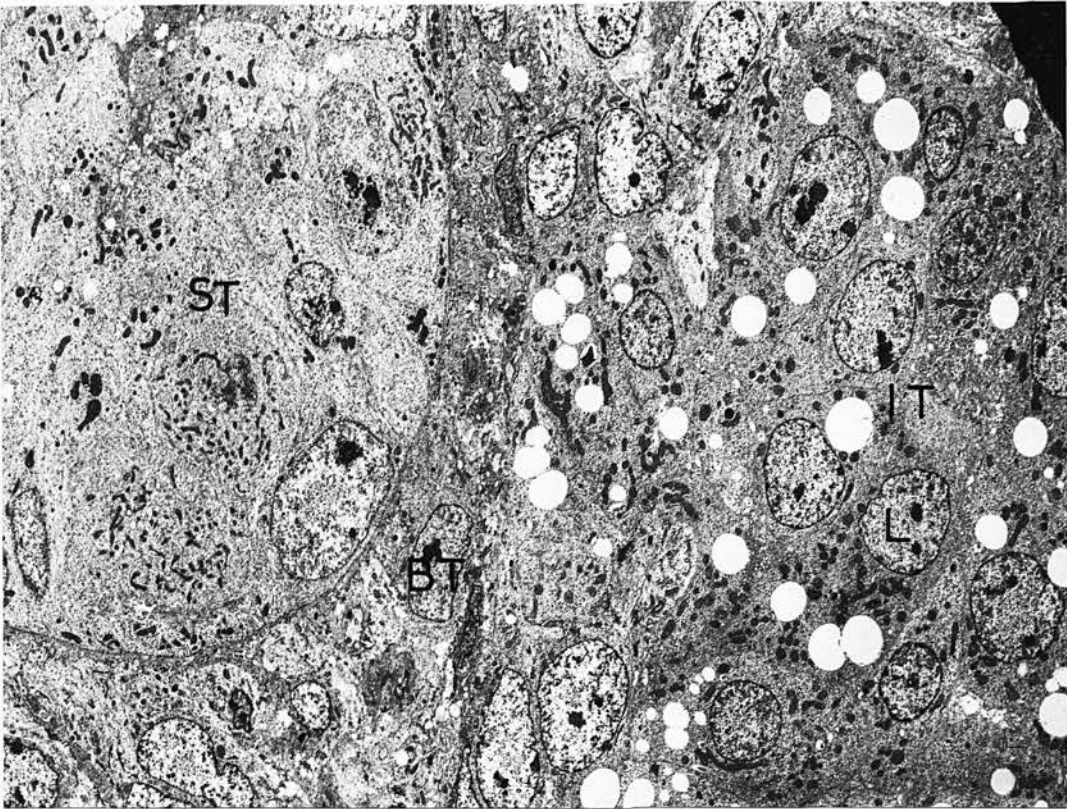




Figure 2.70

6% GTA/2% acrolein/0.1M  $PO_4$

1 week old bird. Demonstrates very good preservation of Leydig cells and mitochondria with tubular cristae. The lipid is not retained. Note prominent Golgi complex.  
X 7,500

Figure 2.71

2.5% GTA/0.1M Millonig's  $PO_4$  venous perfusion.

21 week old bird. Blood capillaries are observed filled with red blood cells. The cells have a dark contracted appearance with perinuclear blistering.  
X 2,000

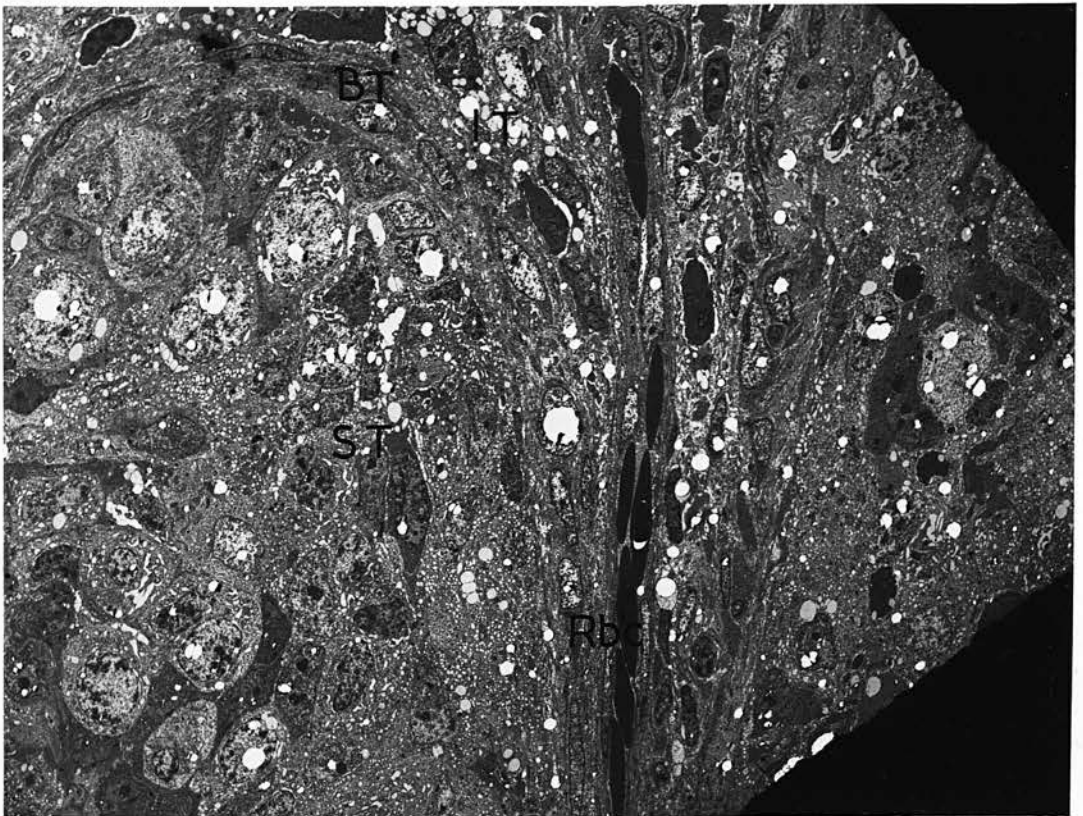
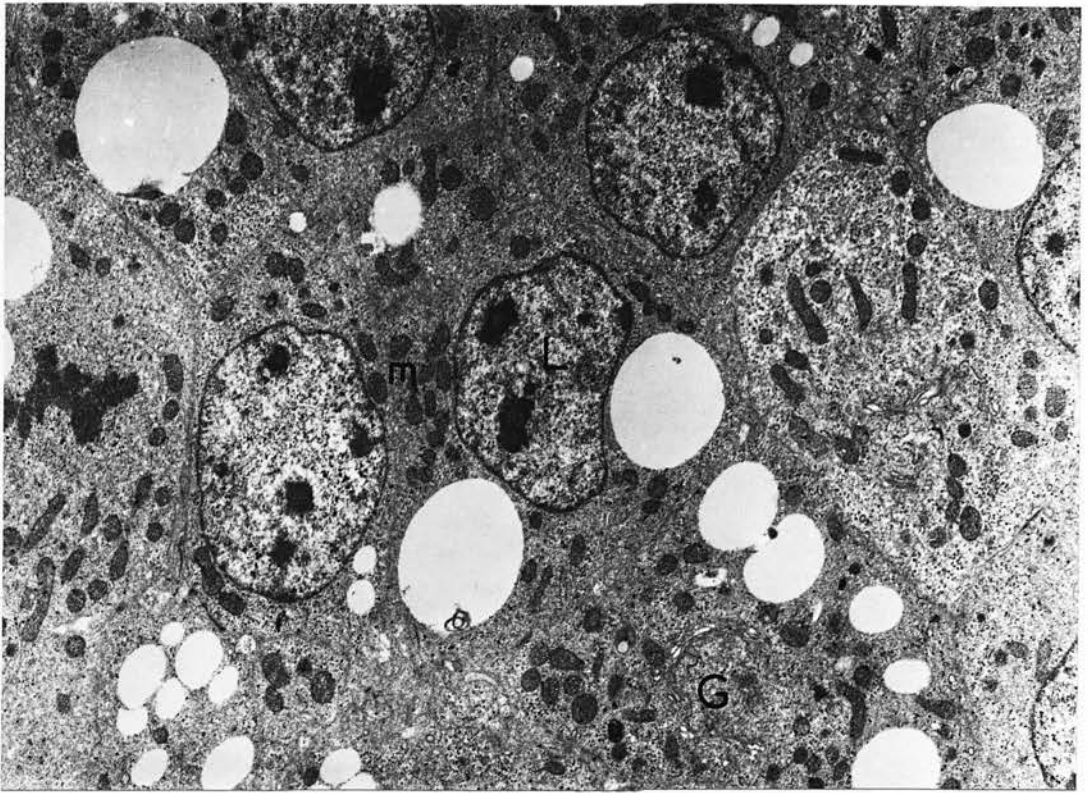


Figure 2.72

1% saline prewash, 4% GTA/0.075M Na cacodylate perfusion.

Collagen fibres are lost from the boundary tissue inter-cellular spaces. The basal lamina has a fuzzy appearance.

X 12,000

Figure 2.73

1.4% saline prewash, 4% GTA/0.075M Na cacodylate perfusion.

Leaching of the ground substance results in an even density of cytoplasm (arrow).

X 6,800

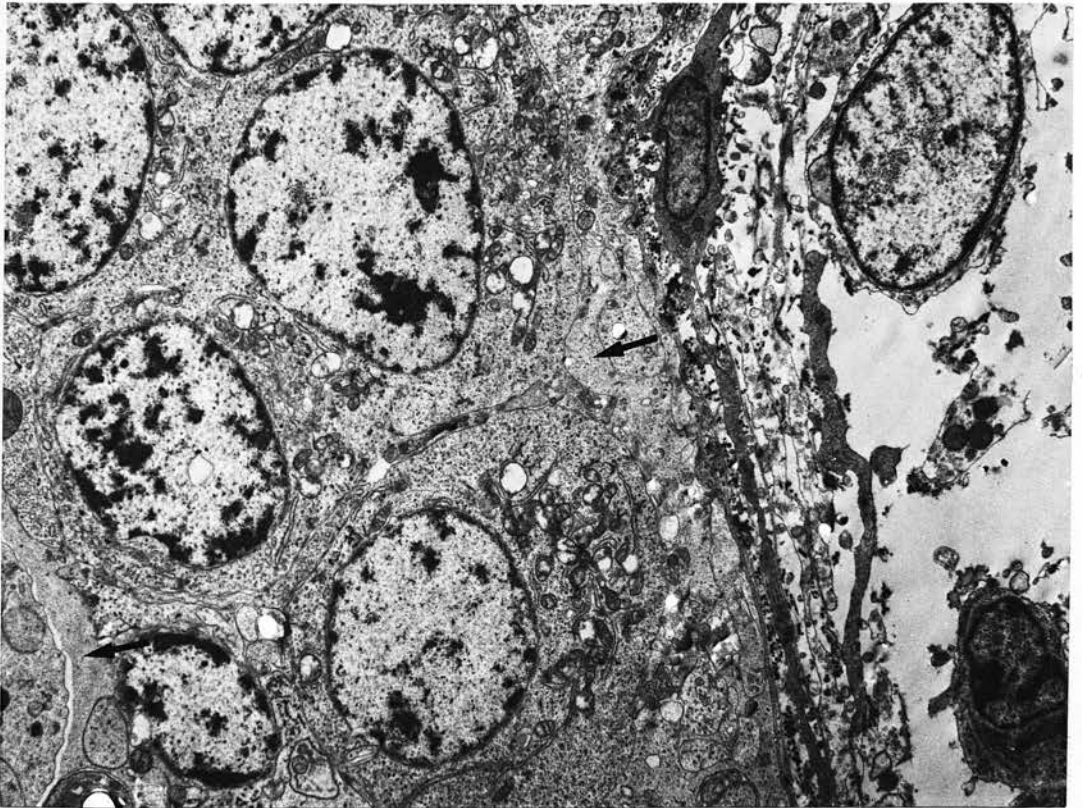
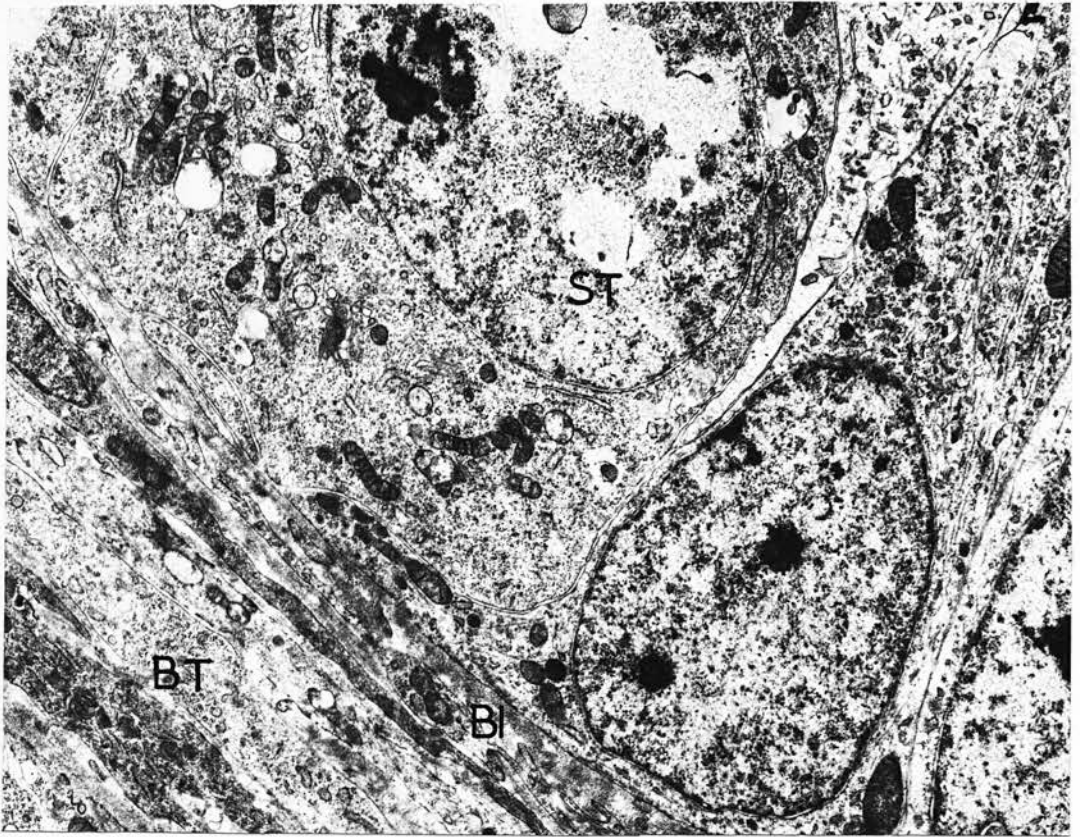


Figure 2.74

Avian Ringer prewash, 4% GTA/0.075M Na cacodylate  
perfusion.

Patches of cytoplasm are lost from the tubule cells.

X 5,800

Figure 2.75

0.075M Na cacodylate prewash, 4% GTA/0.075M Na  
cacodylate perfusion

Leaching of the nucleoplasm is observed with  
clumping of the chromatin.

X 7,500

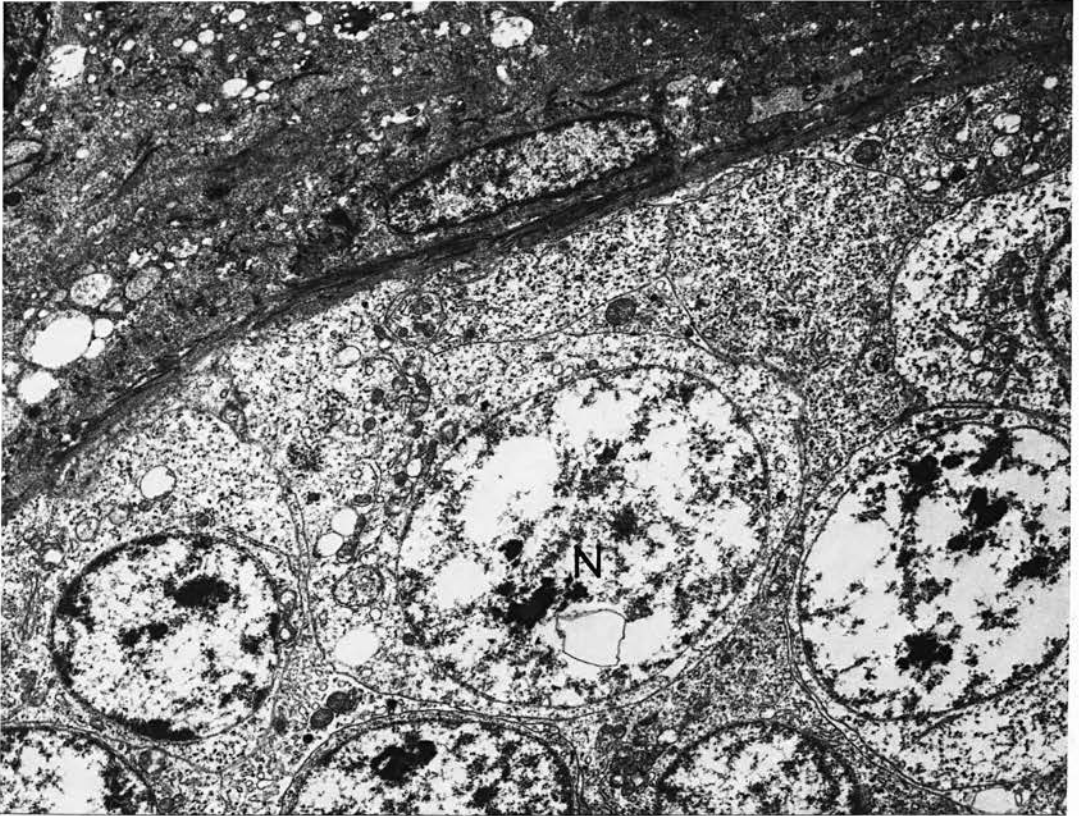
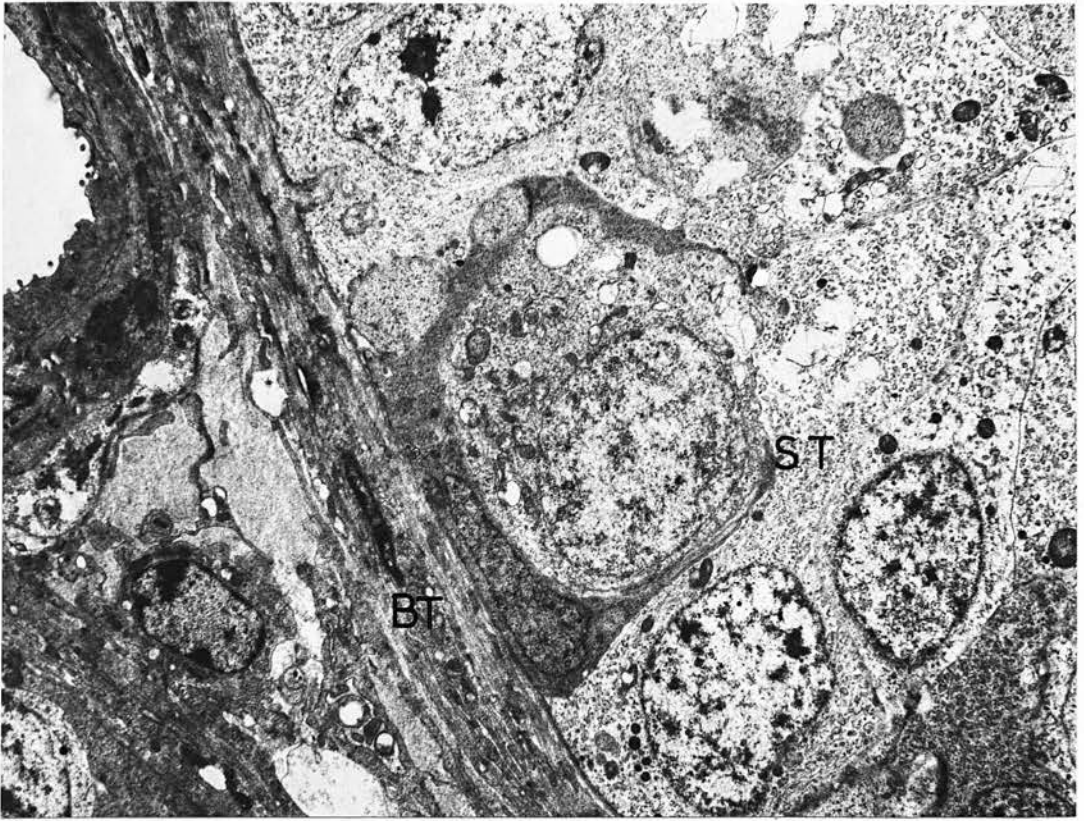


Figure 2.76

4% GTA/0.075M Na cacodylate perfusion.

21 week old bird. Demonstrates a good perfusion fixation image with open lumen blood capillaries and lymph vessels filled with a dense material.

X 900





Figure 2.77

4% GTA/0.075M Na cacodylate perfusion

Demonstrates the destruction of the seminiferous  
epithelium with ~~too~~ high a flow pressure. Only  
the spermatogonial cell layer remains.

X 3,500

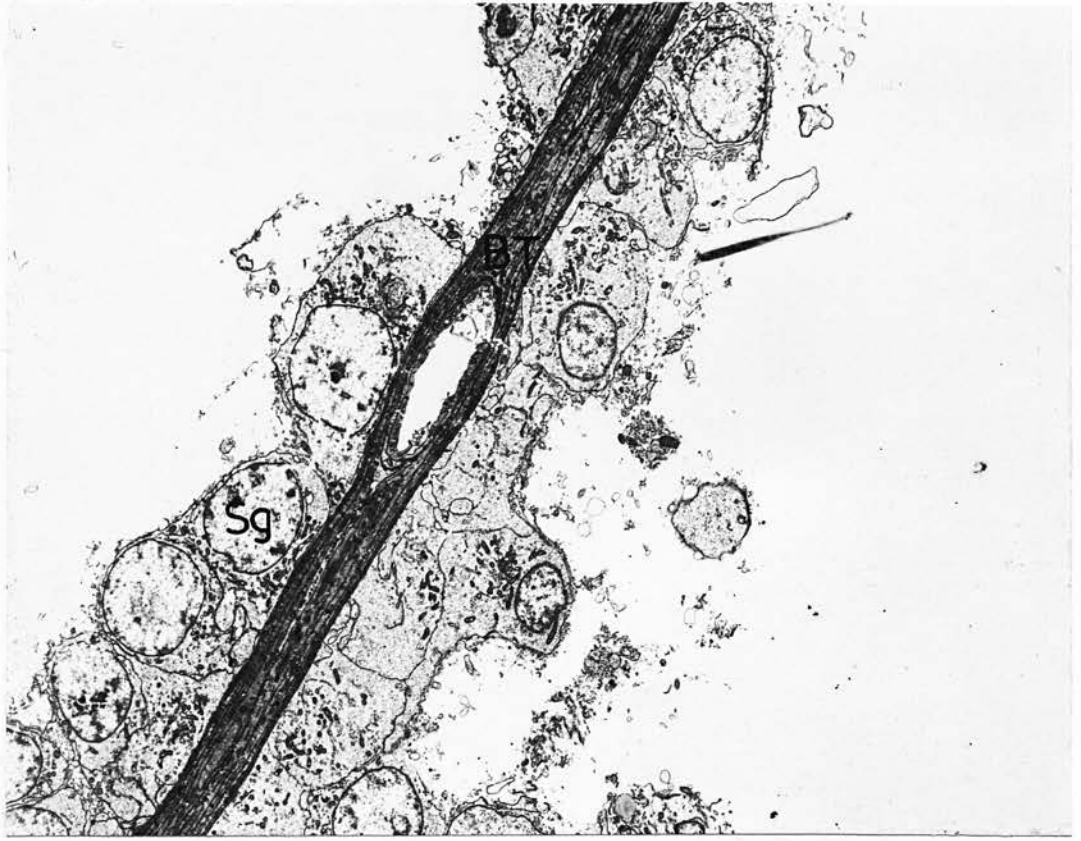


Figure 2.78

2.5% GTA/0.1M Millonig's PO<sub>4</sub> perfusion.

Demonstrates good perfusion fixation preservation  
of intertubular region with a 12 week old bird.

X 1,000



Figure 2.79

4% GTA/0.1M s-collidine perfusion.

15 week old bird. Demonstrated well preserved  
intertubular and boundary tissue, and seminiferous  
tubule. Note prominent appearance of collagen  
fibres in basal lamina (arrow) with s-collidine buffer.

X 3,500



Figure 2.80

6% GTA/2% acrolein/0.1M PO<sub>4</sub> hand perfusion.

1 week old bird.

The intertubular tissue shows widened intercellular spaces and loss of interstitial material (arrow).

X 4,000

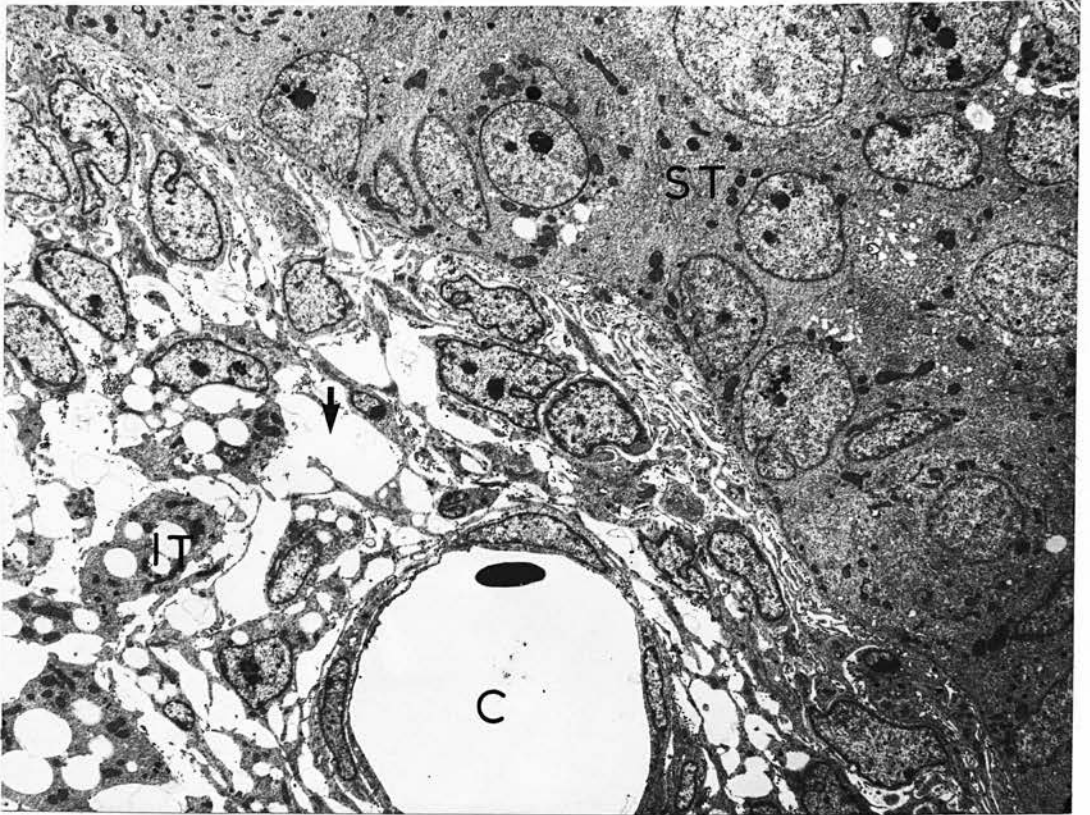




Figure 2.81

2.5% GTA/0.075M Na cacodylate perfusion.

3 week old bird.

Demonstrated <sup>S</sup>good preservation of the intercellular  
tissue with open lumen blood capillaries.

X 2,800

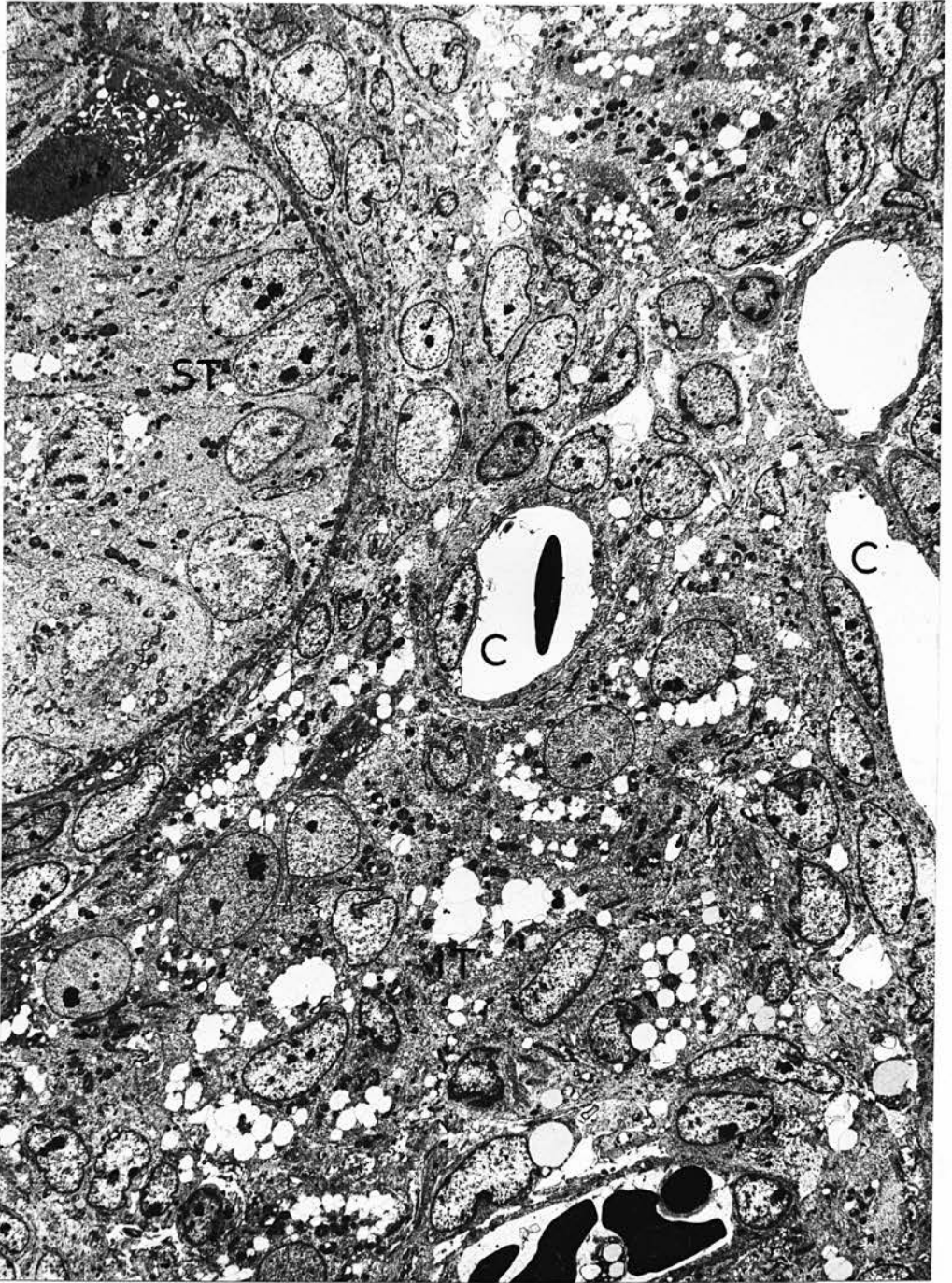


Figure 2.82

1% OsO<sub>4</sub>/modified Dalton's buffer perfusion.  
3 week old bird. Demonstrates poor preservation  
of the tissue as evinced by vesiculated cytoplasm.  
Note red blood cells blocking the capillaries.  
X 3,500

Figure 2.83

1% OsO<sub>4</sub>/modified Dalton's buffer perfusion  
1 week old bird. Demonstrates good preservation of  
the cells but separation of the intertubular tissue  
from the boundary tissue.  
X 6,048

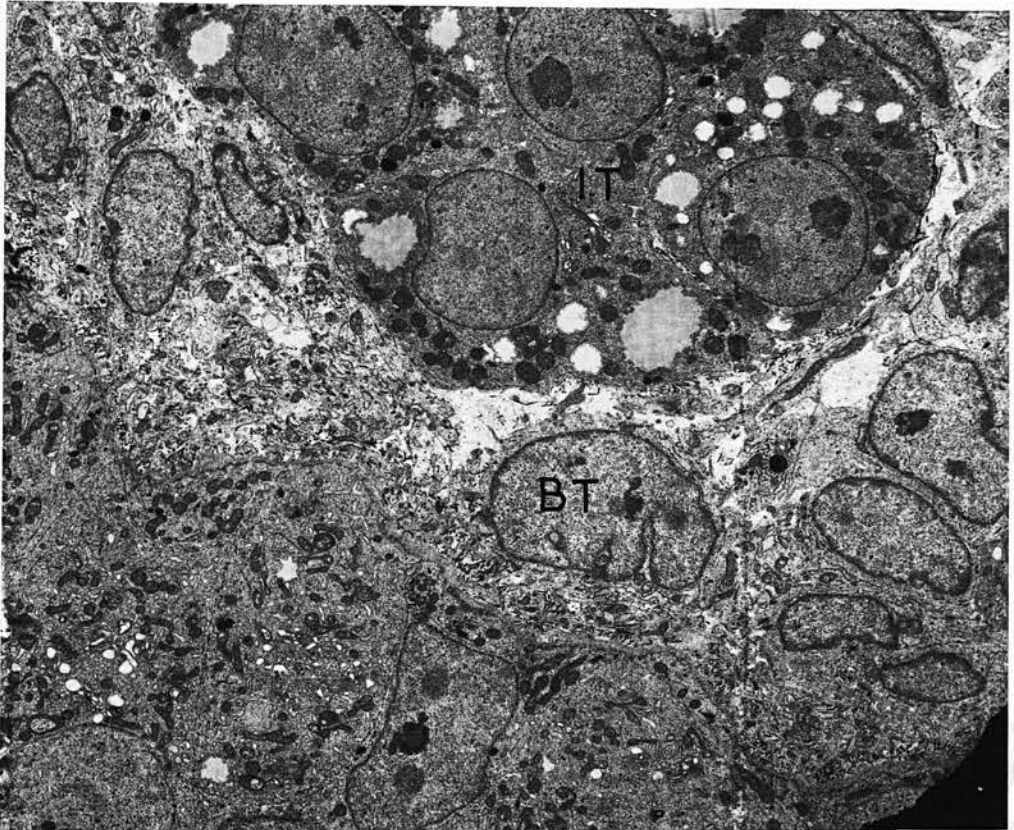
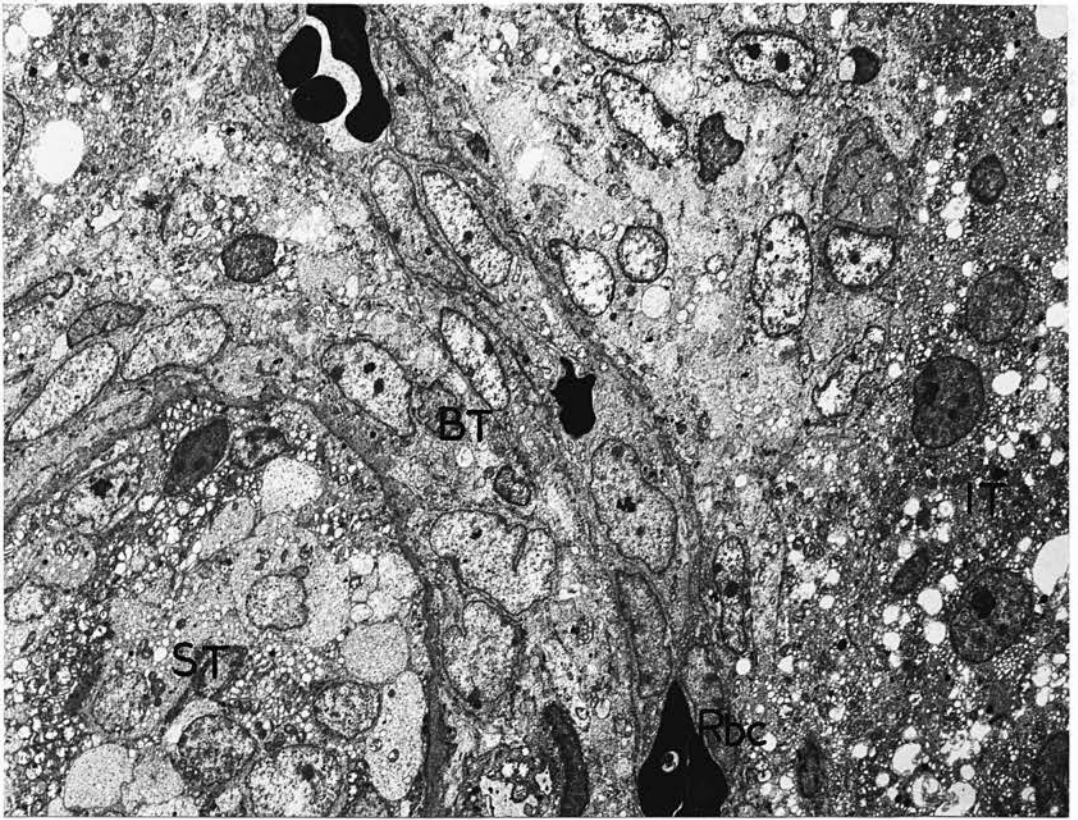


Figure 2.84

2.5% GTA/0.075M Na cacodylate perfusion.

3 week old bird. Demonstrates good overall  
preservation of intertubular and boundary tissue  
and seminiferous tubules.

X 3,500

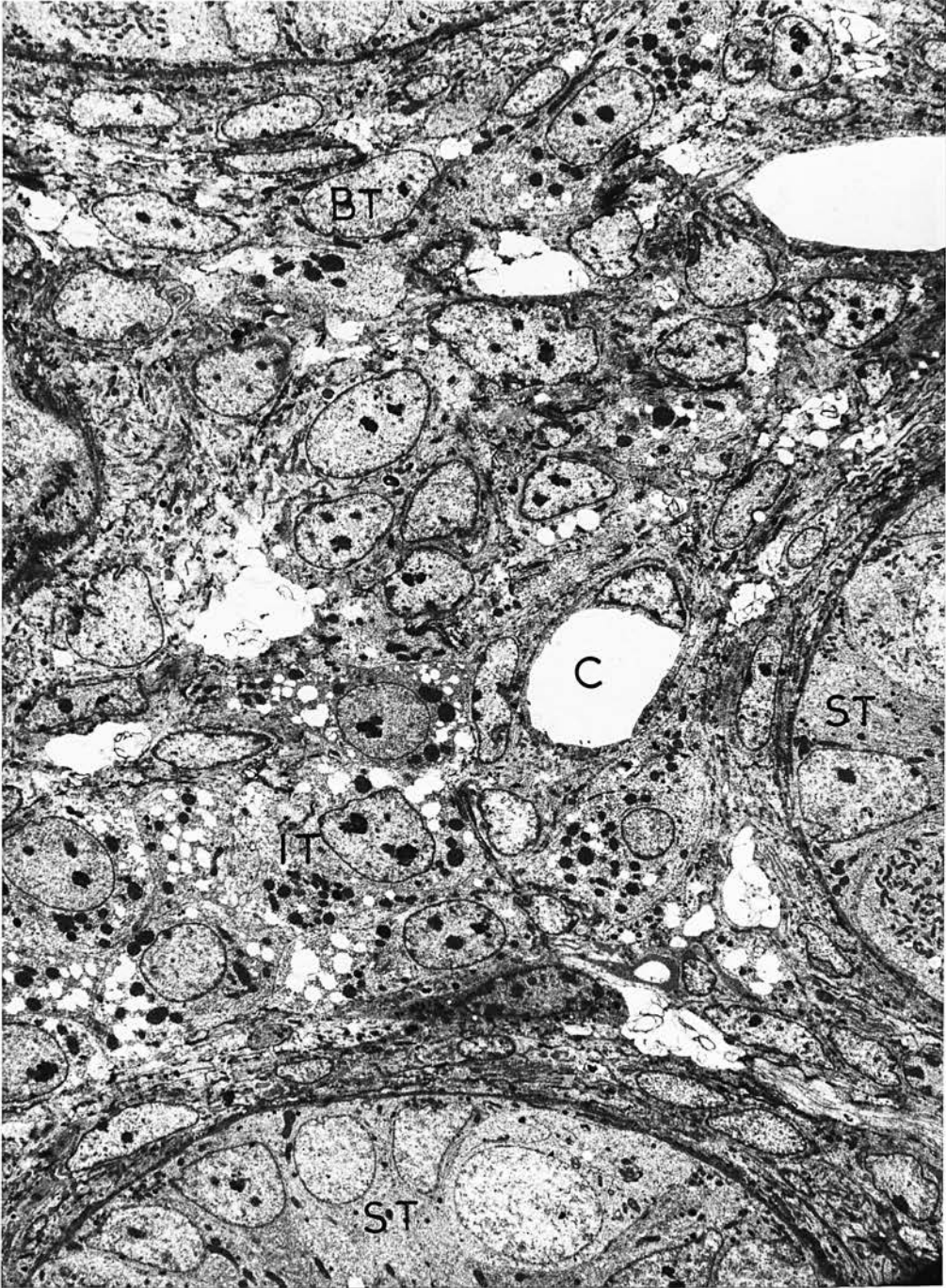


Figure 3.1

6 week old bird. Cross section through a seminiferous tubule showing spermatogonia and Sertoli cells around the basal lamina and Sertoli cell cytoplasm filling the centre of the tubule.  
x 2,800.

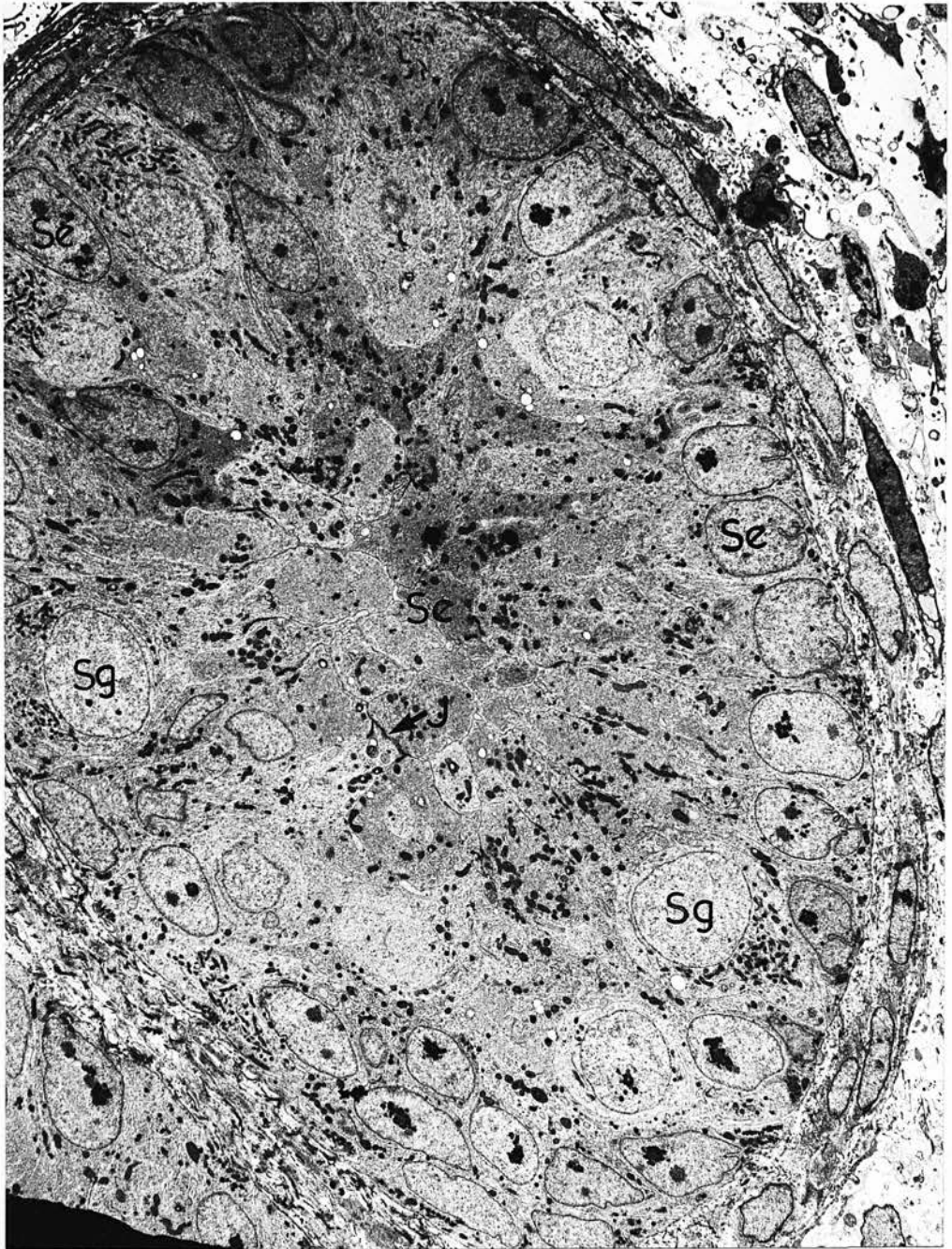




Figure 3.2

3 week old bird. Cross section through a seminiferous tubule.  
x 2,800.

Figure 3.3

Basal portion of Sertoli cell cytoplasm showing the indented nucleus with prominent nucleoli and lysosomes lying in the cytoplasm subadjacent to the basal lamina.  
x 16,000.

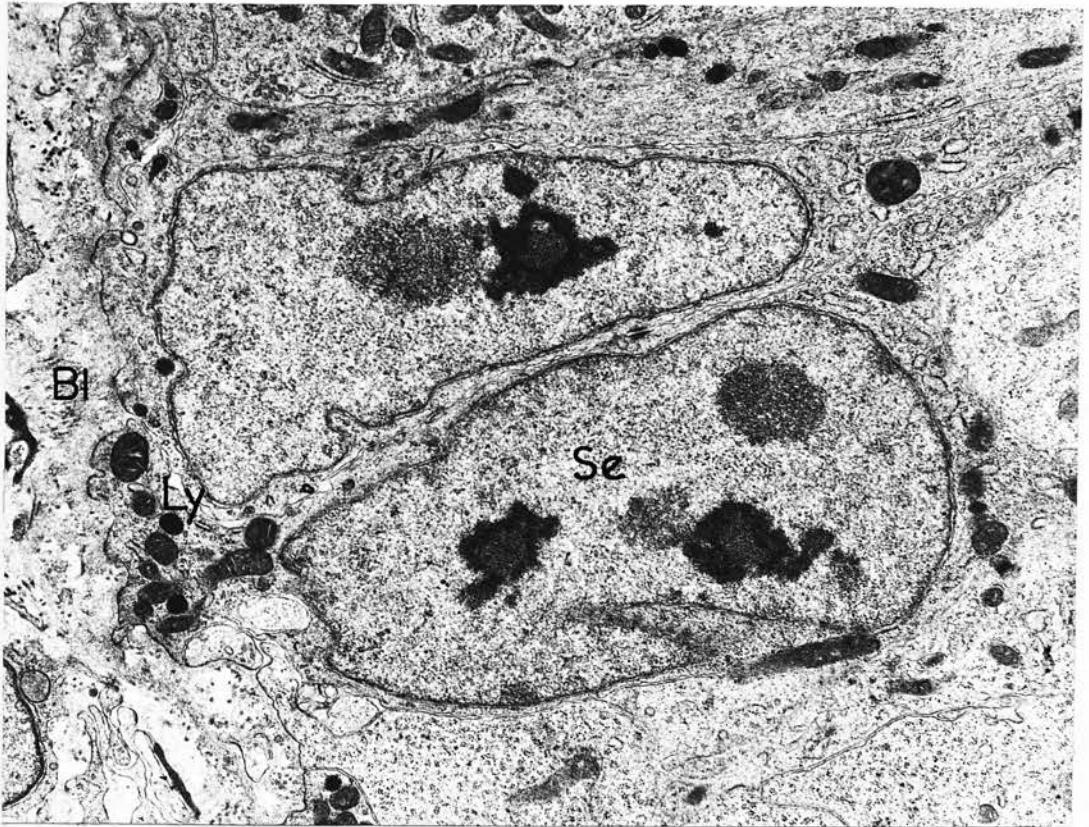
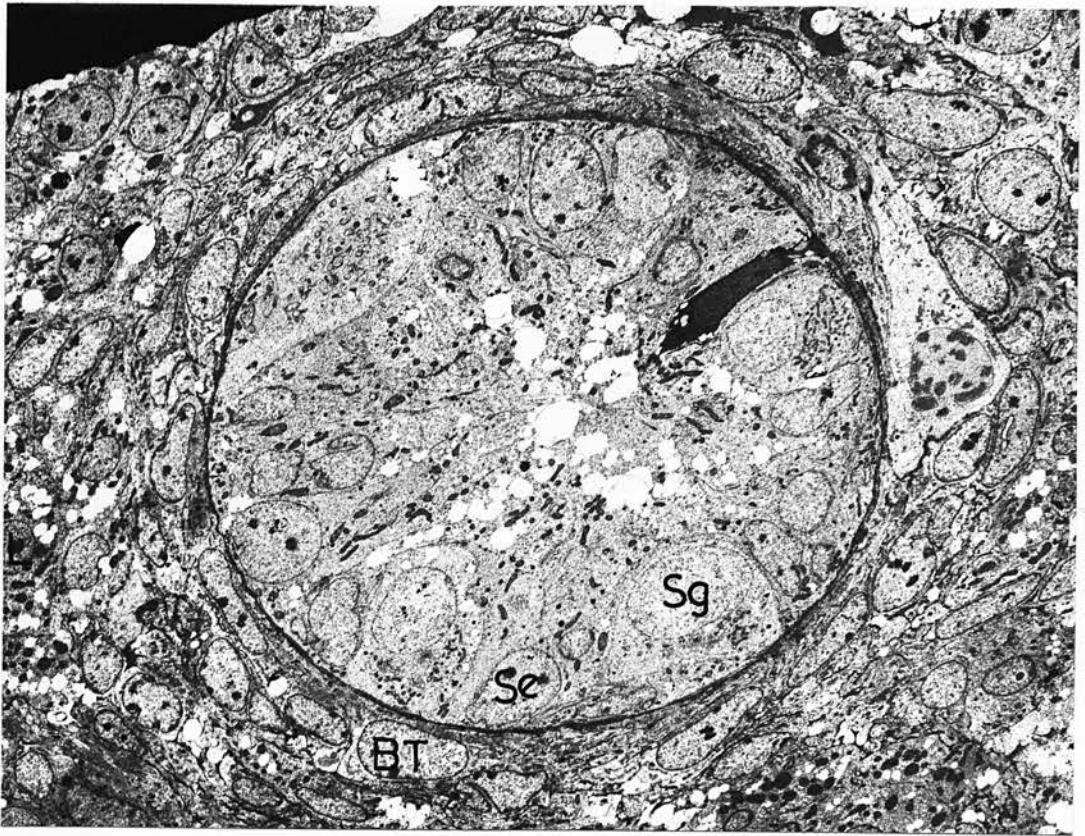


Figure 3.4

Apical portion of Sertoli cell cytoplasm demonstrating the junctional complexes between Sertoli cells in the centre of the tubule.

x 54,000.

Figure 3.5

A prespermatogonium is seen lying in contact with the basal lamina. The nucleoplasm is pale with a disperse distribution of chromatin. Circular, rod, dumbbell and cup-shaped mitochondria are observed in the basal cytoplasm. A few long cisternae of rough ER are observed. Note the micropinocytotic vesicle formation.

x 105,000.

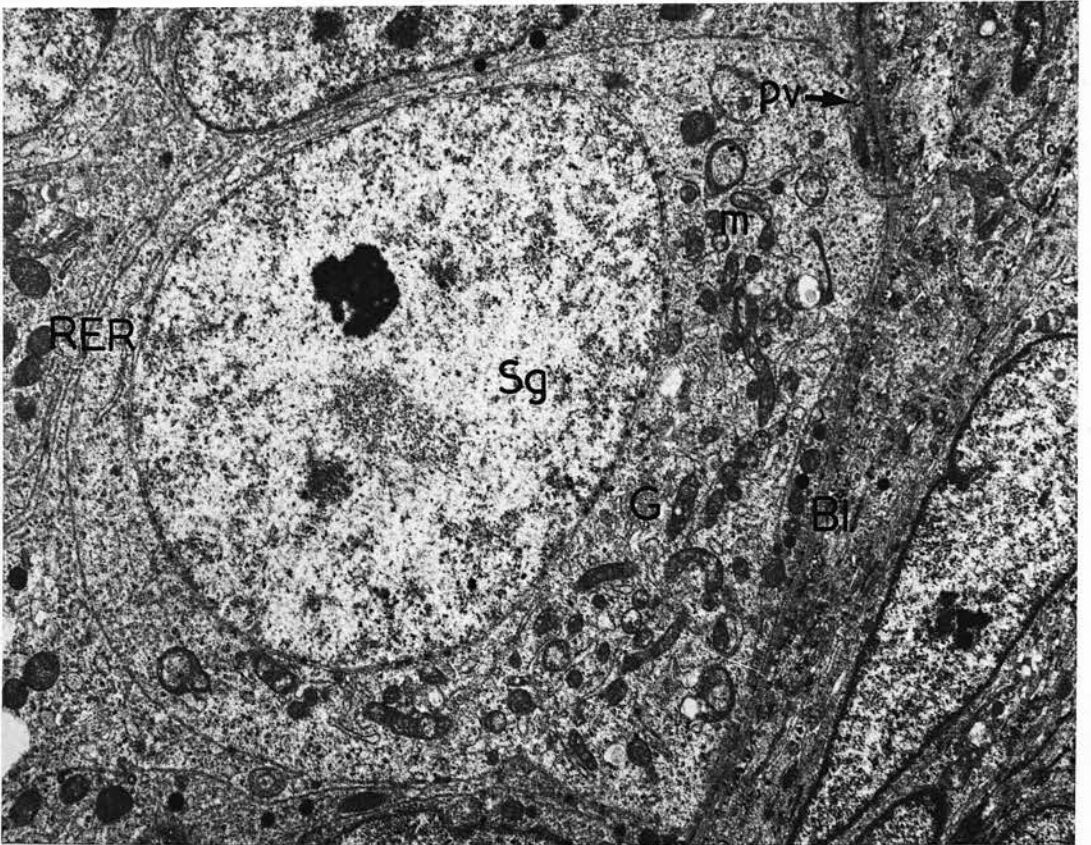
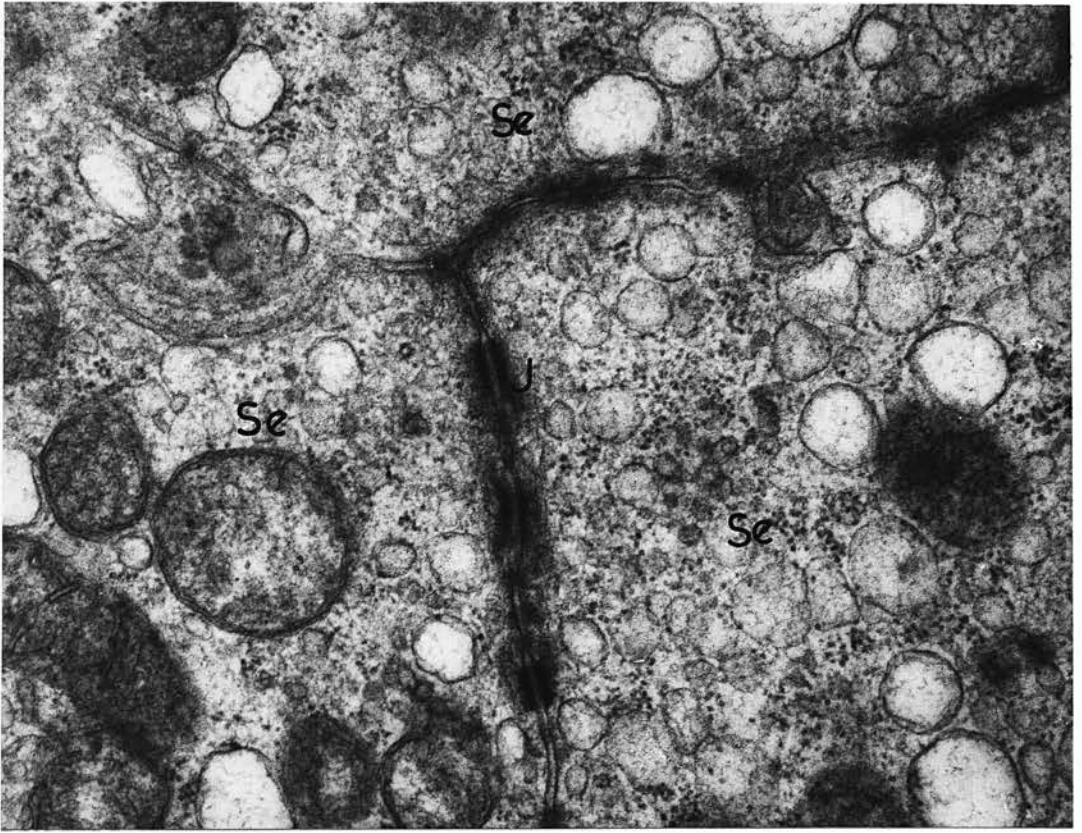


Figure 3.6

A prespermatogonium is seen with the cell organelles grouped in the basal cytoplasm. Note the Golgi complex and pinocytotic vesicle formation.

x 10,000.

Figure 3.7

A prespermatogonium with mitochondria showing round, dumbbell, rod and cup-shapes and a centriole located in the basal cytoplasm. Note the lipid bodies in the adjacent Sertoli cell cytoplasm.

x 11,000.

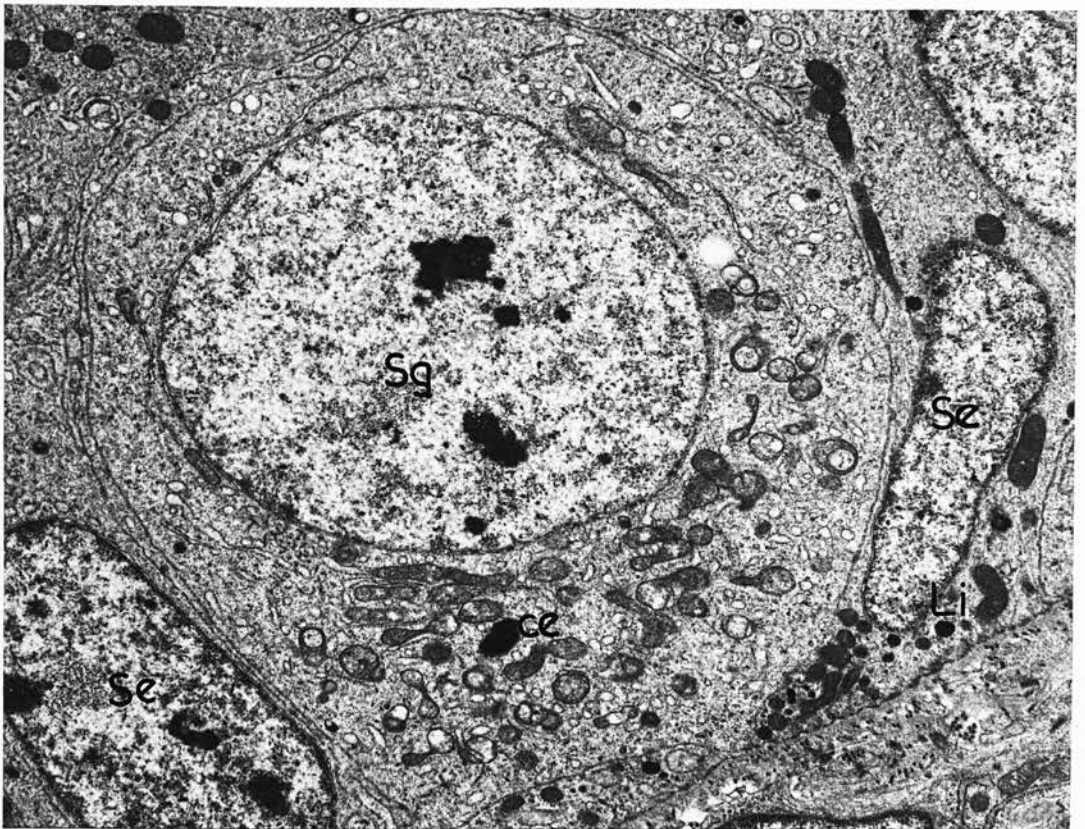
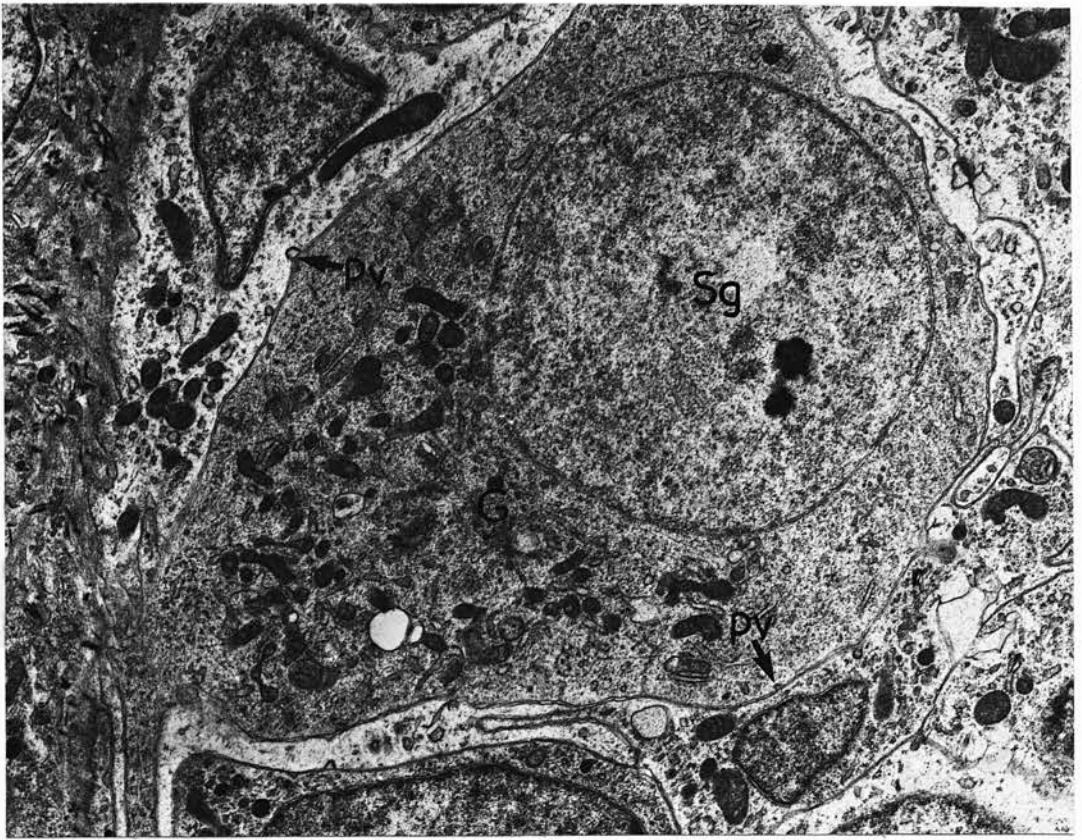


Figure 3.8

A Sertoli cell is seen undergoing mitosis. Note the Sertoli-Sertoli junction of the apical lateral plasma membrane.  
x 6,000.

Figure 3.9

Spermatogonia are seen undergoing mitosis. Note the perpendicular axis of the metaphase plate of the spermatogonium that is in contact with the basal lamina.  
x 6,000.

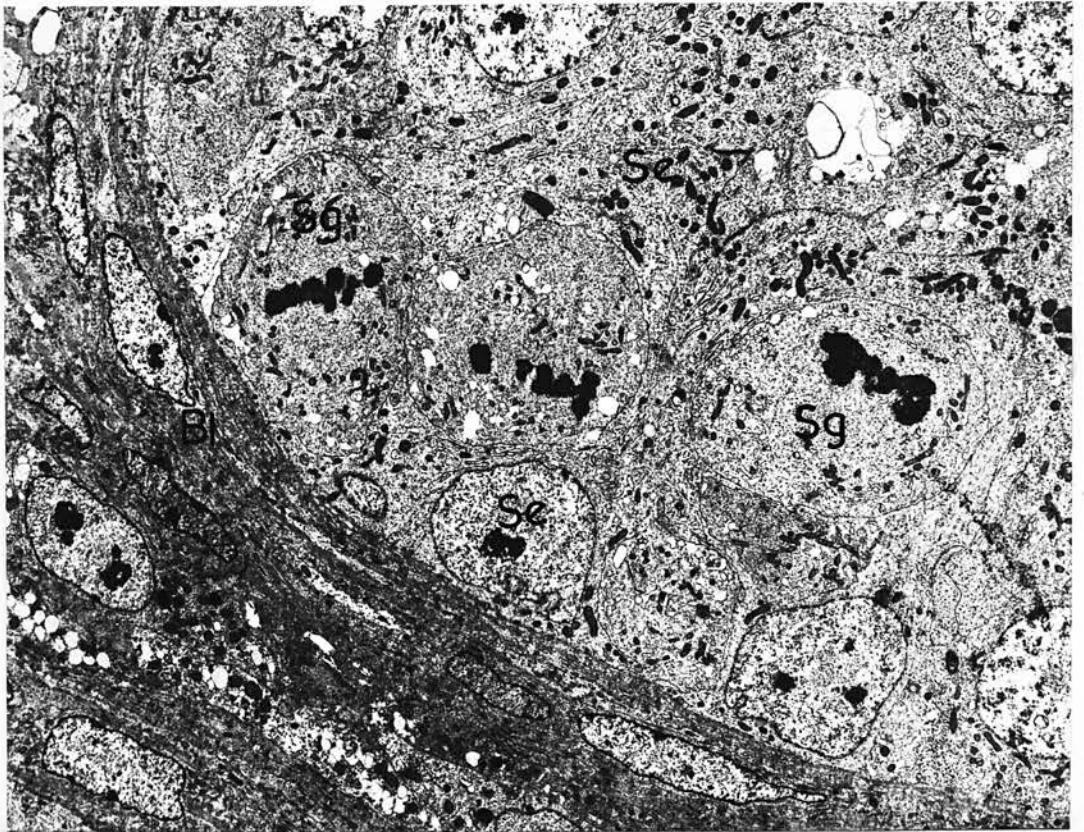
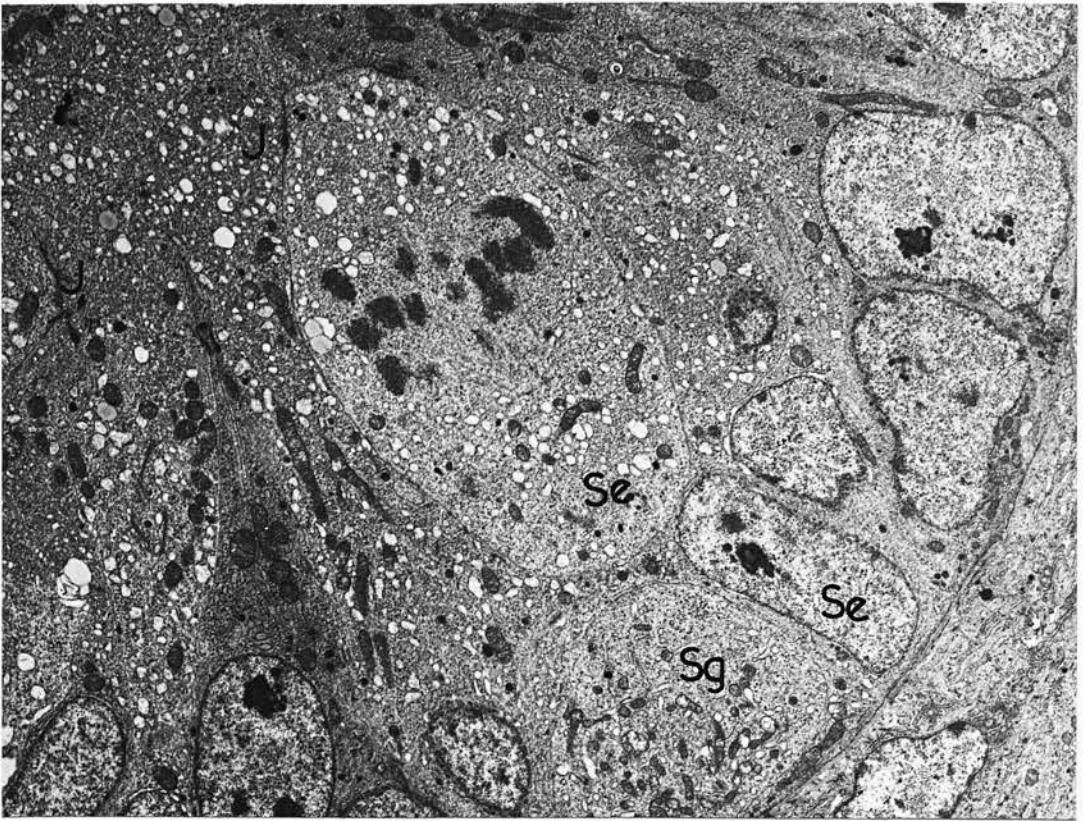




Figure 3.10

A cytoplasmic bridge between two A-type spermatogonia is observed with remains of the spindle fibres within the bridge. Note the electron dense material along the inner surface of the plasma membrane indentations.

x 10,000.

Figure 3.11

An A-type spermatogonium and two B-type spermatogonium are observed lying in contact with the basal lamina.

x 6,800.

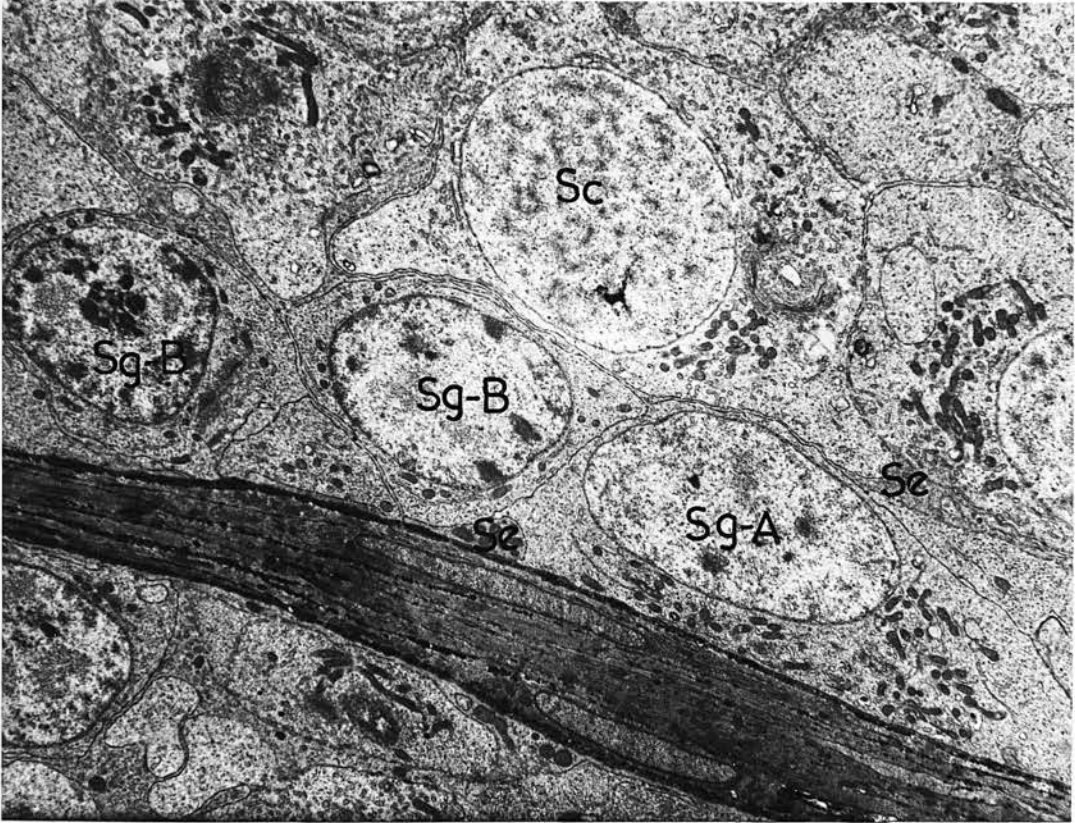
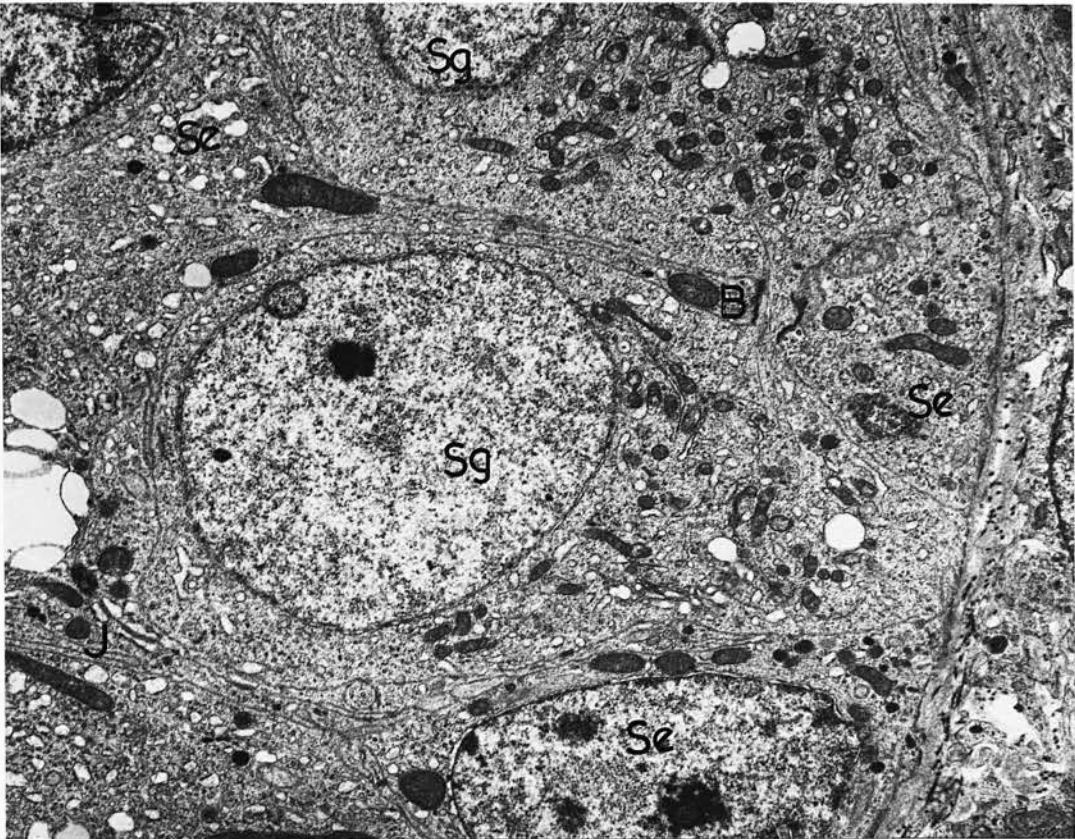


Figure 3.12

Shows an A-type spermatogonium having an ovoid nucleus with an even distribution of chromatin | mitochondria are clustered in the basal cytoplasm.

M

x 7,500.

Figure 3.13

The basal cytoplasm of an A-type spermatogonium showing a Golgi complex, clusters of centriole and mitochondria with a dense inter-mitochondrial substance. The mitochondria are circular, rod or dumbbell-shaped and easily distinguished from the larger ovoid Sertoli cell mitochondria. A few cisternae of rough ER and many free ribosomes can be seen.

x 15,000.

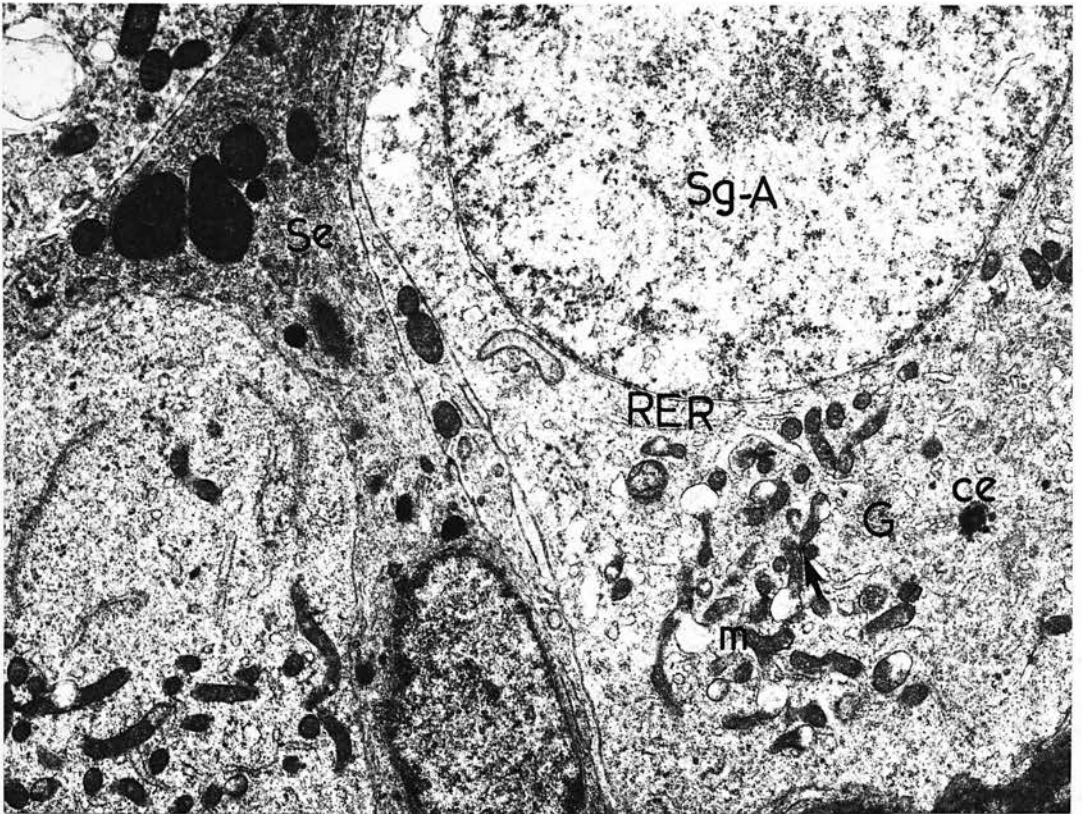
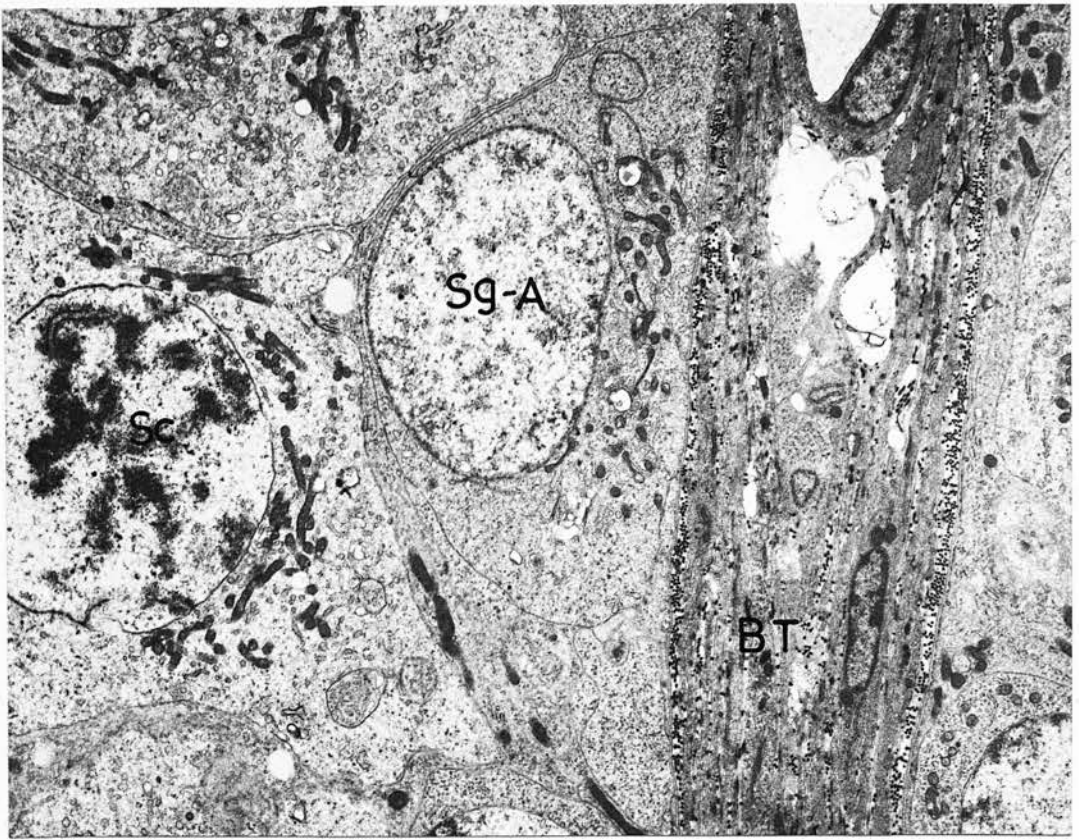


Figure 3.14

Shows a B-type spermatogonium having a spherical nucleus with perinuclear clumping of the chromatin. Only a small portion of the basal plasma membrane is in contact with the basal lamina. (single arrow). A cytoplasmic bridge is observed between spermatogonia (double arrow). Spherical or rod-shaped mitochondria are evenly dispersed through the cytoplasm.

x 8,200.

Figure 3.15

A B-type spermatogonium showing evenly dispersed rough ER, mitochondria and ribosomes. Note intercellular bridge.

x 20,000.

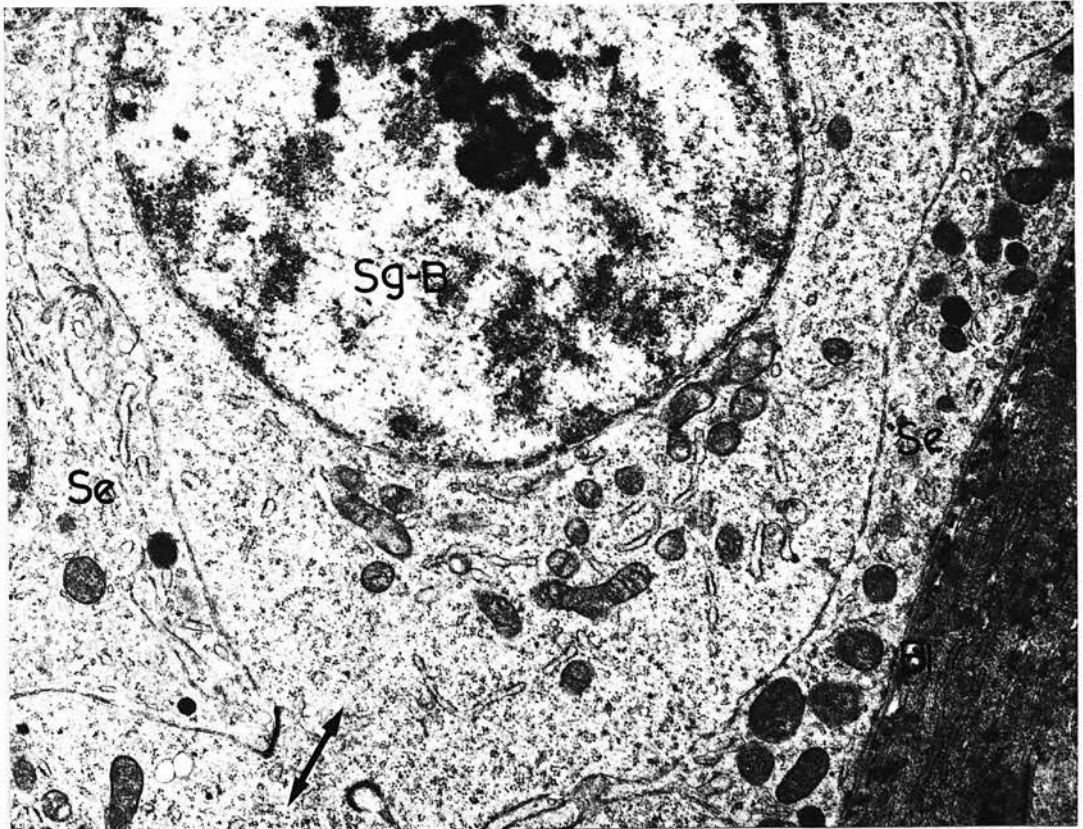
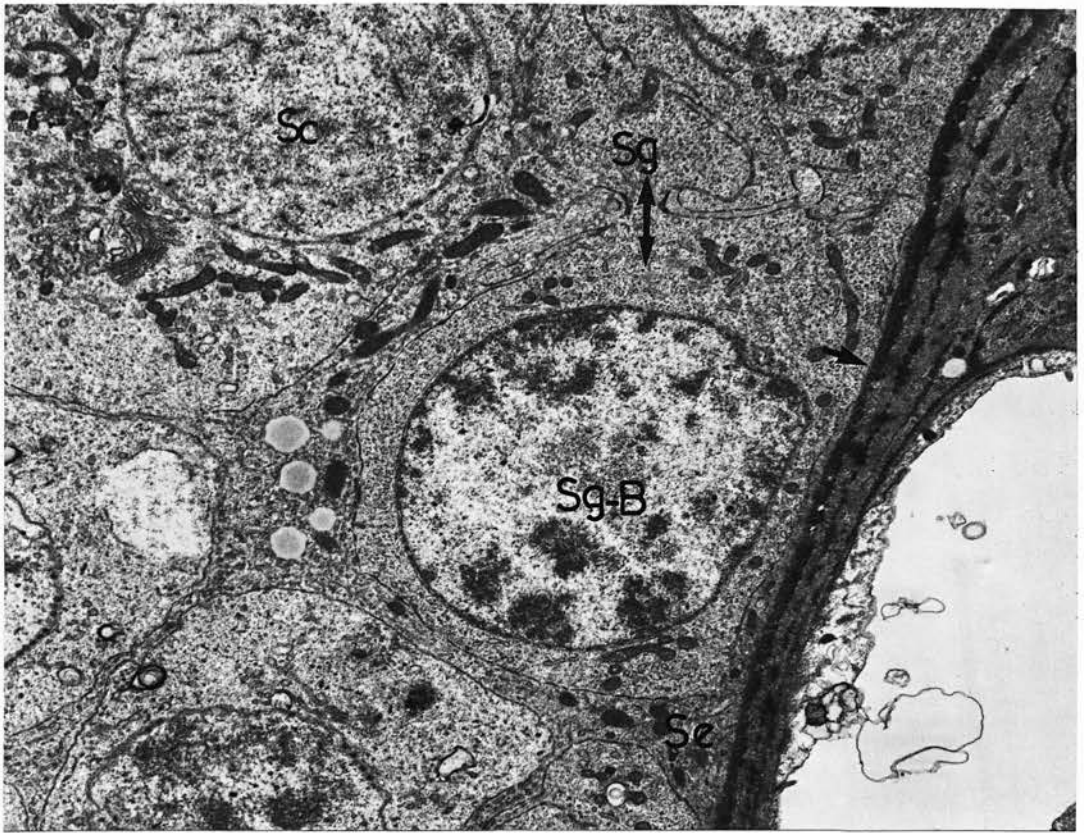


Figure 3.16

A B-type spermatogonia is seen in contact with the basal lamina and a resting spermatocyte in an apical position.

x 12,000.

Figure 3.16A

An almost completed cytoplasmic bridge between two spermatocytes (arrow). Note the dense material between the plasmalemma indentations.

x 26,000.

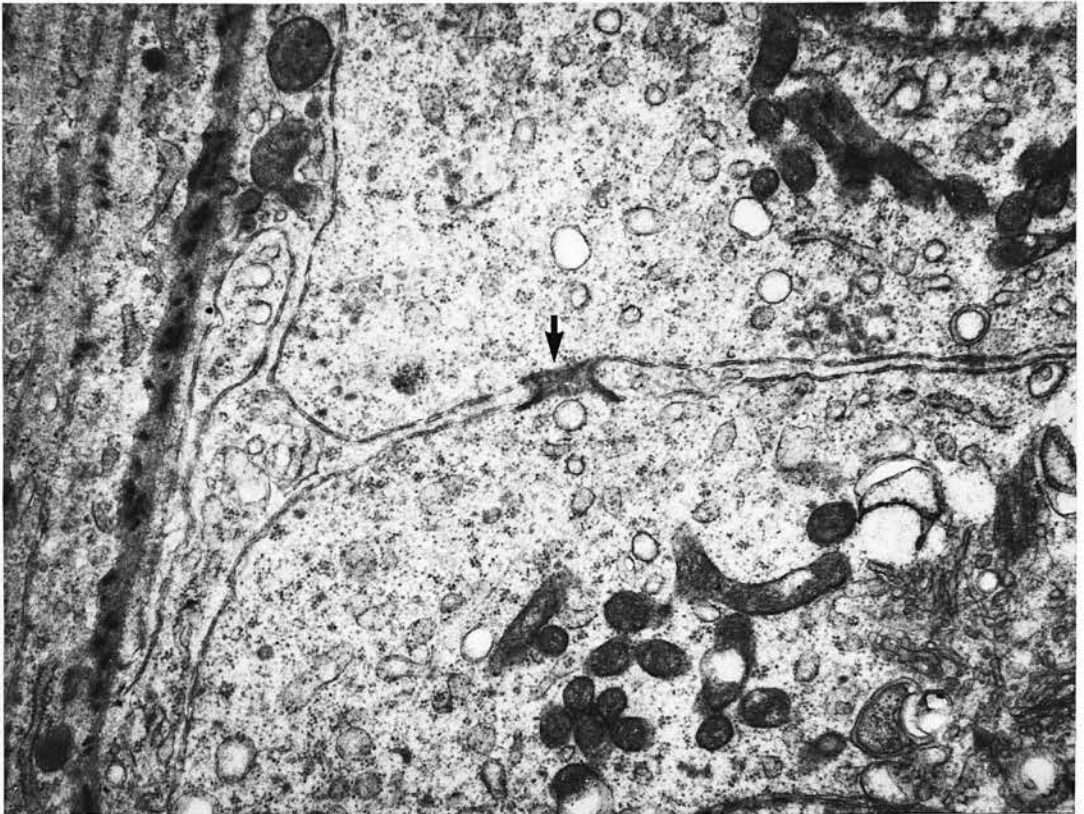
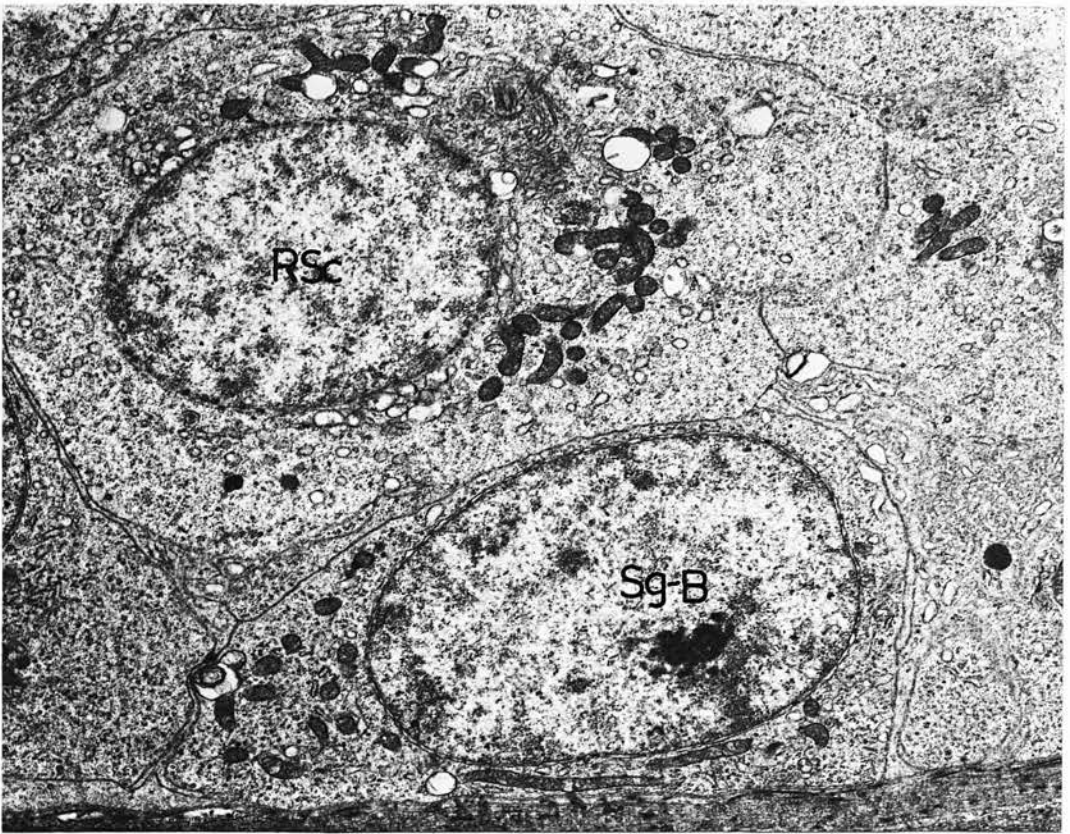




Figure 3.17

A cytoplasmic bridge between a spermatogonium and a resting spermatocyte (arrow).

x 3,000.

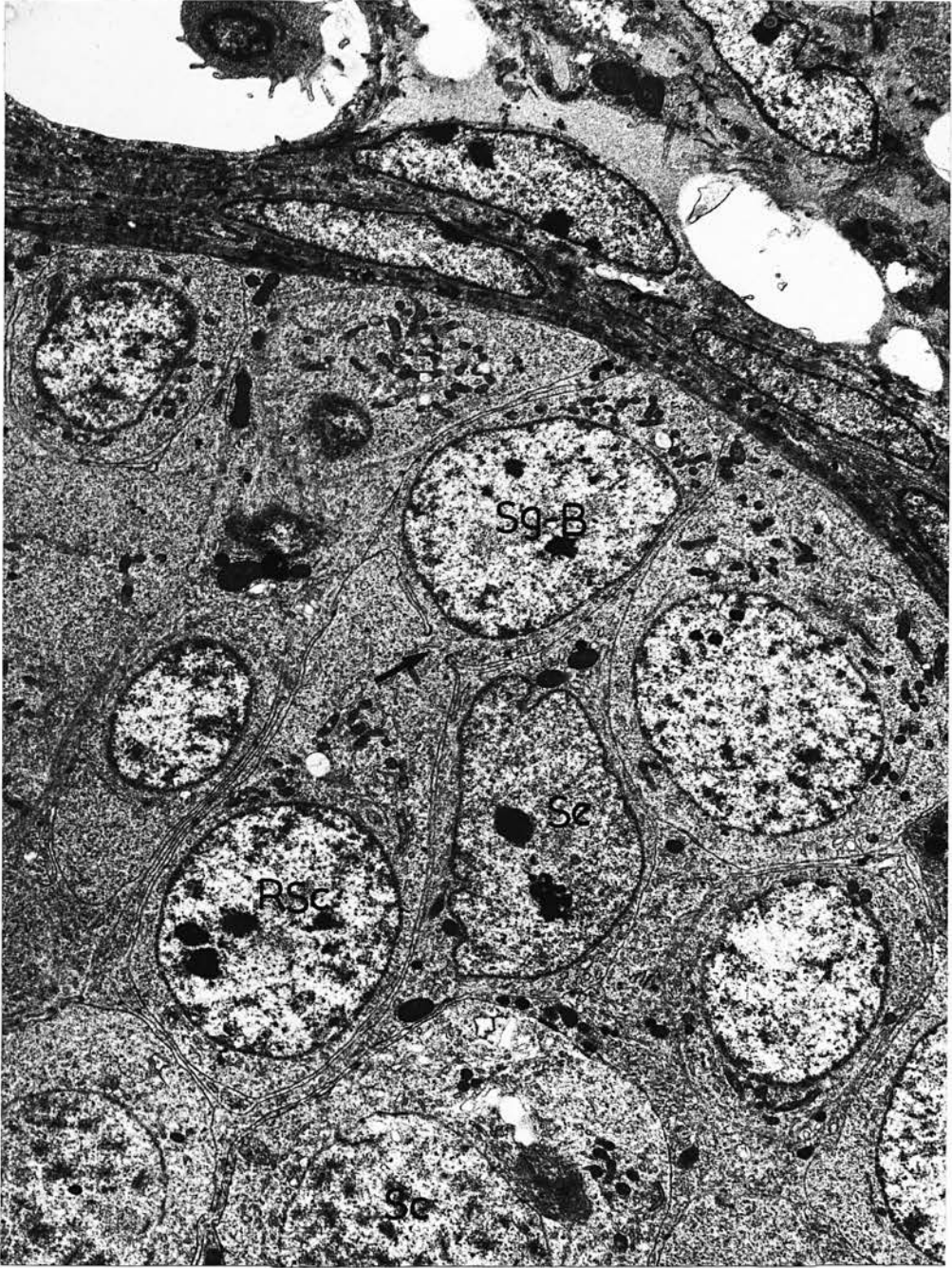


Figure 3.18

A spermatocyte with a leptotene stage nucleus showing dispersed chromatin and chromosomal cores or their threads. Clusters of mitochondria are observed adjacent to a horseshoe-shaped Golgi complex and centrioles.

x 7,500.

Figure 3.19

A leptotene spermatocyte showing the large vesicles and cisternae of ER in a juxtannuclear position and a prominent Golgi complex. Note electron dense chromosomal cores in the nucleus.

x 26,000.

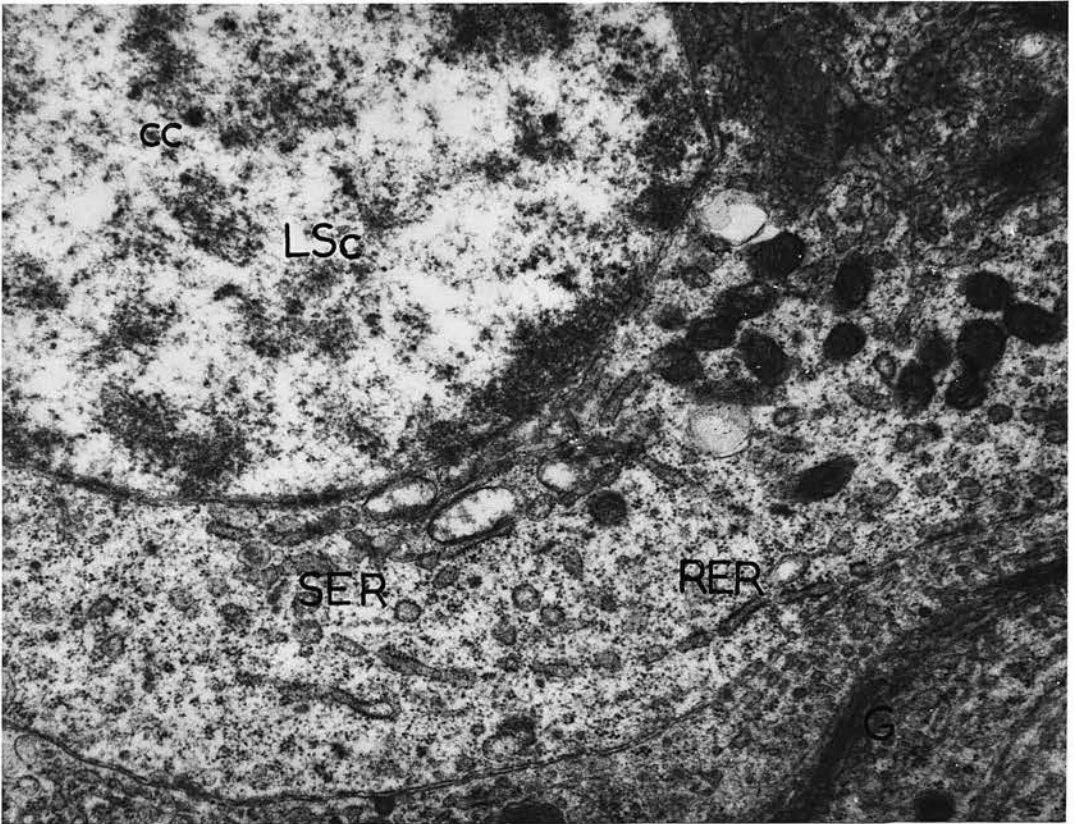
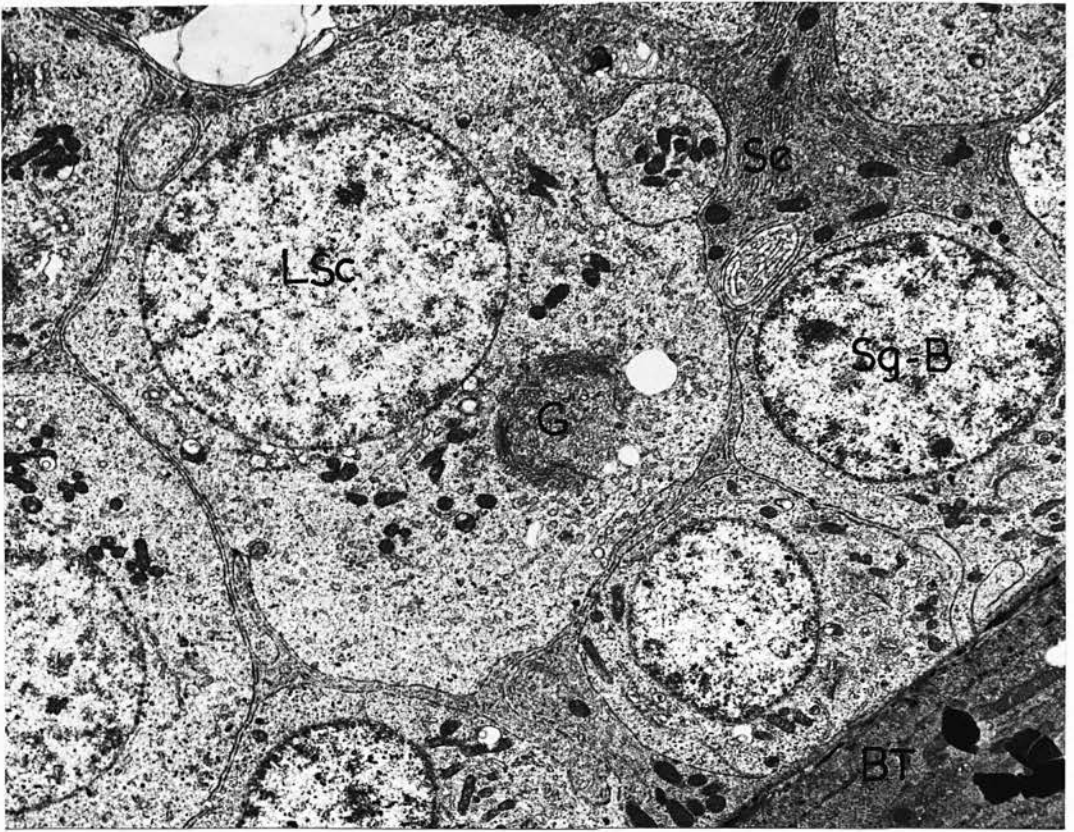


Figure 3.20

A leptotene spermatocyte showing chromosomal cores some of which are seen attached to the nuclear membrane. Two centrioles are observed in close proximity to the Golgi complex. Note the clusters of mitochondria and the differentiation of tubular smooth ER.

x 7,560.

Figure 3.21

A leptotene spermatocyte showing the horseshoe-shaped Golgi complex and associated centriole. Note the dense inter-mitochondrial substance (arrow).

x 21,000.

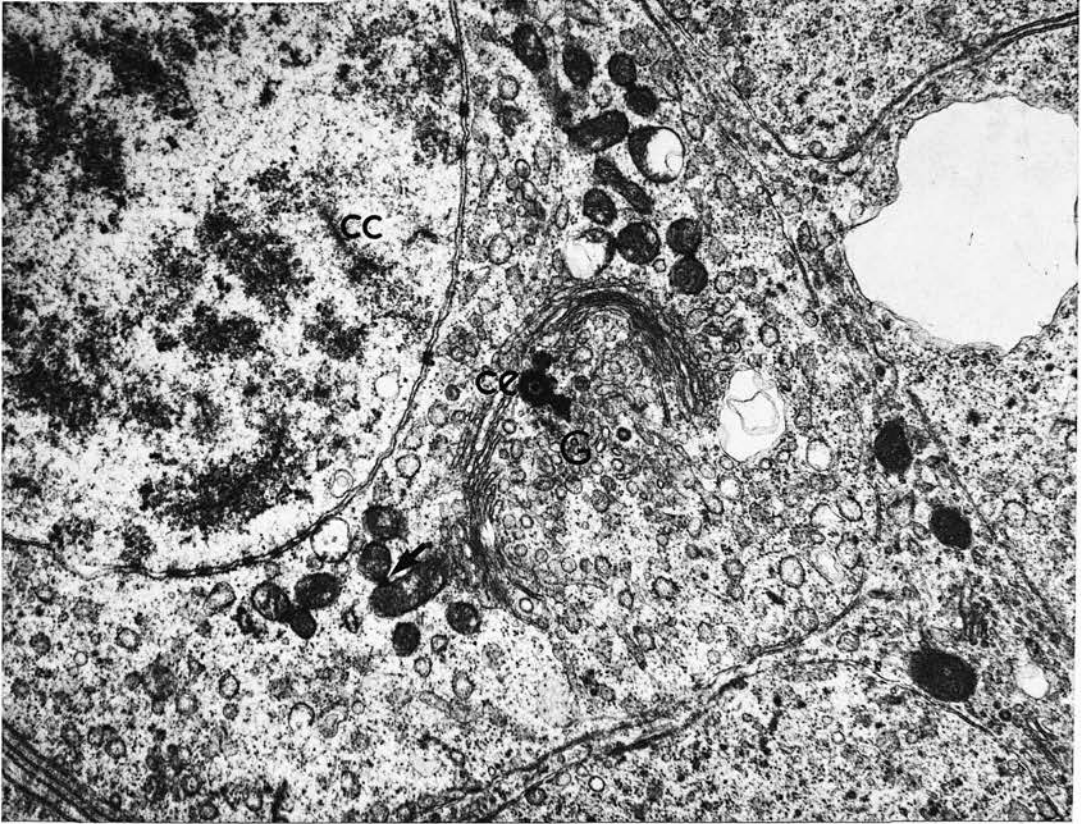
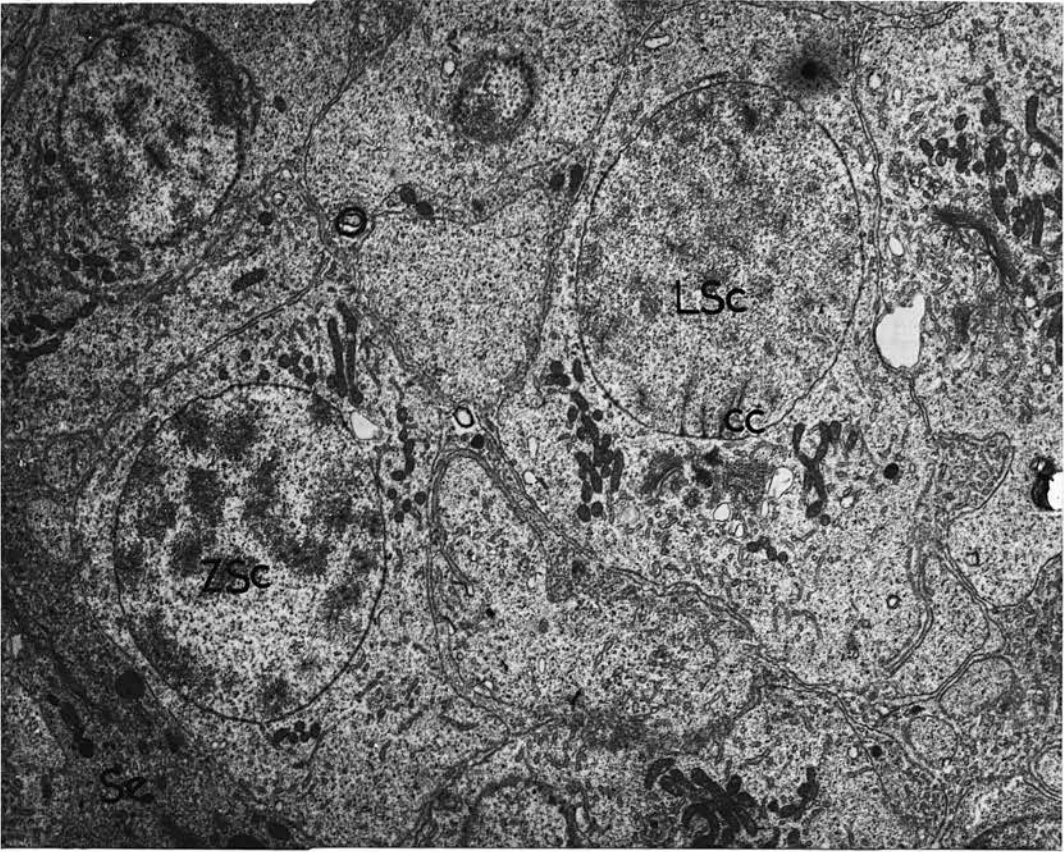


Figure 3.22

A zygotene spermatocyte showing chromatin massed around the chromosomal cores in the nucleus. Note the difference in chromatin density of the sex vesicle and around the chromosomal cores.

Vesicle

x 9,000.

Figure 3.23

A zygotene spermatocyte showing some single chromosomal cores and some paired cores forming the synaptonemal complex.

Note long rod-shaped mitochondria.

x 13,600.

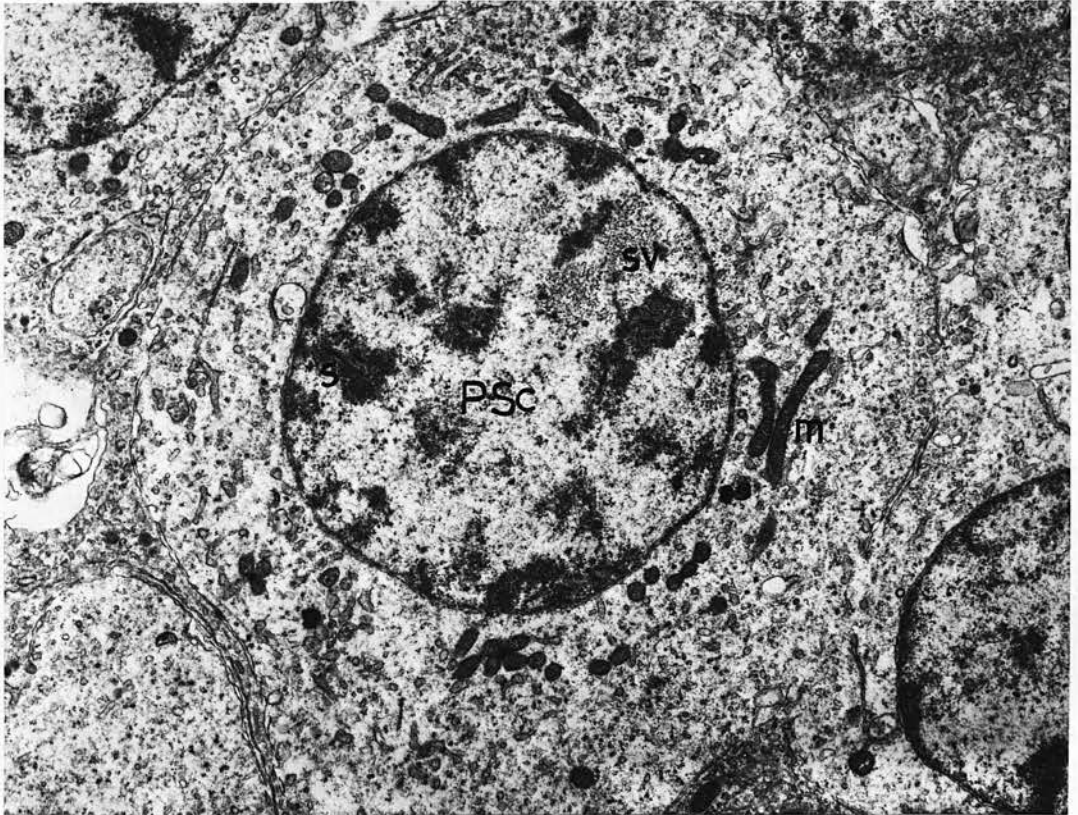
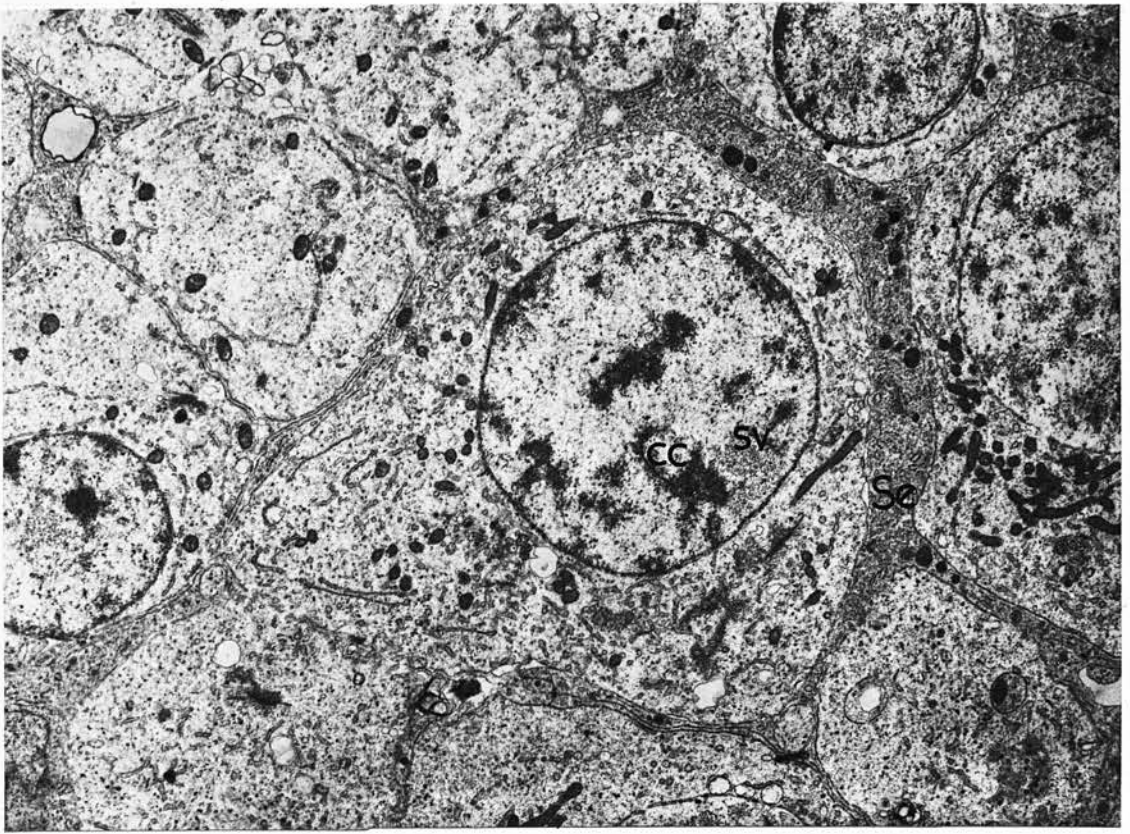




Figure 3.24

A pachytene spermatocyte showing long lengths of synaptonemal complex in the nucleus.

x 9,500.

Figure 3.25

A pachytene spermatocyte showing synaptonemal complexes in the nucleus and a prominent Golgi complex with two associated centrioles in a juxtannuclear position. Note long lengths of ER.

x 12,000.

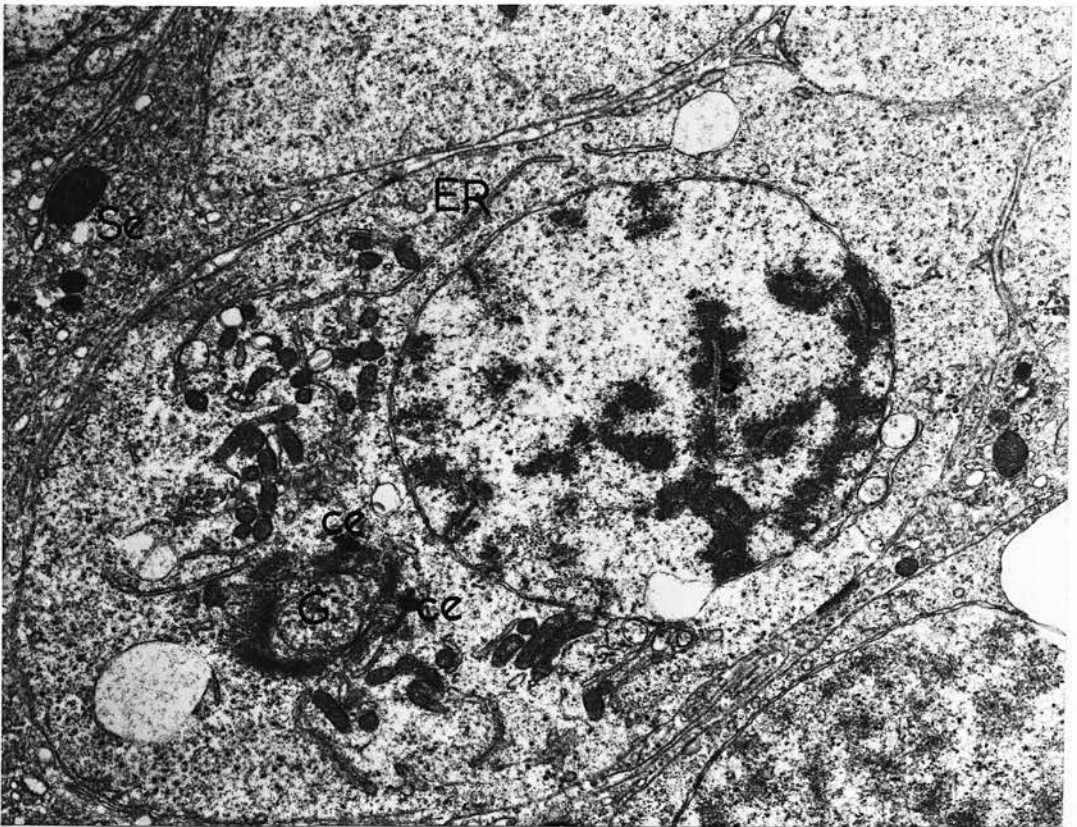
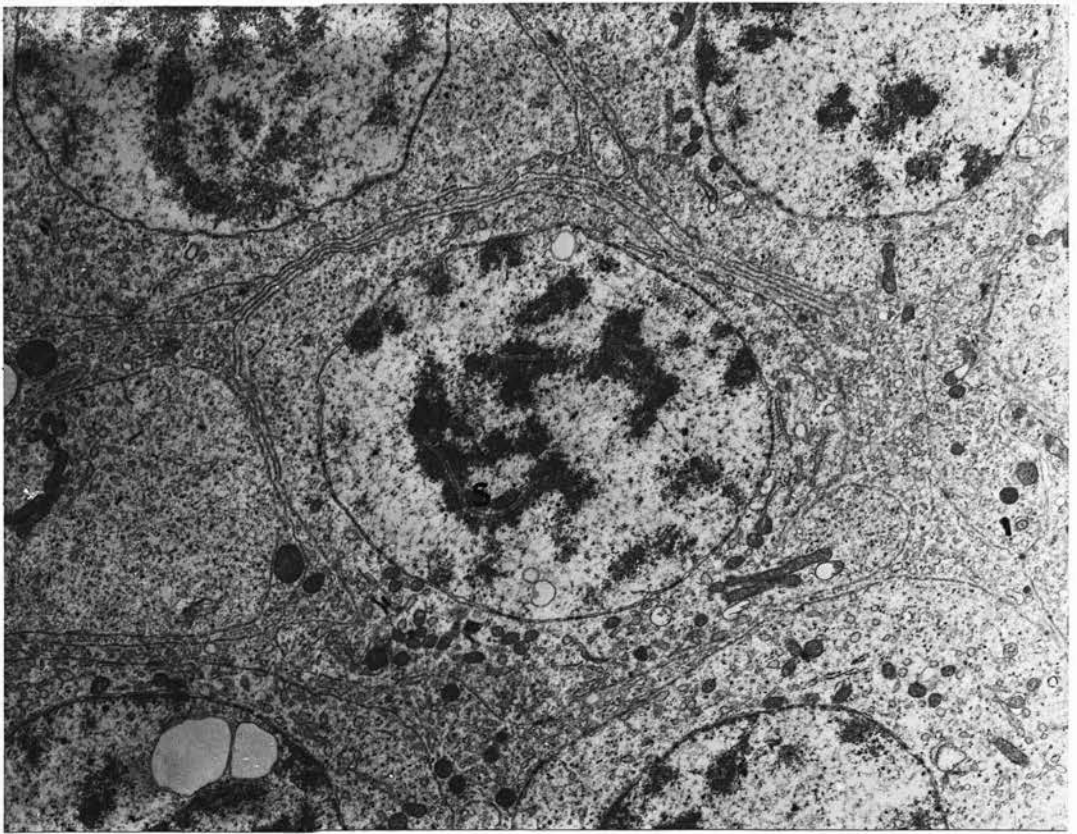


Figure 3.26

A higher magnification of pachytene nucleus of figure 3.24 showing the two dense lateral parallel filaments (arrows) and the central medium dense element (double arrow) of the synaptonemal complex. A condensed mass of chromatin fibrils is seen around the lateral filaments.

x 69,100.

Figure 3.27

A higher magnification of the Golgi complex of the pachytene nucleus of figure 3.25 showing its circular formation and associated centrioles. Note the smooth ER in close vicinity to the mitochondria.

x 43,000.

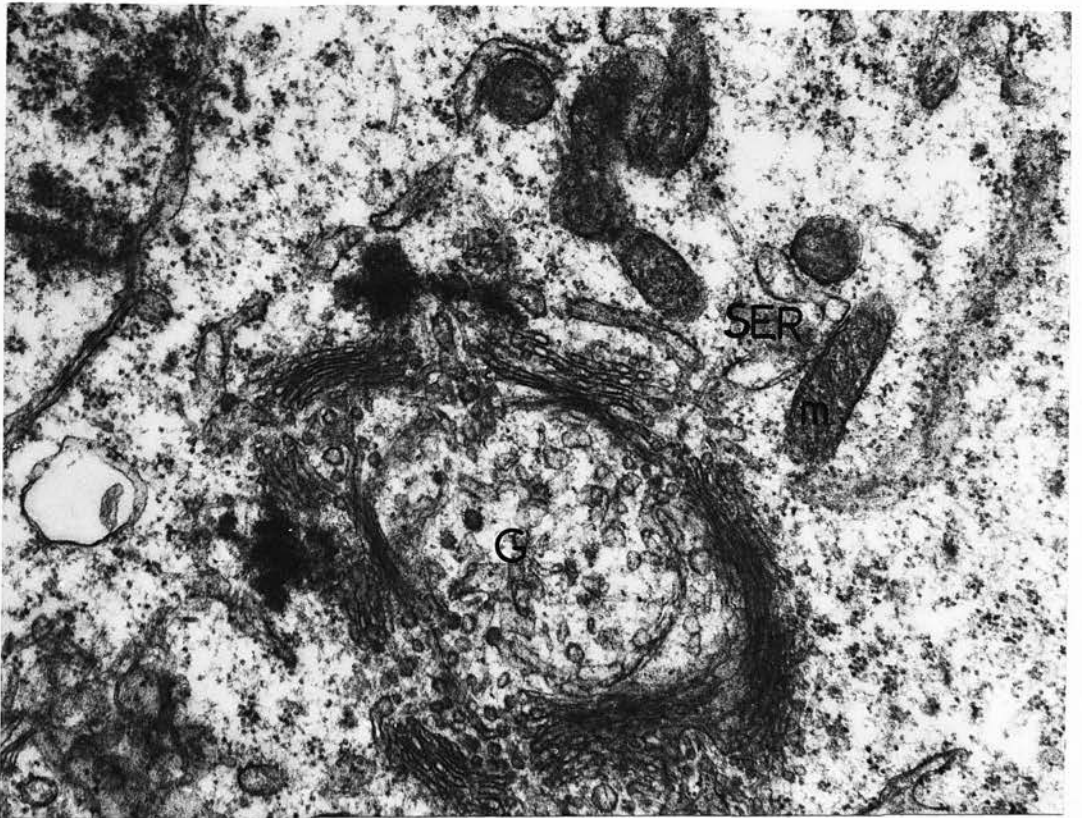
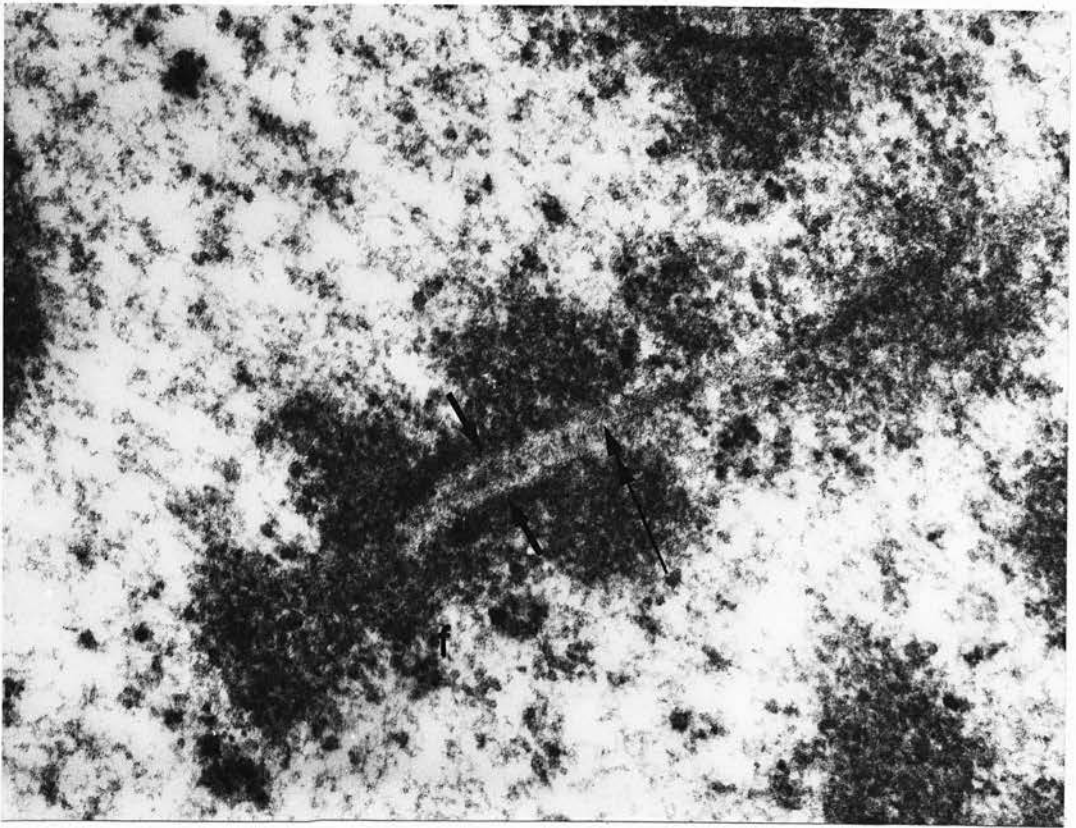


Figure 3.28

A diakinesis spermatocyte showing the perinuclear distribution of the chromatin.

x 12,000.

Figure 3.29

A diakinesis spermatocyte in which vesiculation of the nuclear membranes has begun (arrow).

x 15,000.

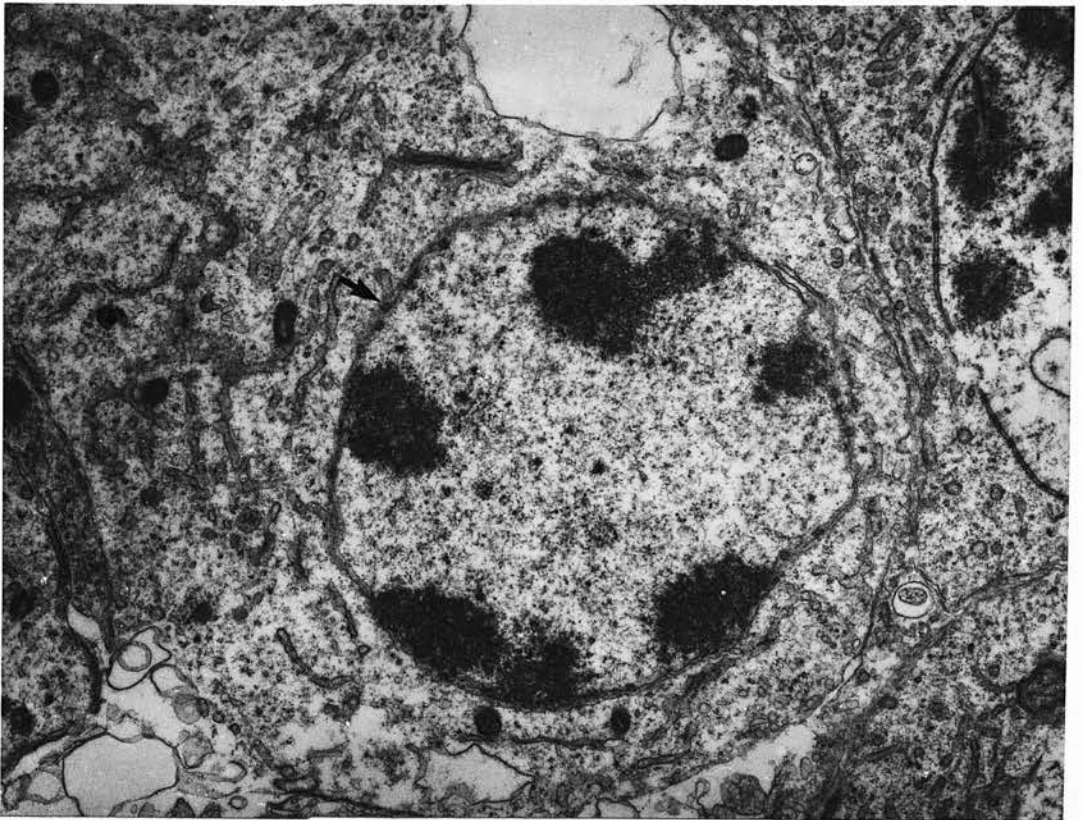
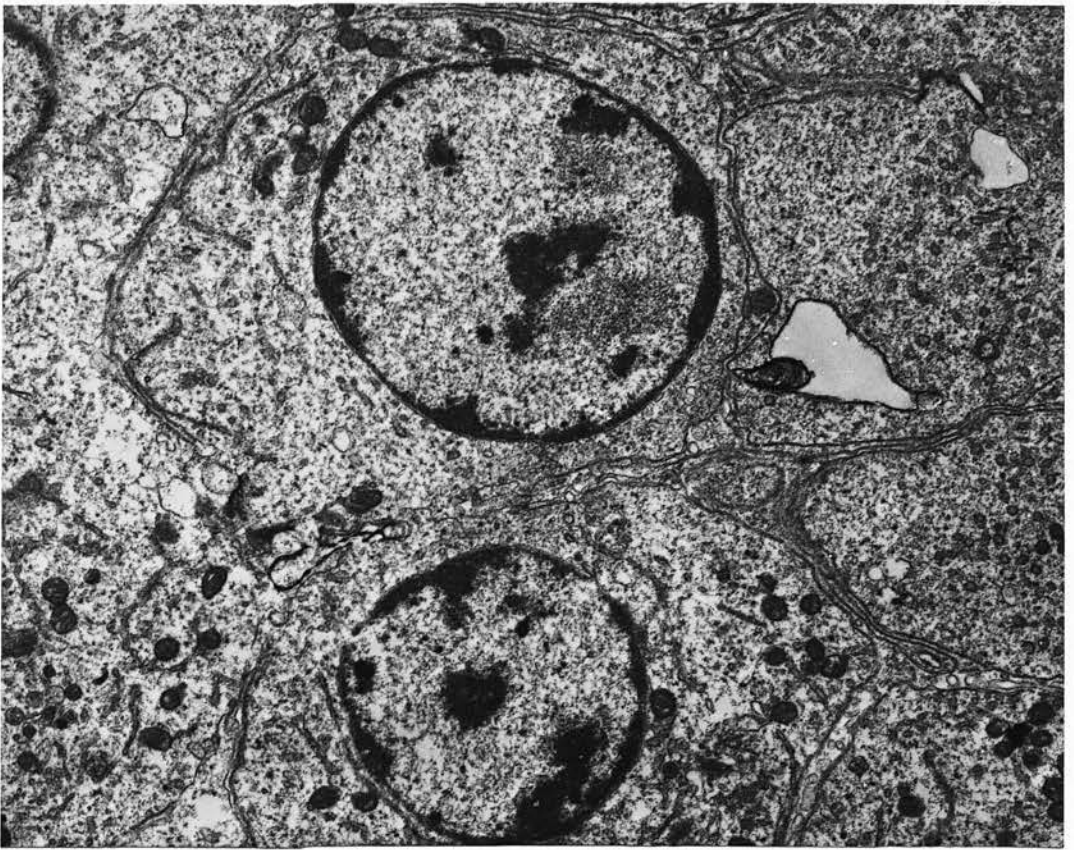


Figure 3.30

A metaphase-anaphase spermatocyte showing the dense chromosomal masses arranged along the equatorial plate of the spindle. Note the long strands of ER arranged around the cell periphery and the mitochondria with dilated cristae.

x 9,500.

Figure 3.31

Metaphase mitochondria showing a dark matrix and very dilated cristae with a clear intracristal matrix.

x 34,000.

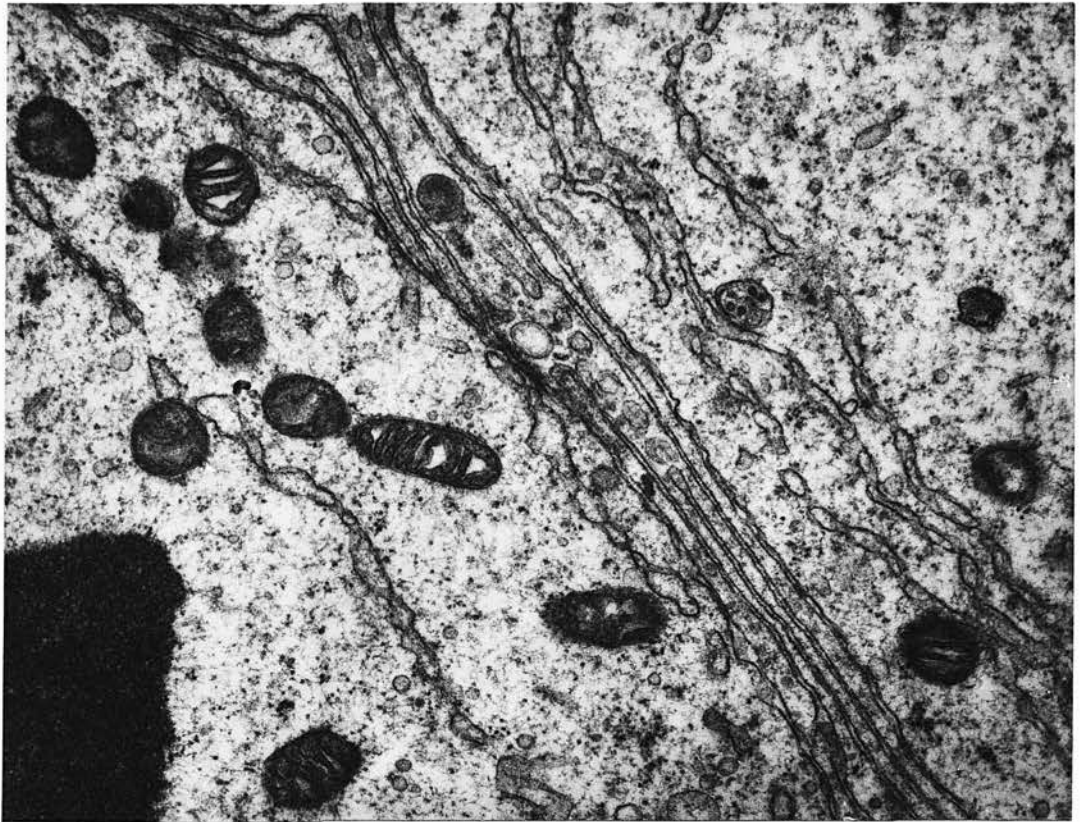
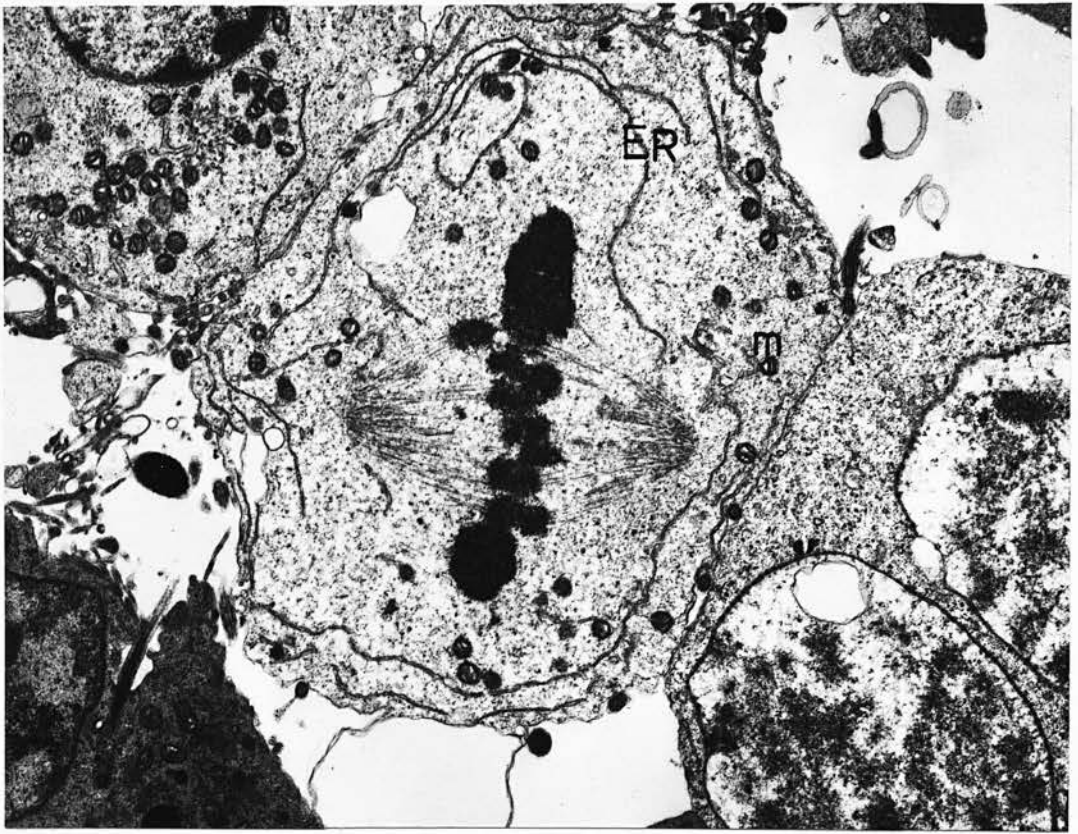




Figure 3.32

Metaphase-anaphase spermatocytes (1) showing the start of the migration of the chromosomal masses to either pole. Note the group of multivesicular and multigranular bodies in the cytoplasm (arrow) and the cytoplasmic bridge between spermatocytes (2) and (3).  
x 10,000.

Figure 3.33

Secondary spermatocytes showing incomplete cytokinesis and the presence of cytoplasmic bridges. Note the nuclear membrane formation by vesicles of a telephase spermatocyte (arrow) and the larger size of the nucleus of the primary spermatocyte compared with the secondary spermatocyte nuclei.  
x 5,800.

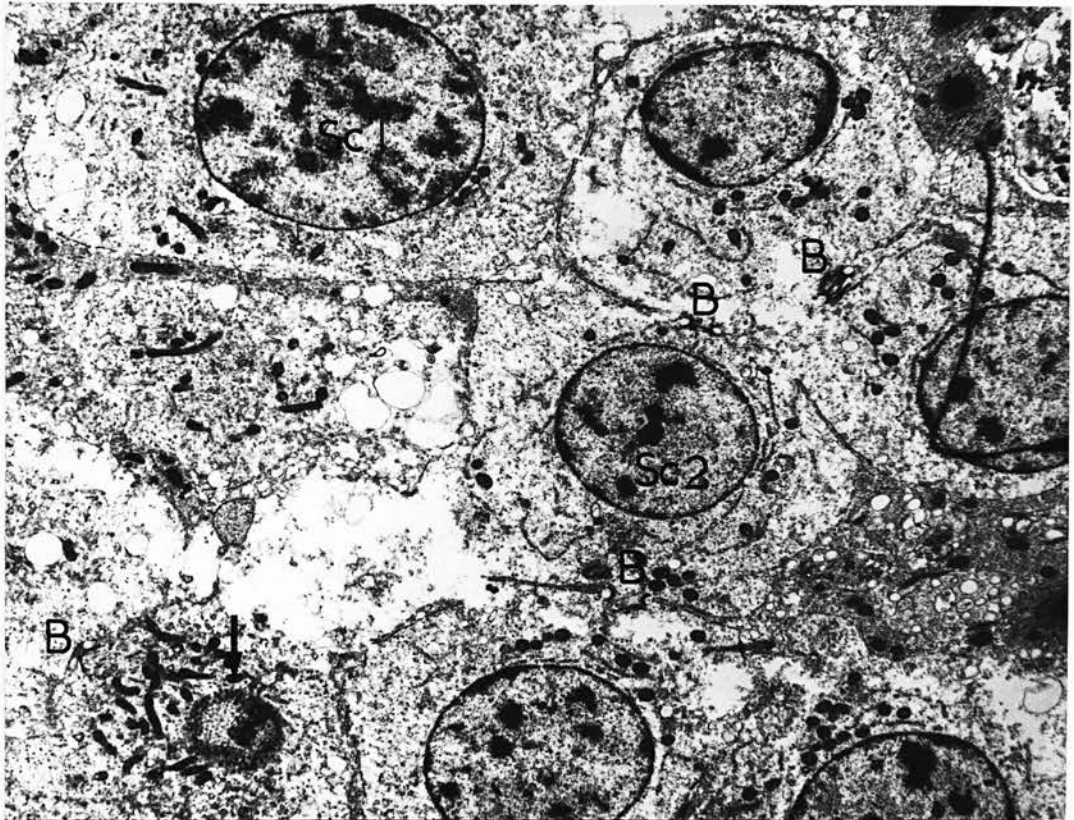
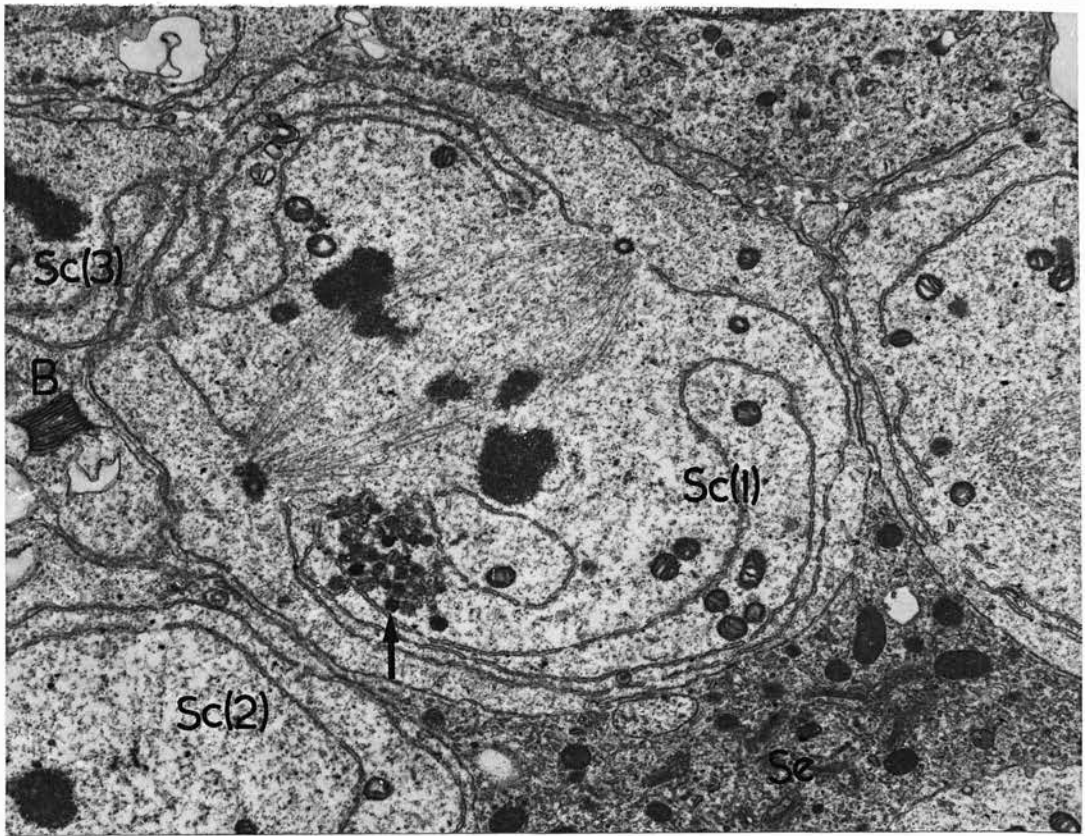


Figure 3.34

A secondary spermatocyte with some mitochondria with dilated cristae and other mitochondria in clusters with a dense inter-mitochondrial substance (arrow). Note the presence of a Golgi complex and multivesicular bodies.

x 16,000.

Figure 3.35

A group of secondary spermatocytes joined by cytoplasmic bridges (double arrows) showing mitochondria with dilated cristae, free ribosomes and long profiles of smooth ER.

x 10,000.

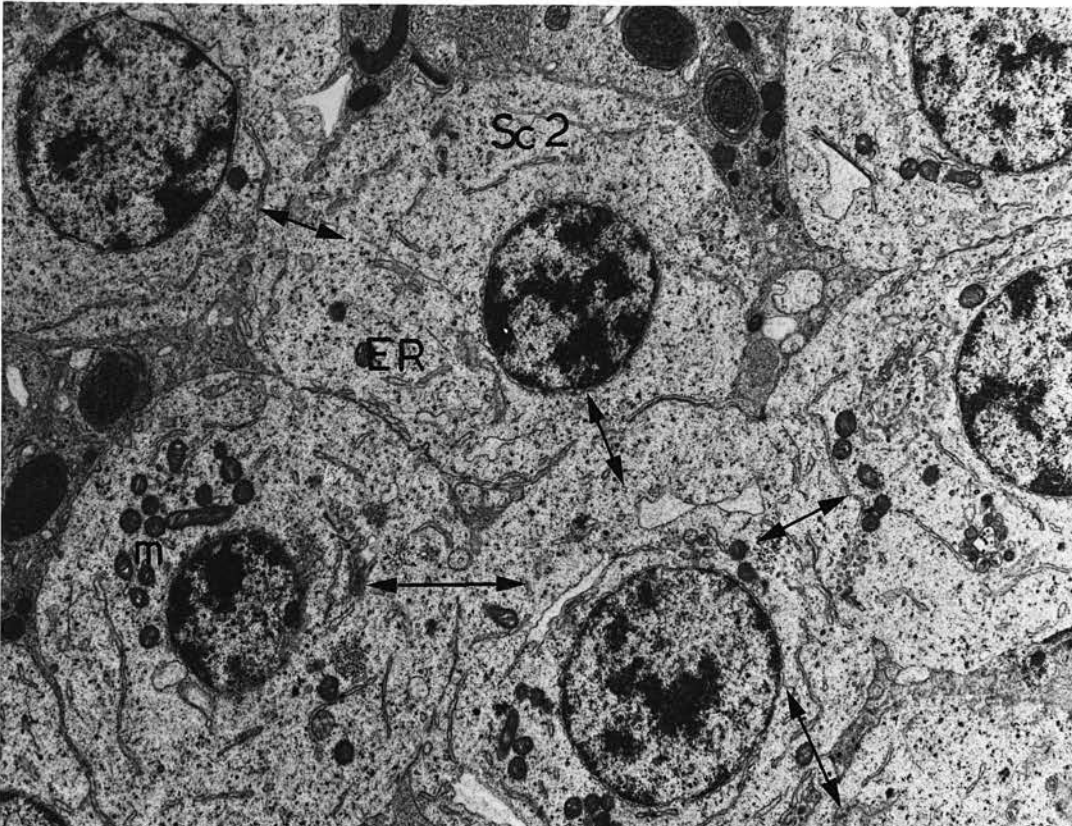


Figure 3.36

An interphase spermatid showing mitochondria with dilated cristae, a large Golgi complex, long profiles of ER and multivesicular bodies. Note cytoplasmic bridge with cisternal structure.  
x 15,500.

Figure 3.37

A higher magnification of the cytoplasmic bridge of figure 3.37 showing cisternal structure.  
x 30,600.

30

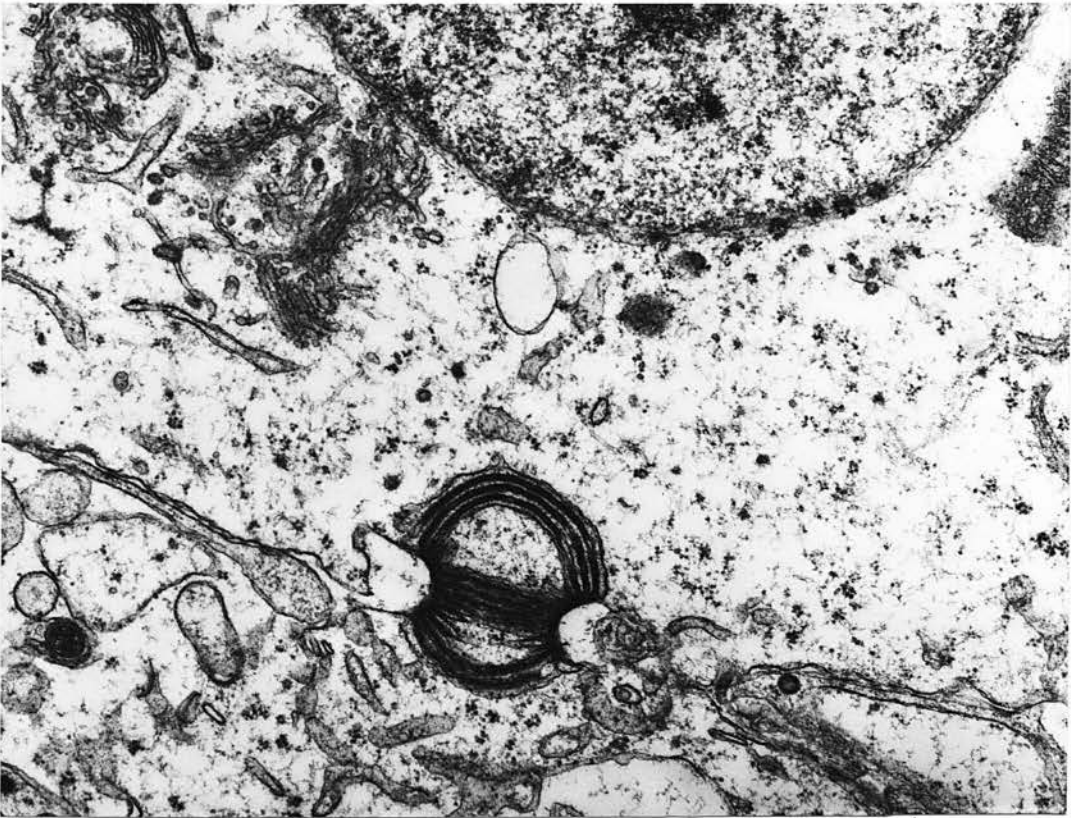
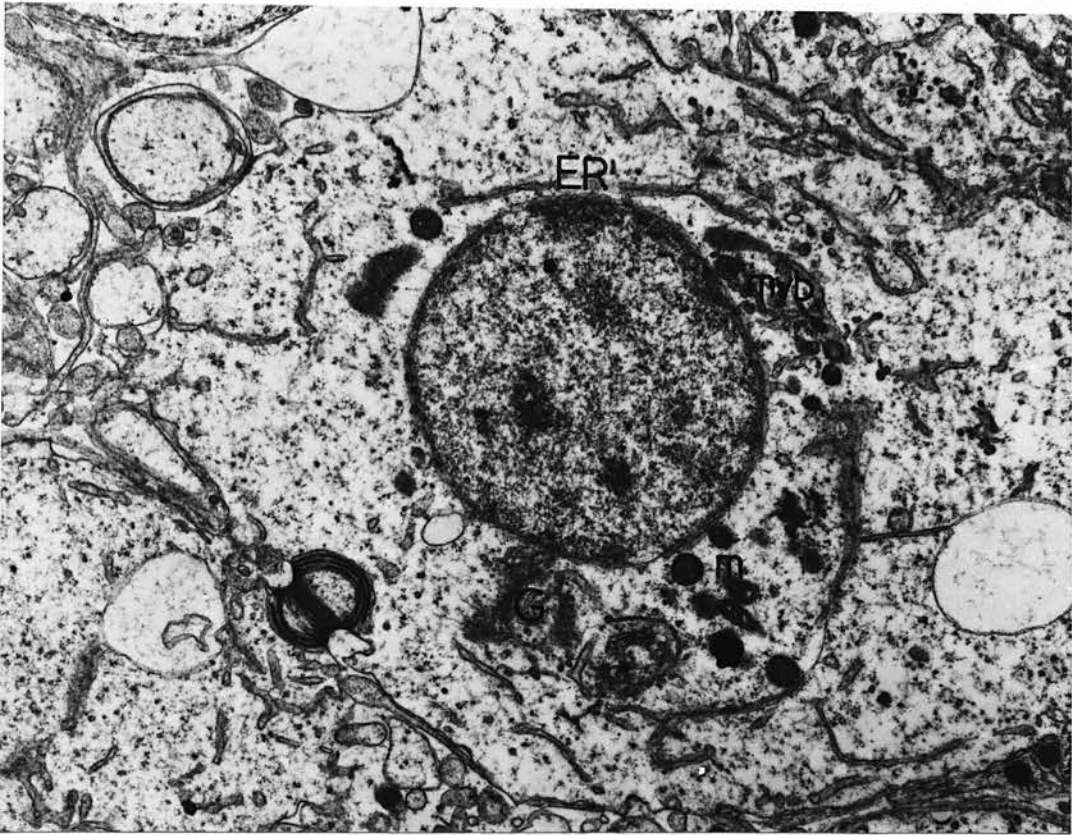


Figure 3.38

R<sub>1</sub> spermatids showing spherical nuclei, long profiles of ER and mitochondria with dilated cristae. Note the cytoplasmic bridge without cisternal structure (double arrow).  
x 15,000.

Figure 3.39

R<sub>2</sub> spermatids showing the proacrosome within Golgi complex. The mitochondria with dilated cristae are no longer seen. Note cytoplasmic bridges (double arrows) and a new organelle that resembles a sunflower in appearance.  
x 12,000.

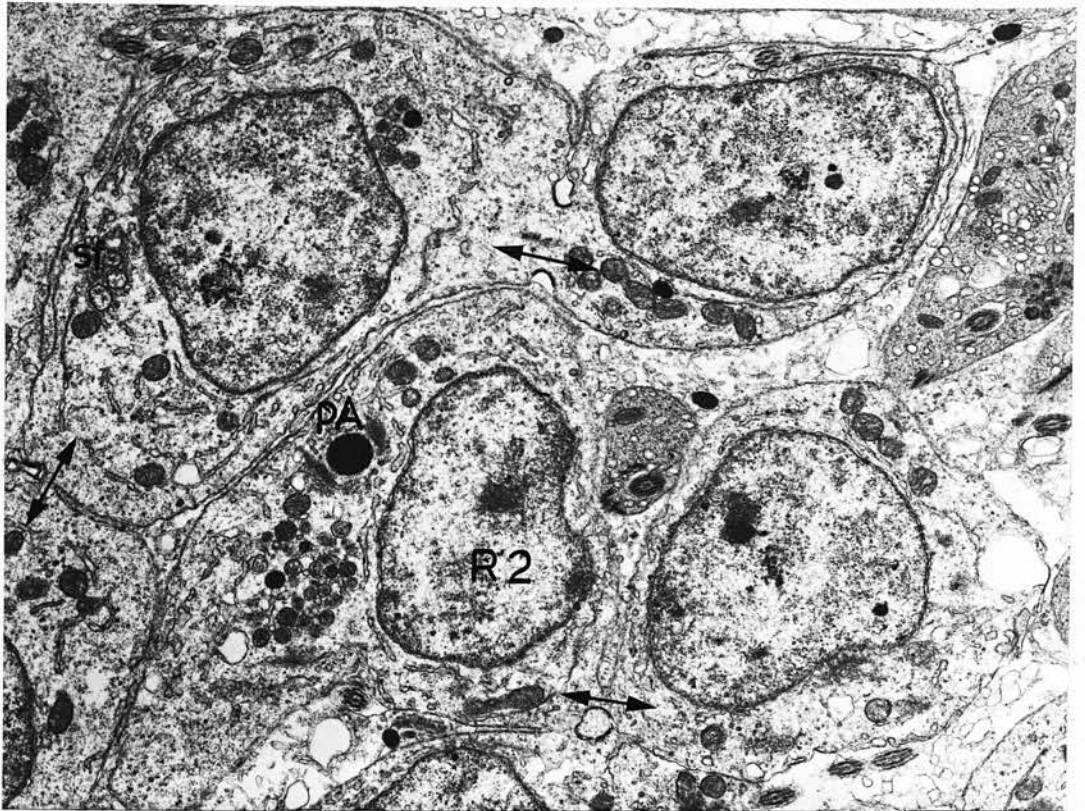
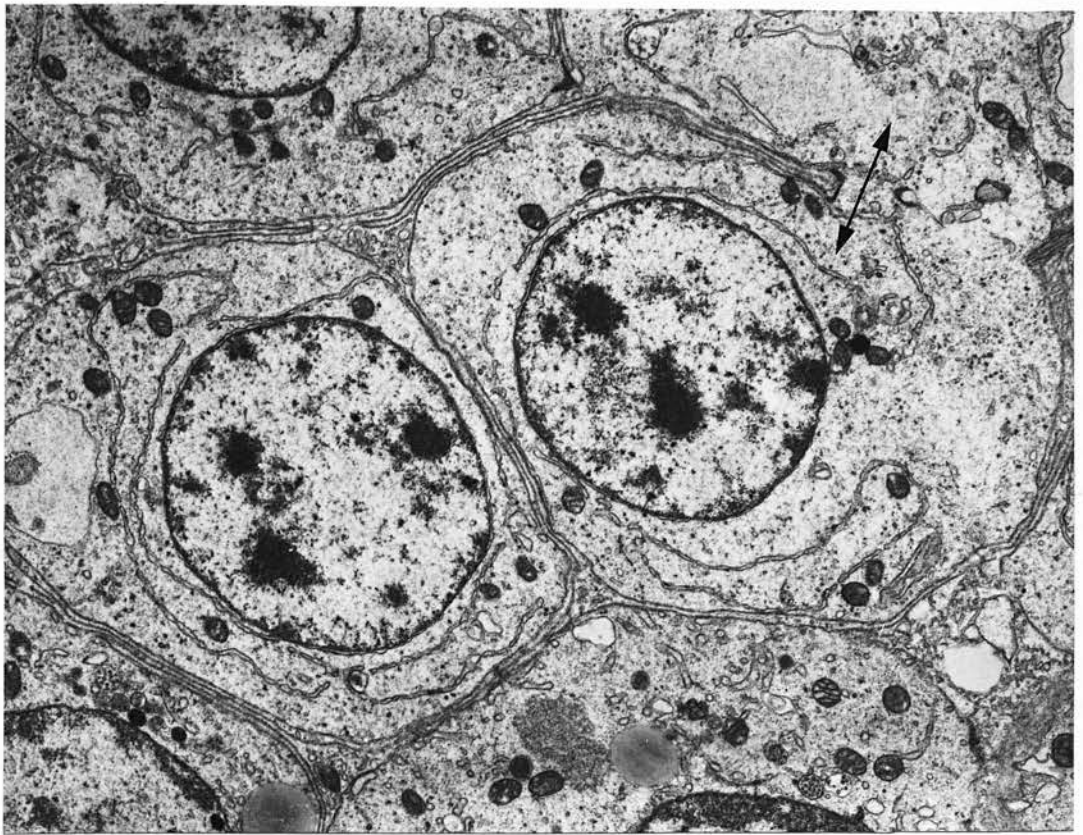




Figure 3.40

The proacrosome is observed surrounded by stacks of Golgi cisternae with tubular smooth ER in close proximity to the Golgi body and mitochondria. Many multivesicular and multigranular bodies are observed.

x 43,000.

Figure 3.41

R<sub>3</sub> spermatids show a perinuclear distribution of chromatin and the proacrosome is seen in contact with the nuclear membrane. An el<sub>4</sub> spermatid shows an even distribution of chromatin in the nucleus and invaginated nuclear membranes in contact with the acrosome. Note 'sunflower' organelle.

x 8,200.

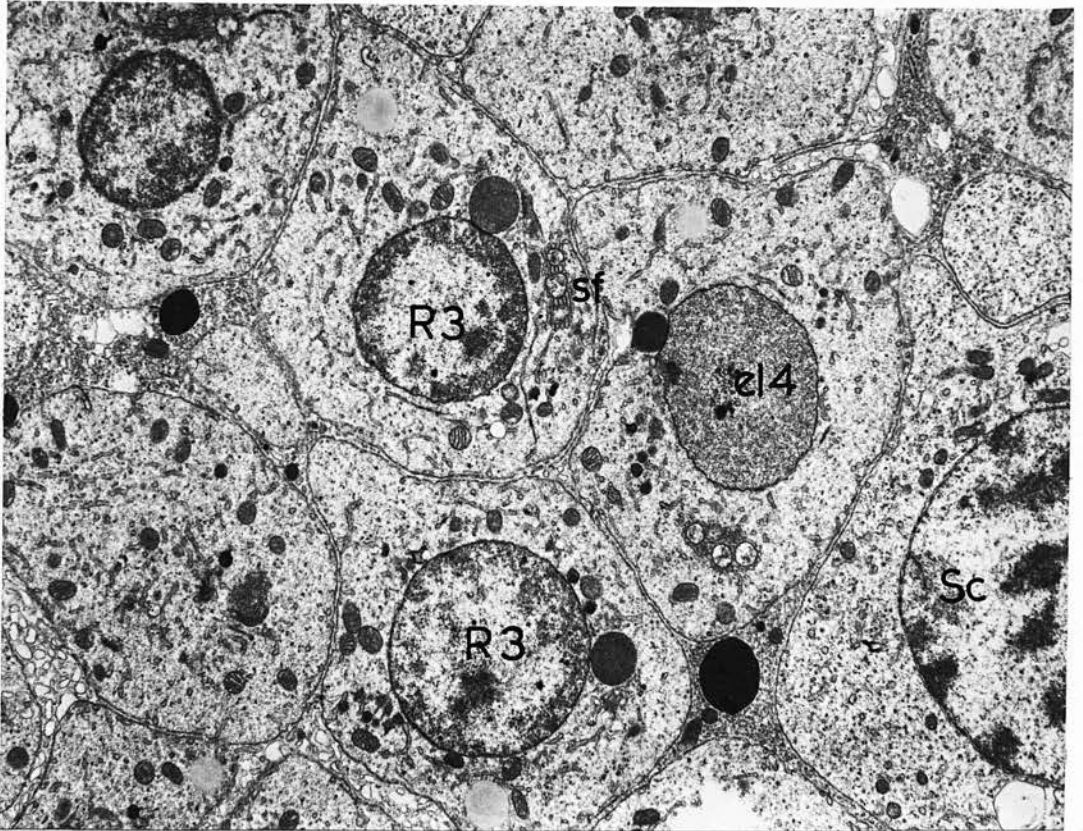
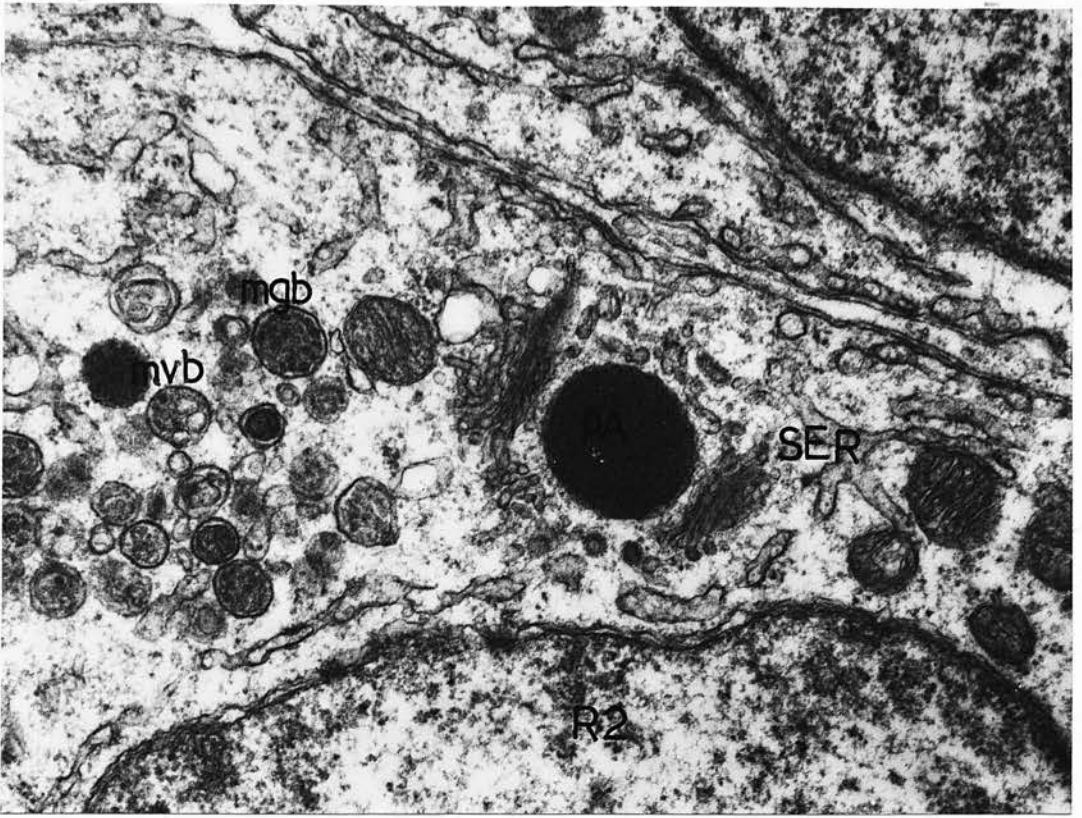


Figure 3.42

An  $el_4$  spermatid showing a spherical nucleus with a wavy nuclear membrane and groups of microtubules in the juxtannuclear cytoplasm (arrow). Mitochondria with diagonal running cristae are seen.

At this late stage of  $el_4$  the 'sunflower' organelle begins to disappear.

x 34,000.



Figure 3.43

An early  $el_4$  spermatid showing the pair of centrioles lying in a juxtannuclear position. Note prominent 'sunflower' organelle.

X 15,000

Figure 3.44

An  $el_5$  spermatid with a hemispherical acrosome. Note the invagination of the nuclear membrane below the acrosome, where the perforatorium will develop.

X 12,000

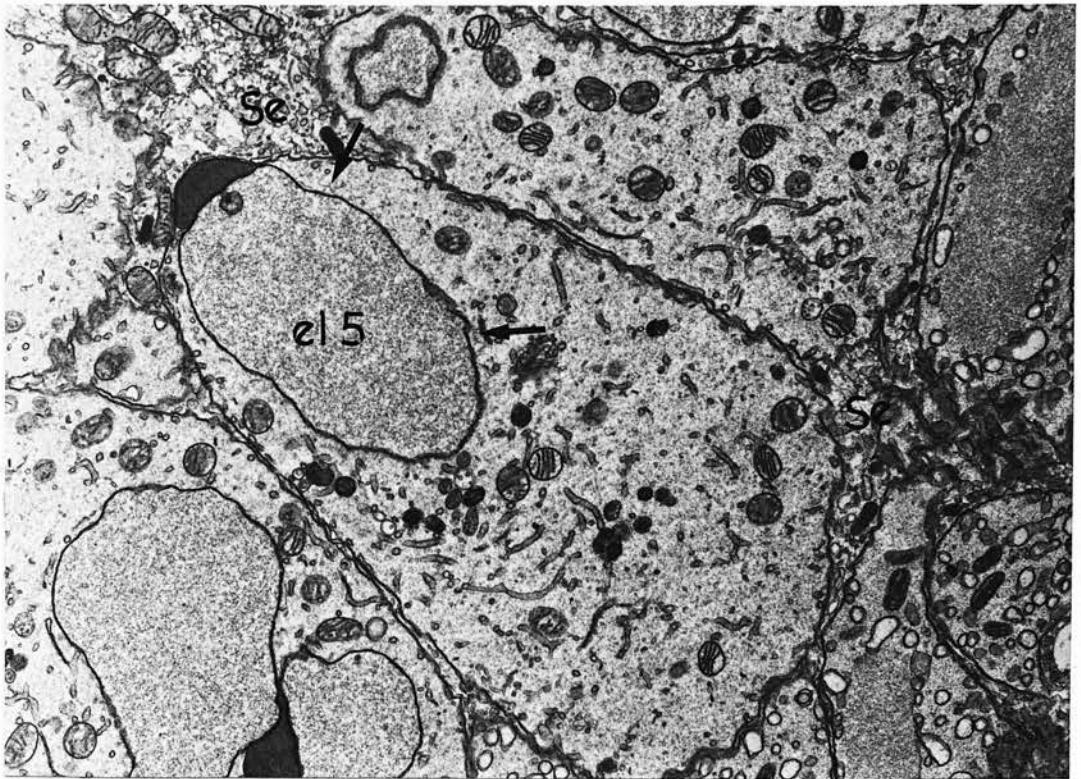
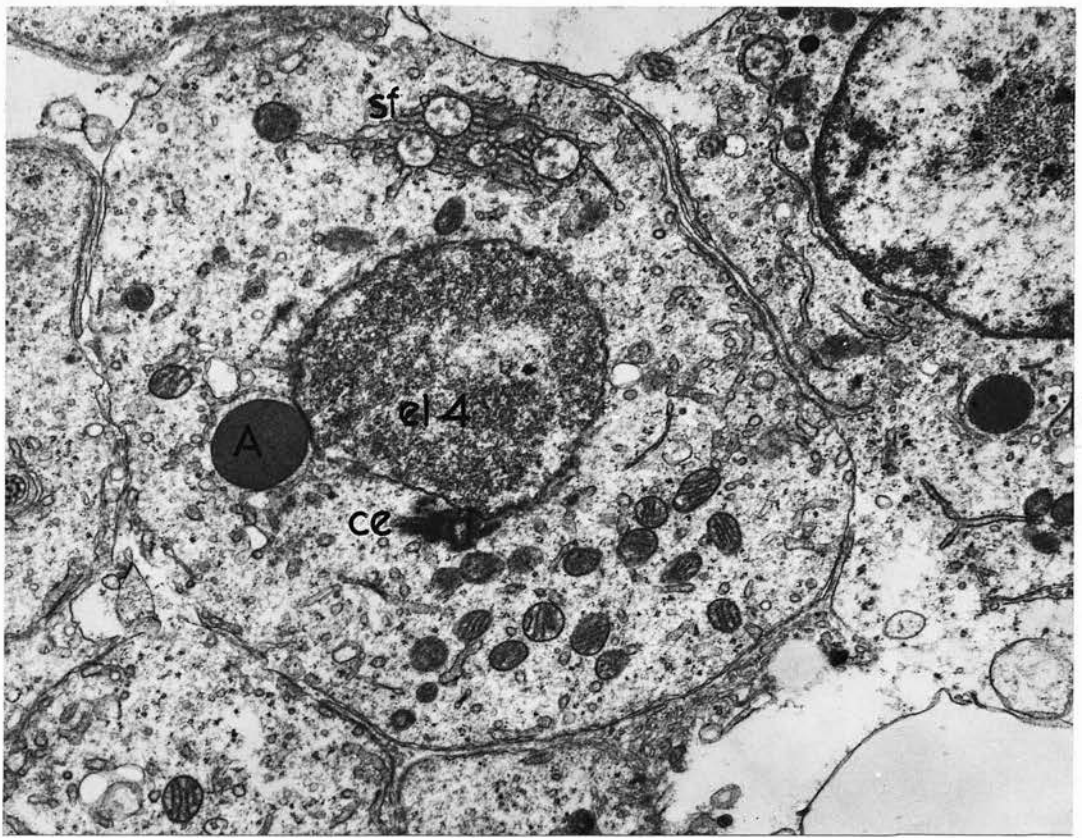


Figure 3.45

El<sub>5</sub> spermatids are seen situated on the luminal surface of the seminiferous tubule with their acrosome oriented towards the basal lamina.

X 1,300





Figure 3.46

A fully elongated  $L_6$  spermatid with the acrosome lying in the Sertoli cell cytoplasm and the tail lying free in the lumen. Note the caudal position of the greater part of the cytoplasm.

X 5,800



Figure 3.47

The acrosome of an  $L_6$  spermatid embedded in Sertoli cell cytoplasm. Note the patches of electron dense amorphous material in the Sertoli cytoplasm subjacent to the acrosome (arrows). A row of microtubules cut in cross section is observed running the length of the spermatid nucleus and where the nucleus is cut obliquely a parallel array of microtubules running across the nucleus is seen (double arrow). Note longitudinal cristae of the  $L_6$  mitochondria.  
X 15,500

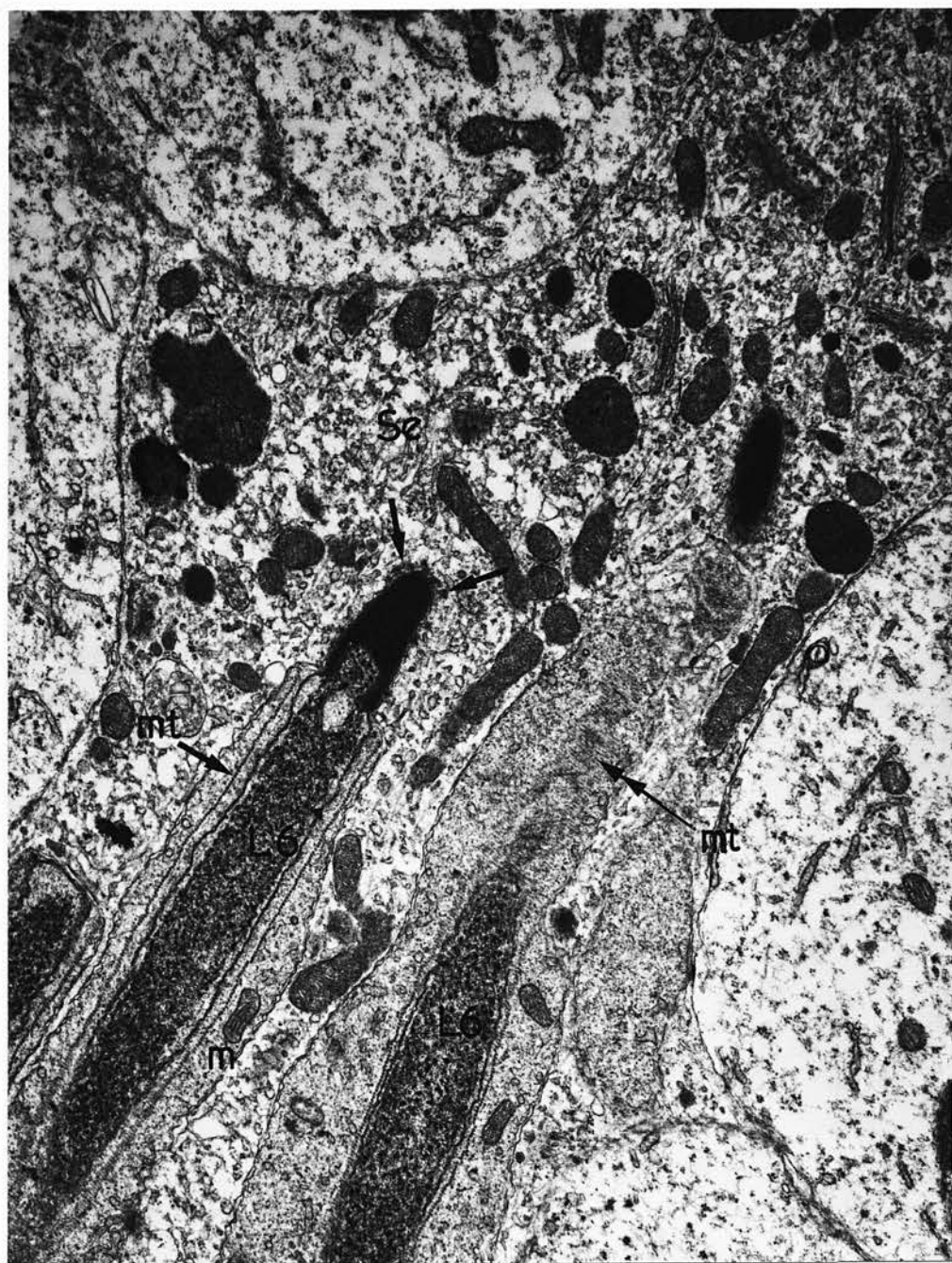


Figure 3.48

Cross-section through L<sub>6</sub> and L<sub>7</sub> spermatids.

The cross-section through the L<sub>6</sub> spermatid at the level of the acrosome shows the amorphous electron dense material in the subjacent Sertoli cell cytoplasm (arrows).

?L7.

The cross-section at the level of the perforatorium shows the subacrosomal cavity containing the perforatorium.

The L<sub>6</sub> nuclei are seen surrounded by bands of microtubules.

L<sub>7</sub> nuclei are seen surrounded by a new array of microtubules lying parallel to the long axis of the spermatid.

These microtubules are observed cut in cross-section.

X 30,600

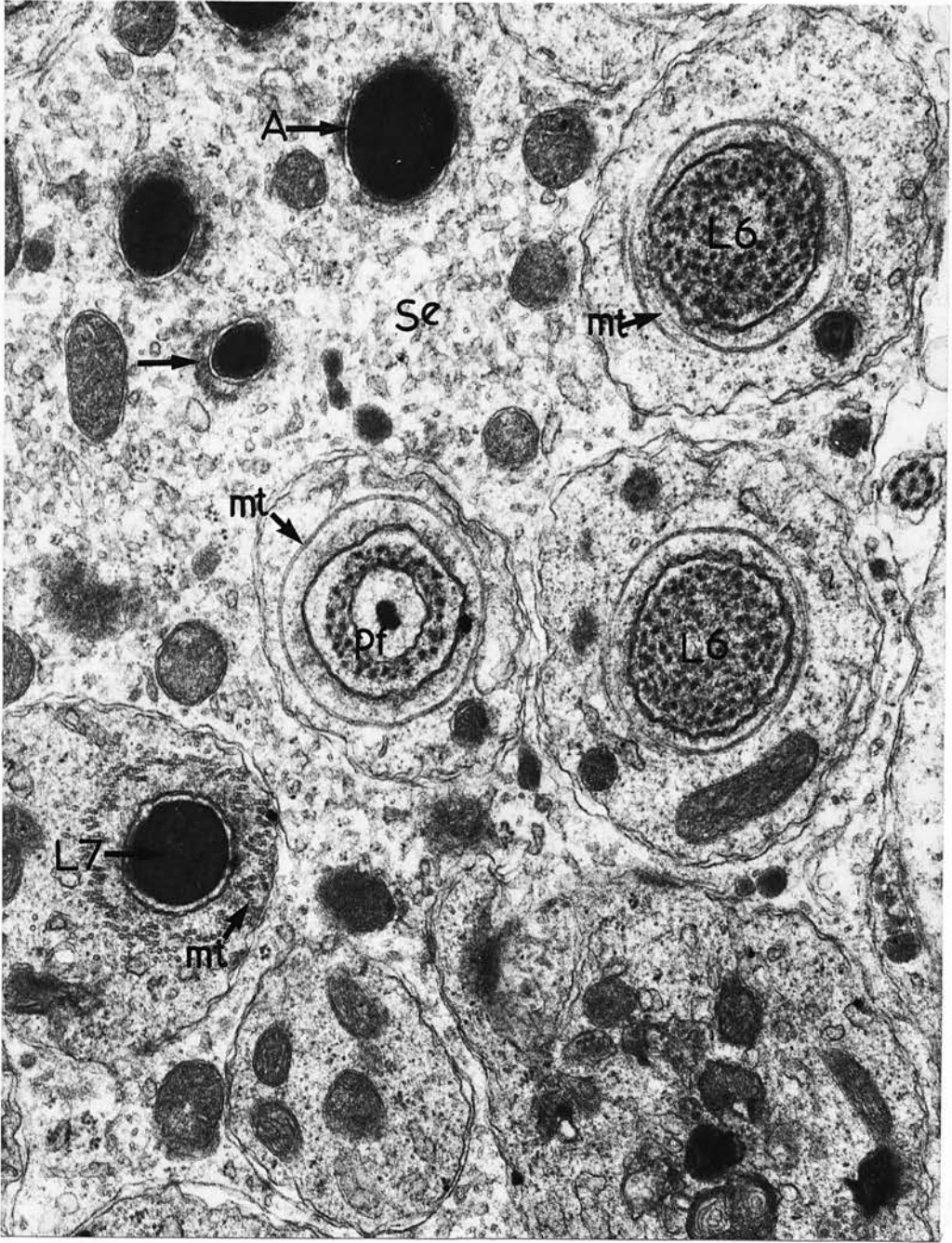


Figure 3.49

Caudal portion of an  $L_6$  spermatid showing proximal centriole lying perpendicular to the long nuclear axis. Note the five non-striated connecting pieces (arrow and inset). The distal centriole lies in the plane of the nucleus between the proximal centriole and the axonemal complex. Note the plasmalemma indentations to the level of the distal centriole (double arrow). A dense homogenous material is seen in the cytoplasm adjacent to the distal centriole.

X 41,000

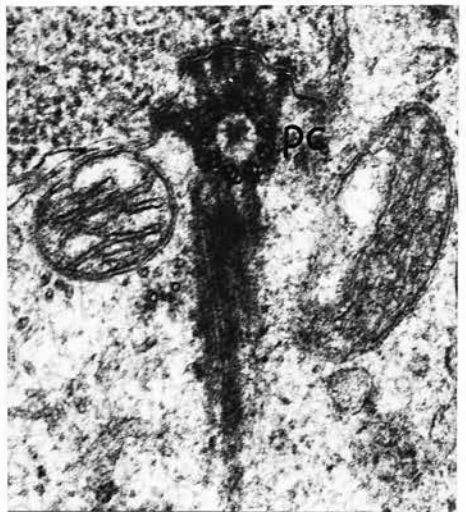
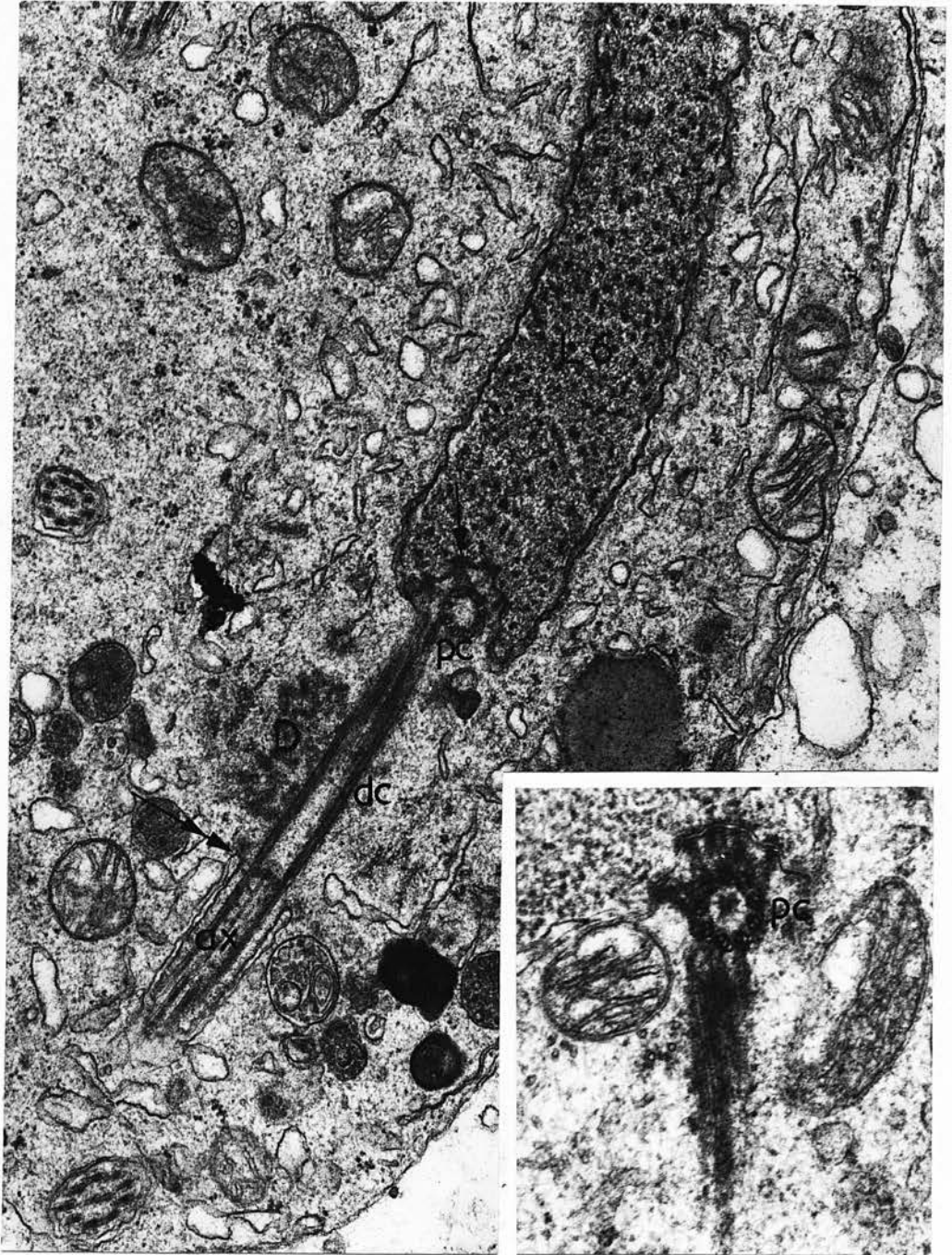




Figure 3.50

The acrosome portion of an L<sub>7</sub> spermatid showing the microtubules of the manchette running the length of the nucleus. Short wide cisternae and vesicles of smooth ER are seen lying at random in the cytoplasm.

X 15,500

Figure 3.51

Higher magnification of figure 3.50 showing the acrosome/Sertoli junction complex. An invagination of Sertoli cytoplasm surrounds the acrosome. The acrosome curves out of the plane of section so that the Sertoli cell invagination appears separated from the bulk of the Sertoli cell. Wings of L<sub>7</sub> cytoplasm are observed on either side of the acrosome/Sertoli complex filled with mitochondria, multivesicular and multigranular bodies.

X 38,000

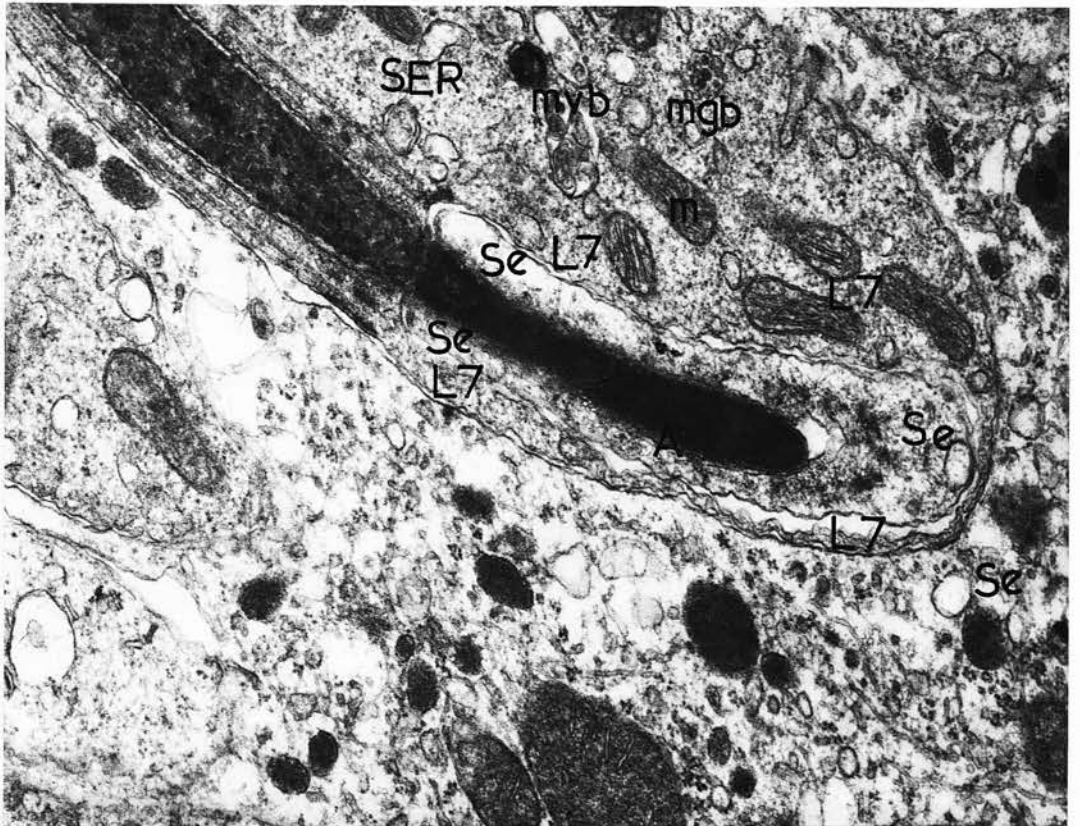
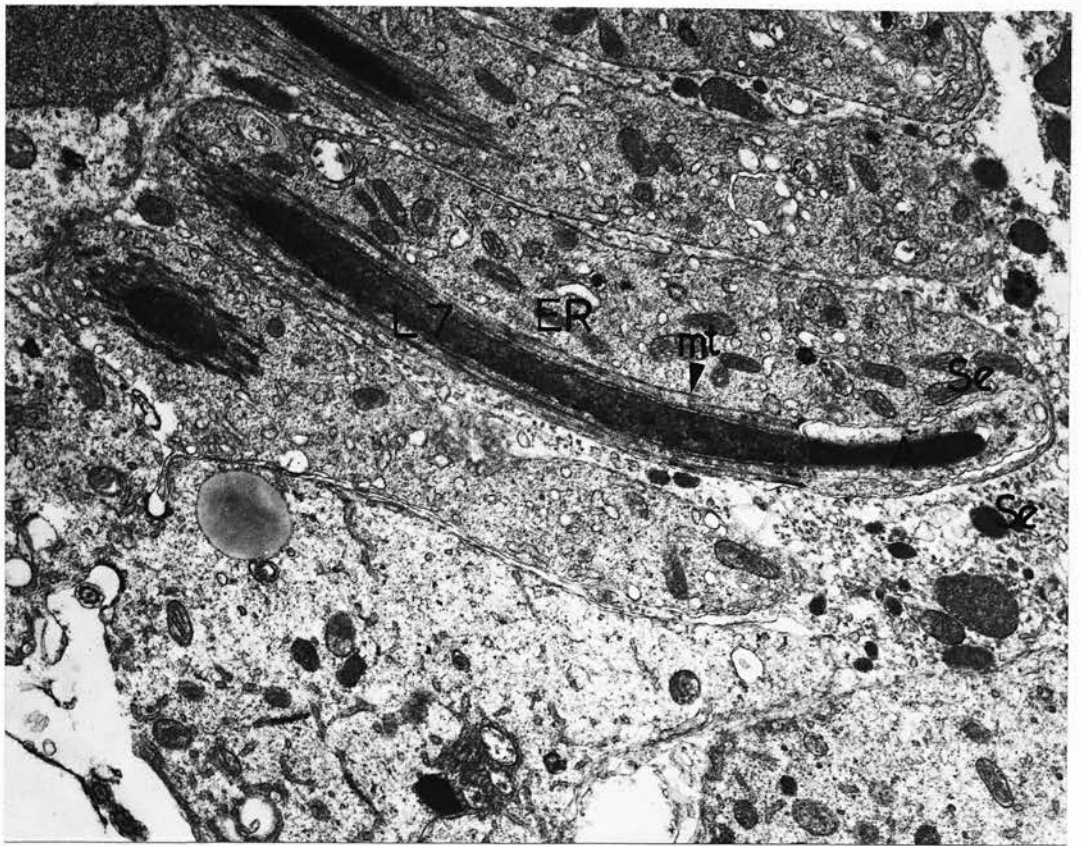


Figure 3.52

Shows an invagination of Sertoli cell cytoplasm around the acrosome and wings of L<sub>7</sub> spermatid cytoplasm on either side. Sertoli cell cytoplasm is characterised by abundant tubular smooth ER. Short wide cisternae or vesicles of smooth ER are seen in the spermatid cytoplasm.  
X 15,000

Figure 3.53

Caudal cytoplasm of an L<sub>7</sub> spermatid showing mitochondria with longitudinal cristae lying around the tail complex.  
X 25,000

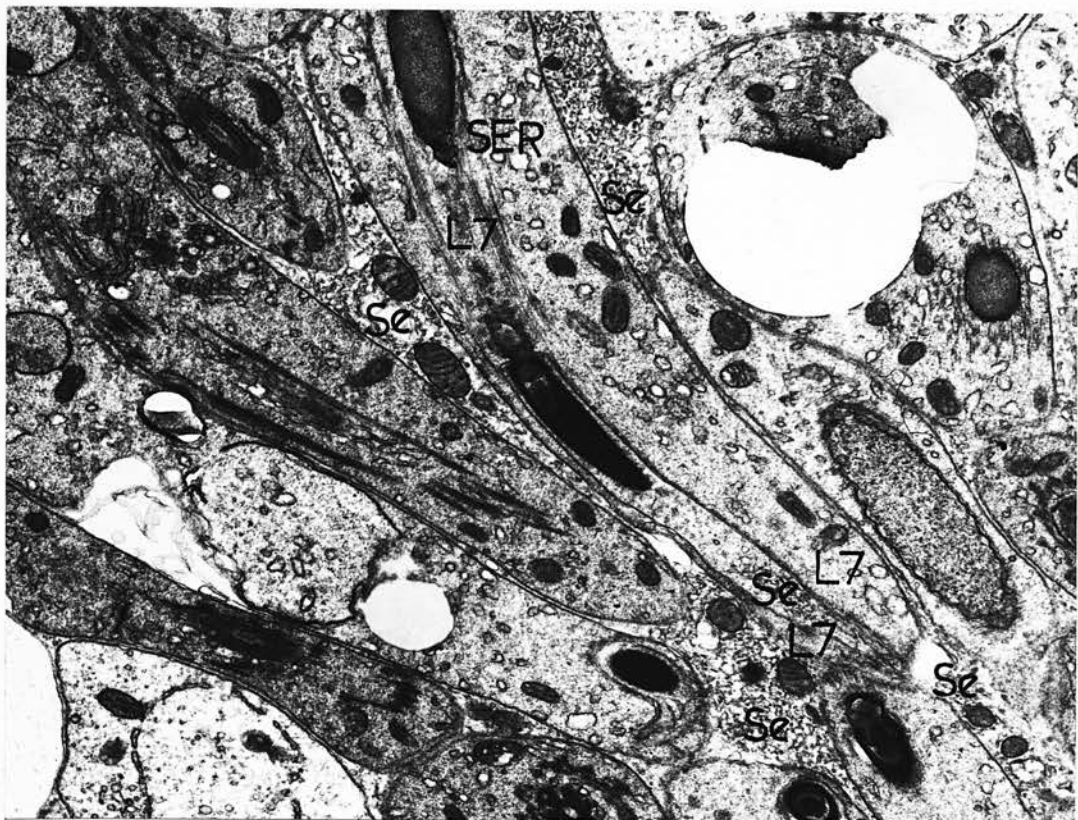


Figure 3.54

Cross-section through the caudal cytoplasm of L<sub>7</sub> spermatids. Mitochondria are seen surrounding the distal centriole. Microtubules of the manchette are cut in cross-section. Vesicles of smooth ER lie subjacent to the plasmalemma.

X 25,000

Figure 3.55

Shows L<sub>8</sub> spermatids with a completely electron dense nucleus. The cytoplasm is filled with vesicles of smooth ER (many of which are invaginated to form C-shaped vesicles). The remains of the manchette lies apart from the nucleus.

X11,000

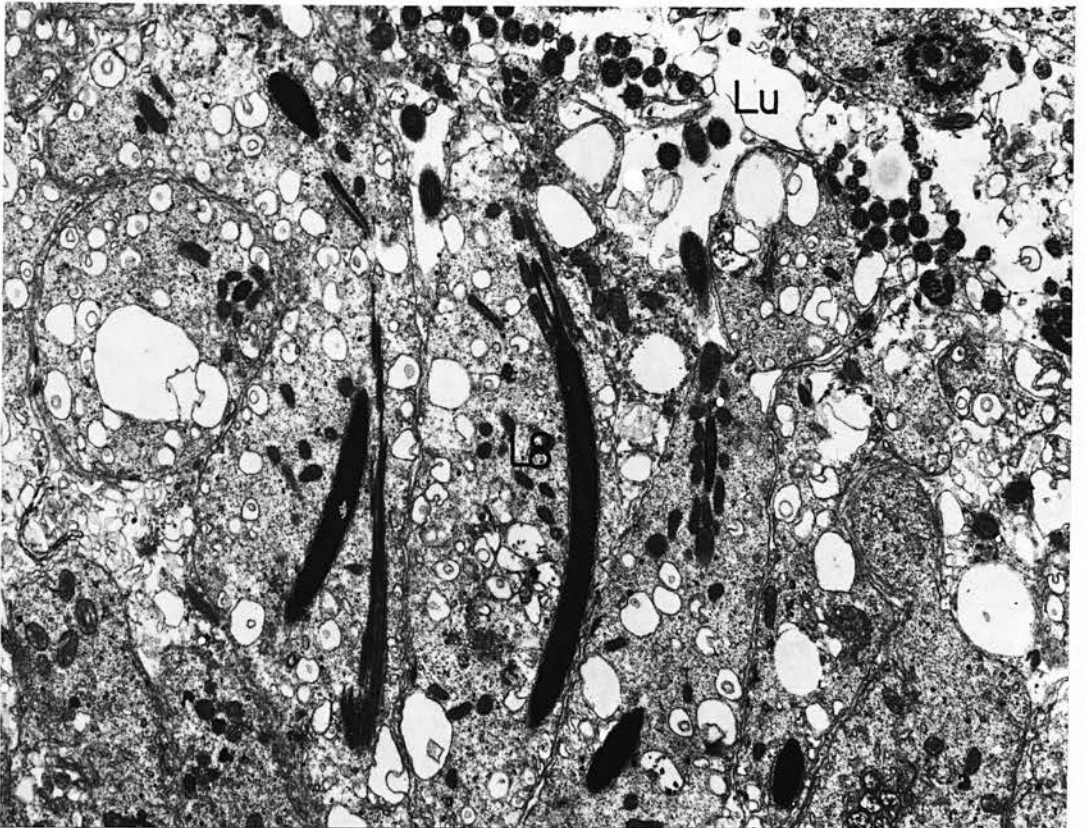
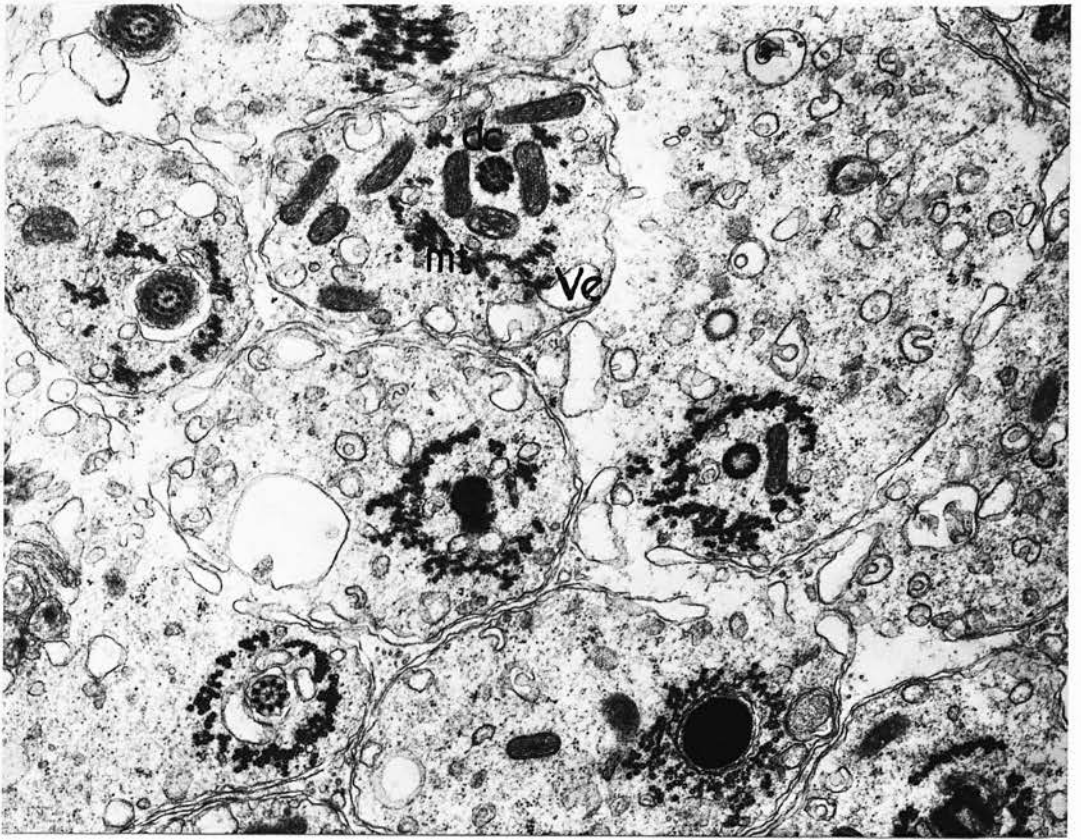


Figure 3.56

L<sub>8</sub> spermatids are seen lying at the luminal surface.

The cytoplasm contains many smooth ER vesicles.

X 6,000

Figure 3.57

A single membrane bound vesicle is seen opening into the spermatid-Sertoli intercellular cleft. There is no invagination of Sertoli cytoplasm.

X 52,000

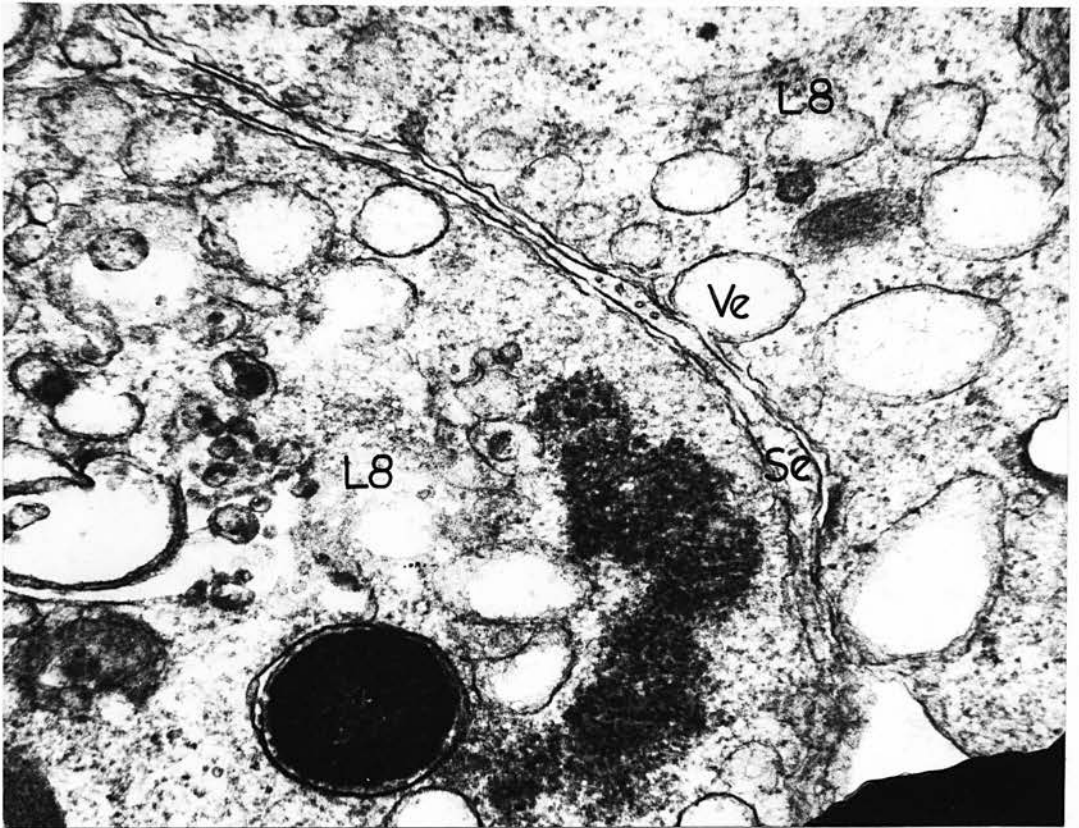
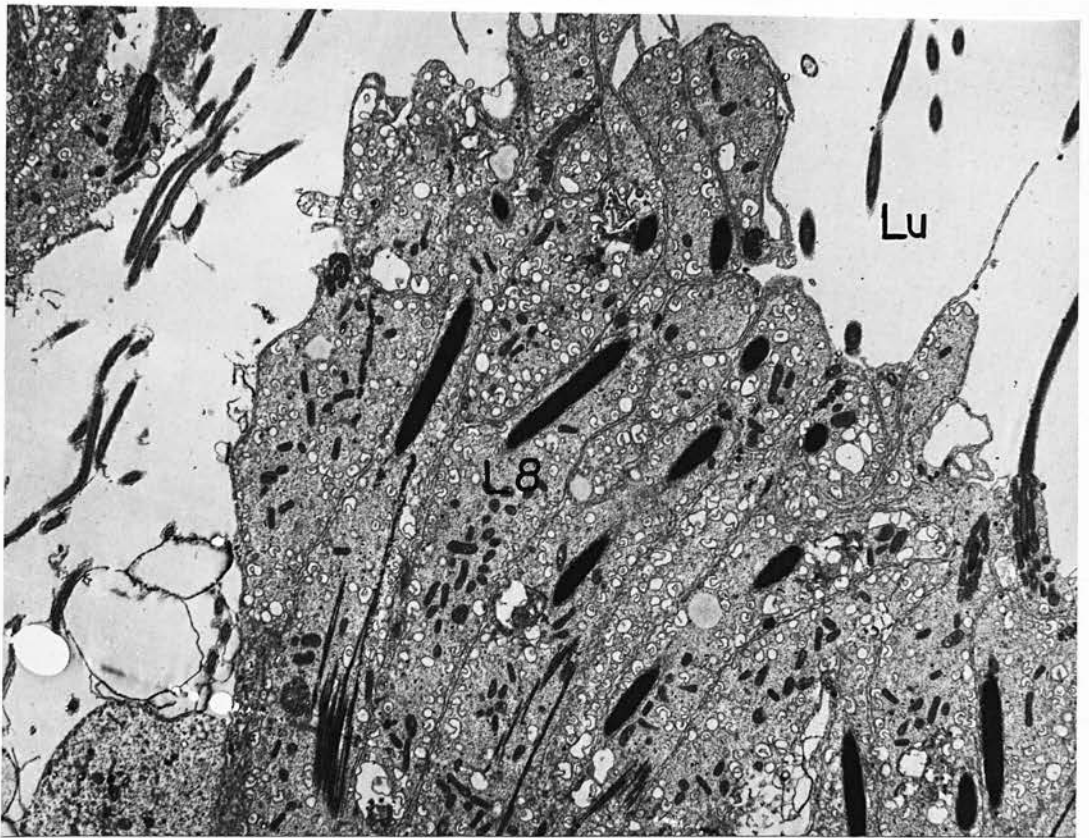




Figure 3.58(a)

Vesicles of smooth ER lie subjacent to the spermatid plasmalemma. Note vesicle (Ve) which appears closed.

X 24,000

Figure 3.58(b)

The same area as in Figure 3.58(a) but with the section tilted through  $40^{\circ}$ . The vesicle (Ve) is now seen to be open with the spermatid-Sertoli intercellular space.

X 24,000.

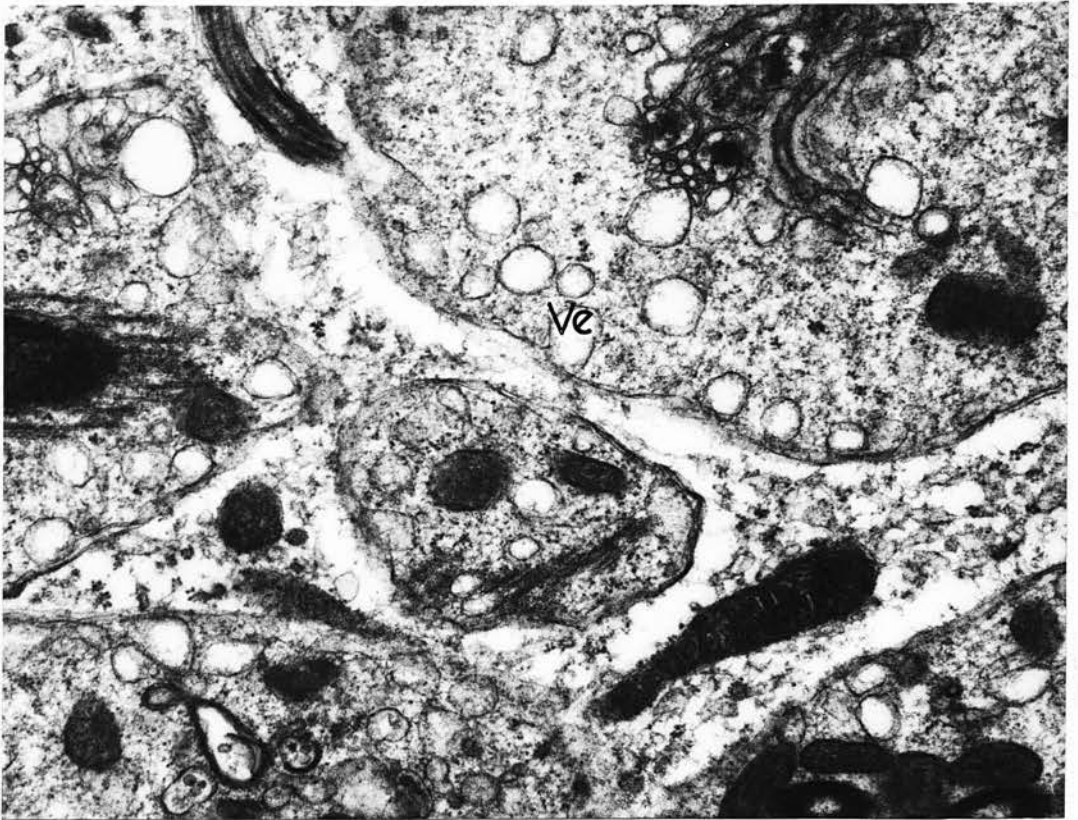
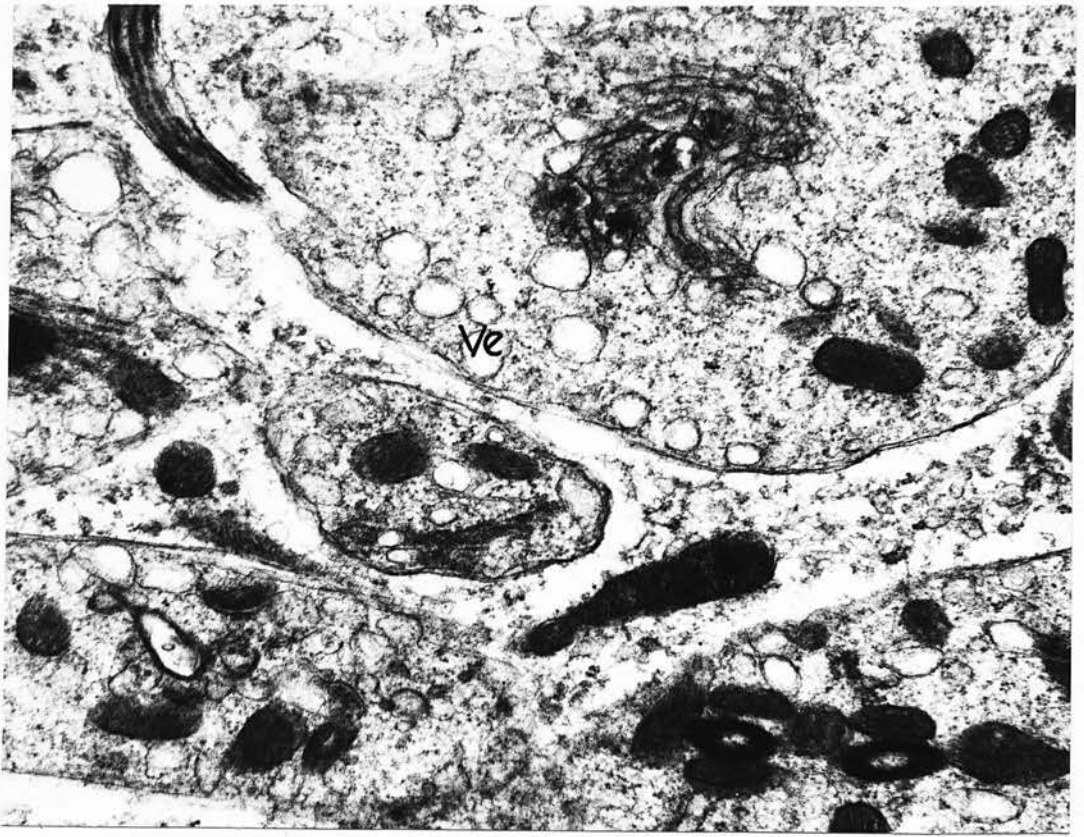


Figure 3.59

The vesicles invaginate to form C-shaped vesicles, *and* centrally located vesicles appear to coalesce around the nucleus to form a large vesicle.

X 40,000

Figure 3.60

An L<sub>8</sub> spermatid is seen lying in the spermatid cytoplasm with part of the nucleus surrounded by large vesicles.

X 10,000

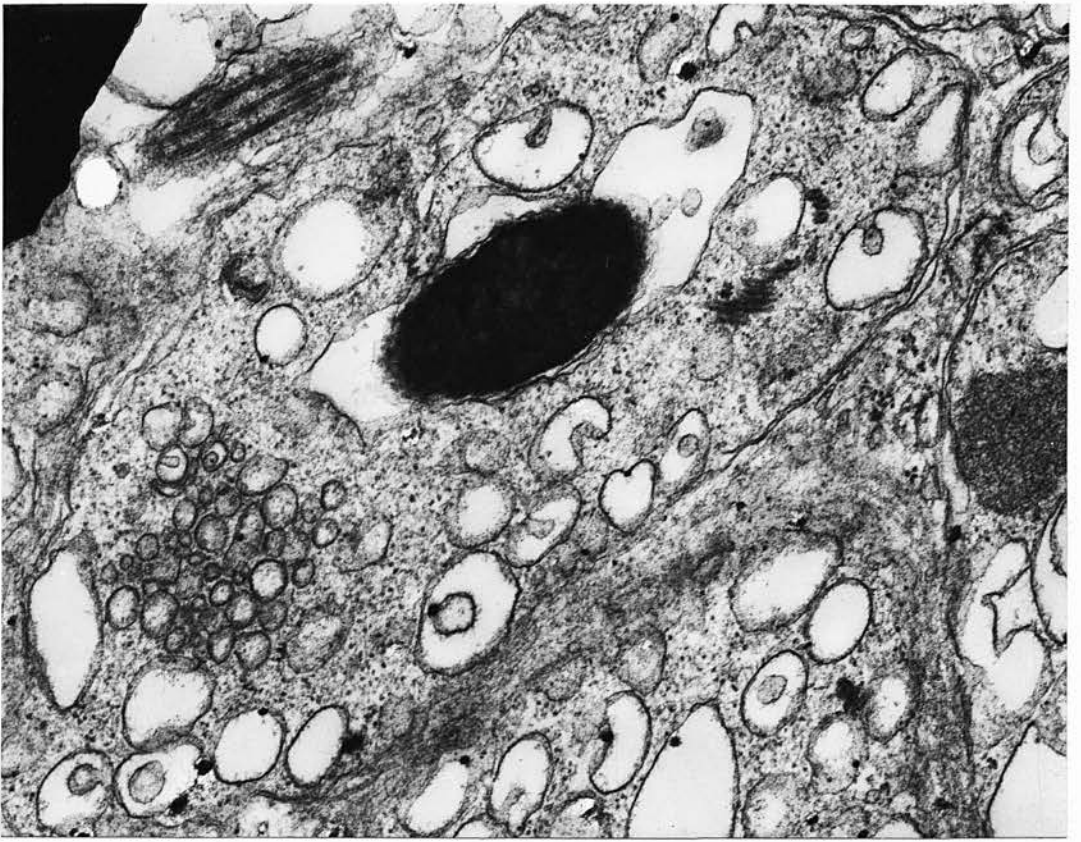


Figure 3.61

A mature spermatid is seen lying in the lumen immediately prior to release. The only remaining point of attachment is between the acrosome and Sertoli cell cytoplasm.

X 25,000

Figure 3.62

Mature spermatids are seen lying free in the lumen and strands of Sertoli cytoplasm lie on the luminal surface and surround the masses of residual vesiculated cytoplasm.

X 7,700

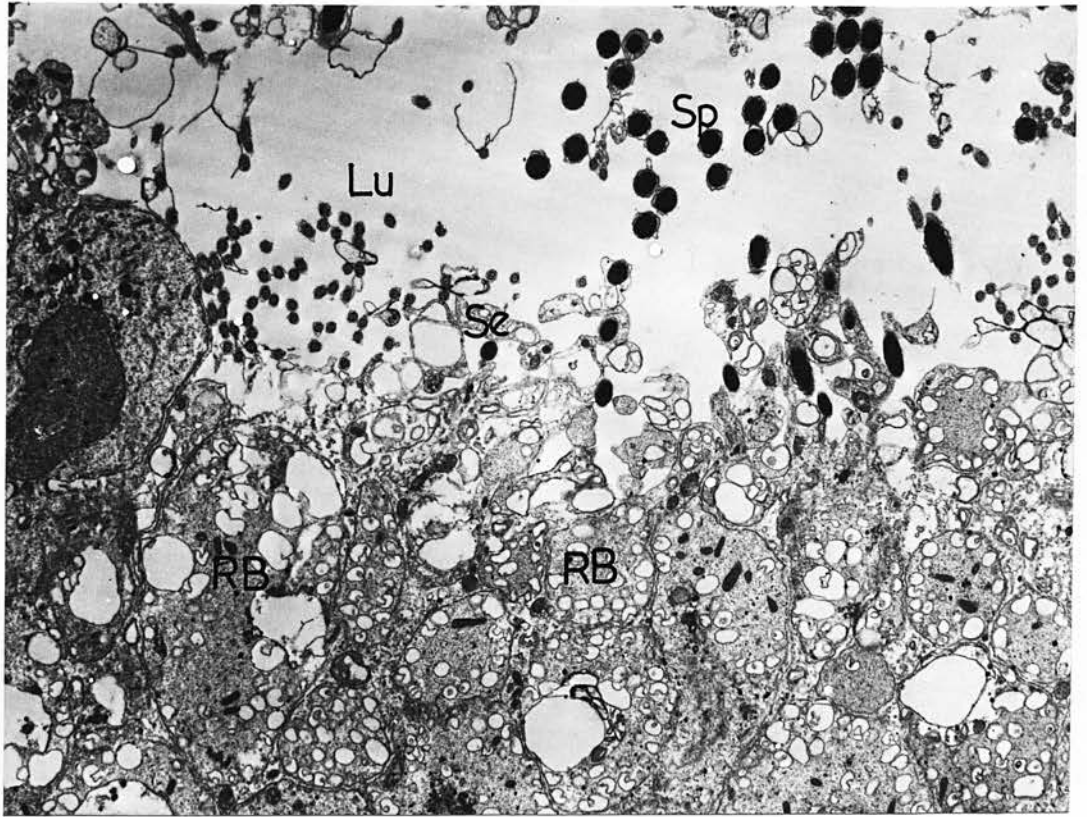
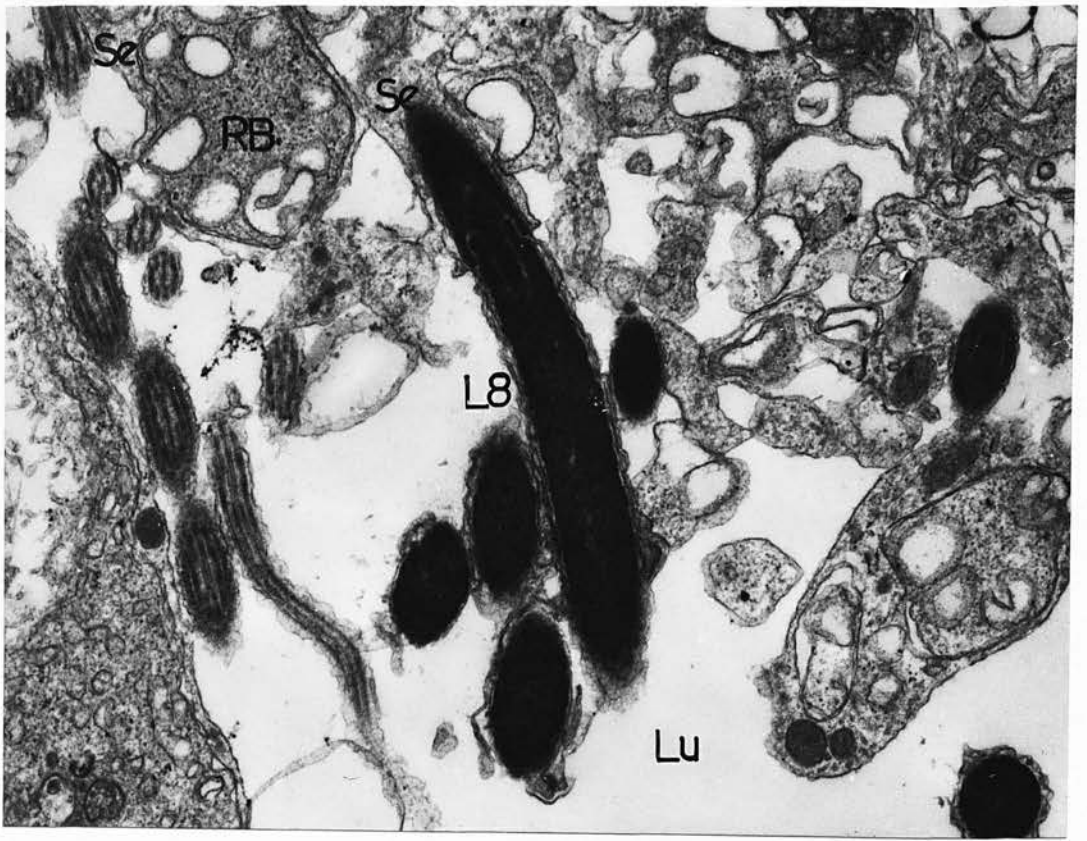


Figure 3.63

Shows the release of the contents of multivesicular  
and multigranular bodies <sup>to</sup> in the residual cytoplasm  
(arrow) ^

X 32,000

Figure 3.64

Shows the formation of double membraned vesicles (arrow)  
enclosing spare organelles in the residual cytoplasm

X 25,000

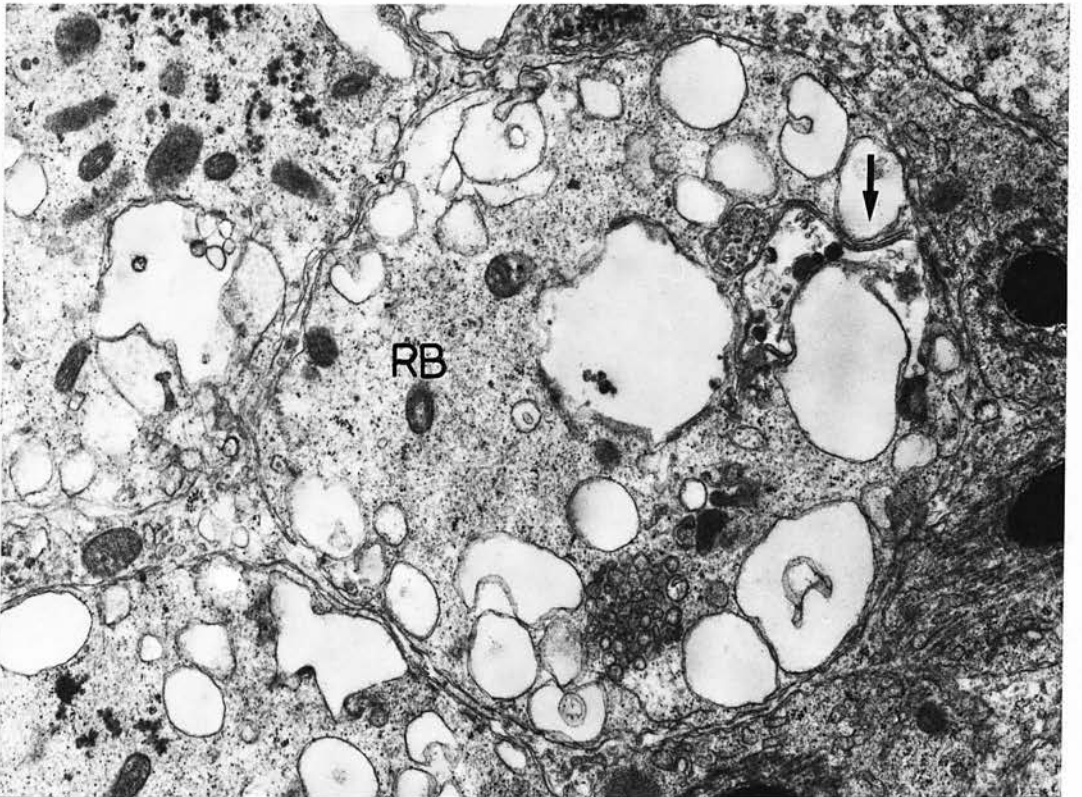
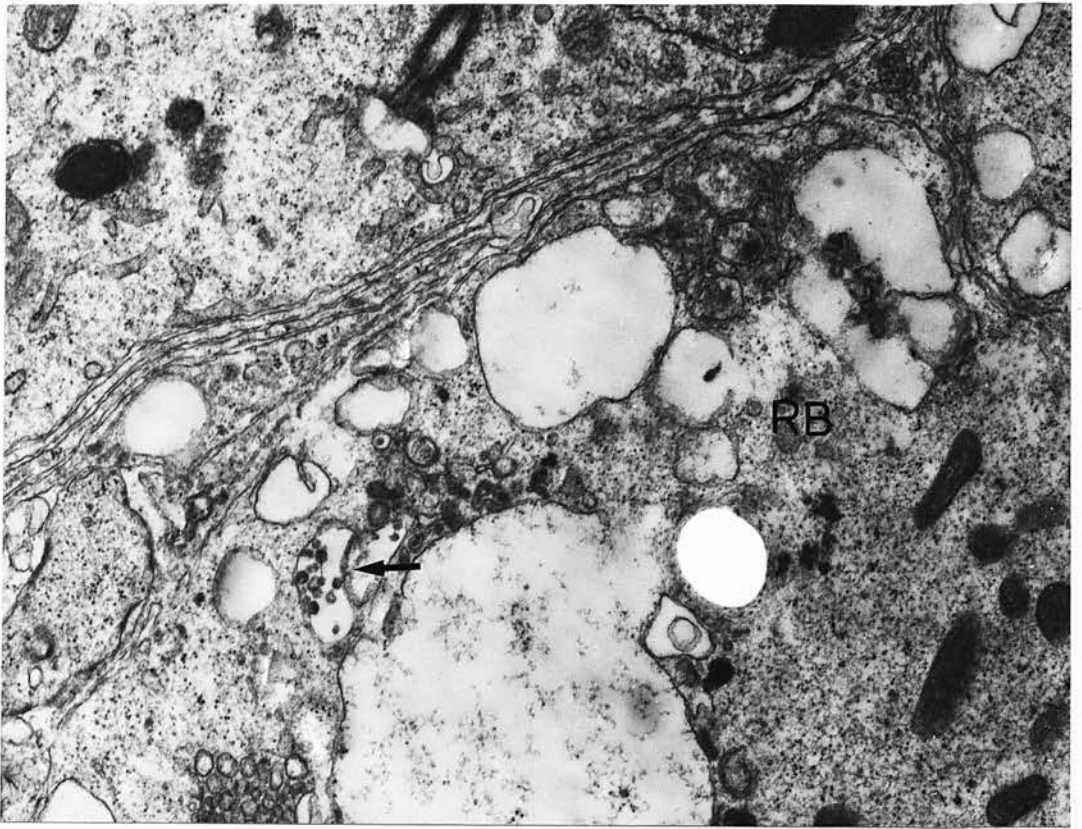




Figure 3.65

The residual cytoplasm and organelles condense to form residual bodies at the luminal surface surrounded by Sertoli cytoplasm.

X 9,100

Figure 3.66

The dark condensed mass of the residual body comes to lie in the Sertoli cytoplasm in the mid portion of the seminiferous epithelium at the level of the round spermatids. Note abundant smooth ER and lipid droplets in the Sertoli cytoplasm.

X 82,000

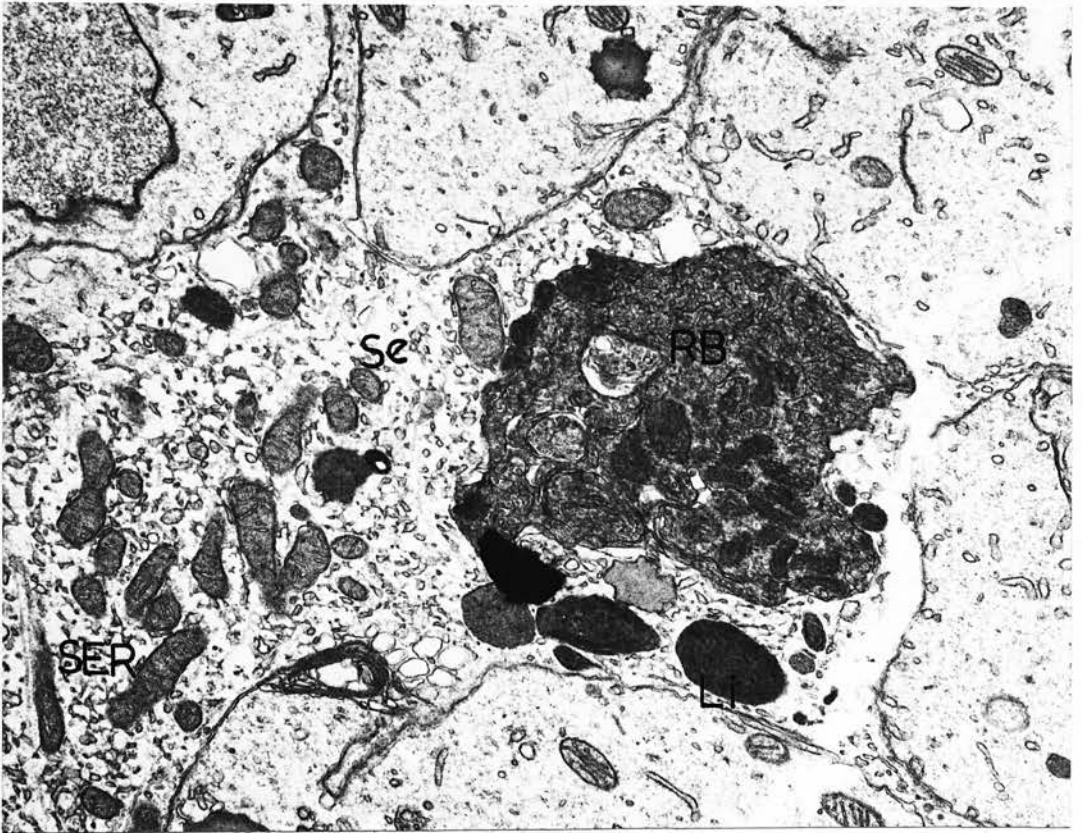
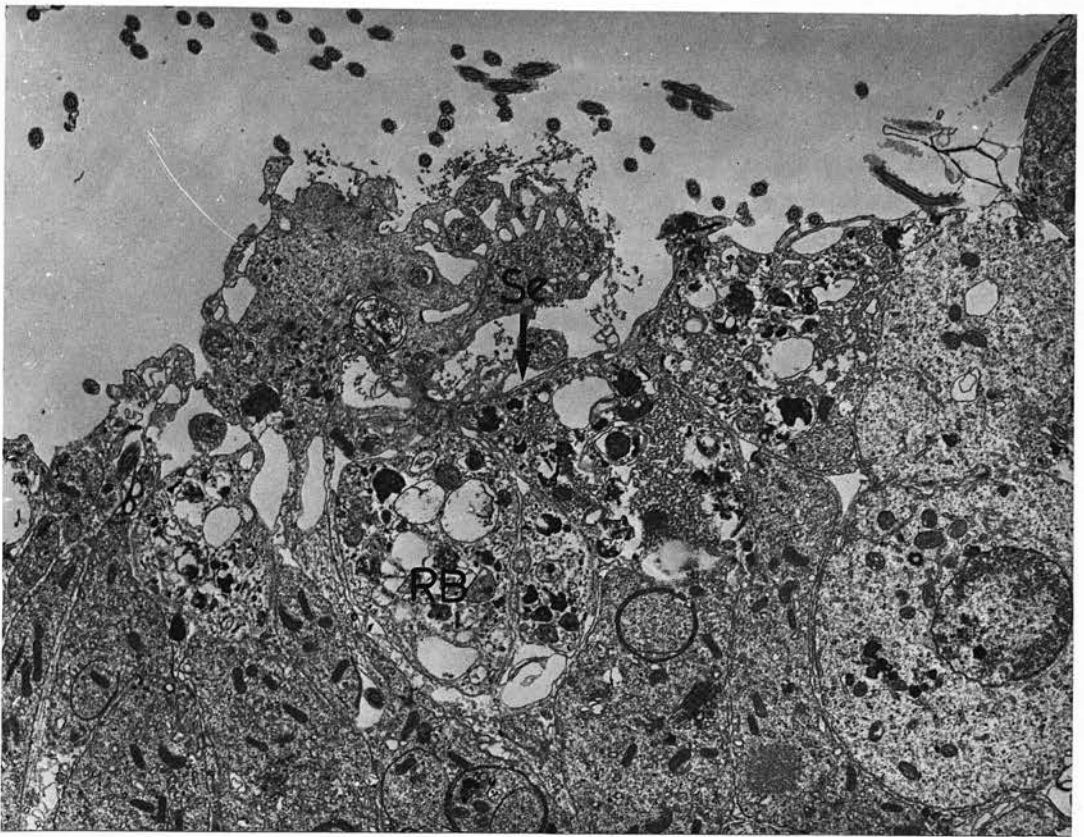


Figure 3.67

20 week old bird. Intertubular region showing single Leydig cell separated from blood capillary by loose connective tissue with large interstitial spaces.

X 2,160

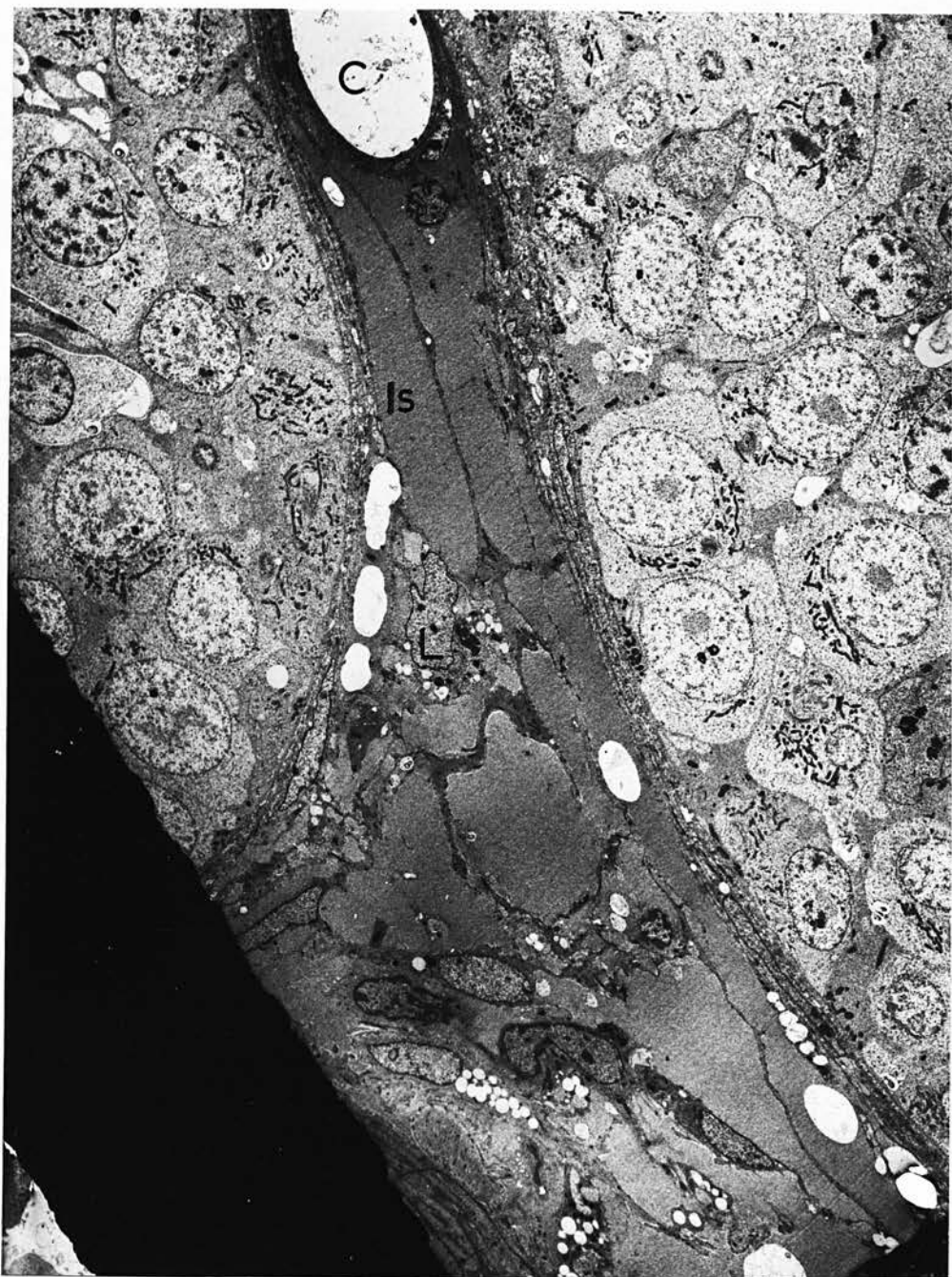


Figure 3.68

20 week old bird. Intertubular tissue showing a large lymphatic vessel lined by endothelial cells that are supported by bundles of collagen fibres. Note the paler appearance of the fine grained precipitate in the lymphatic vessel compared to the precipitate of the interstitial spaces.

X 5,100

Figure 3.69

20 week old bird. Intertubular tissue showing a row of Leydig cells along the boundary tissue. Note the shape of the lymphatic vessel in relation to the angle of the intertubular region.

X 3,800

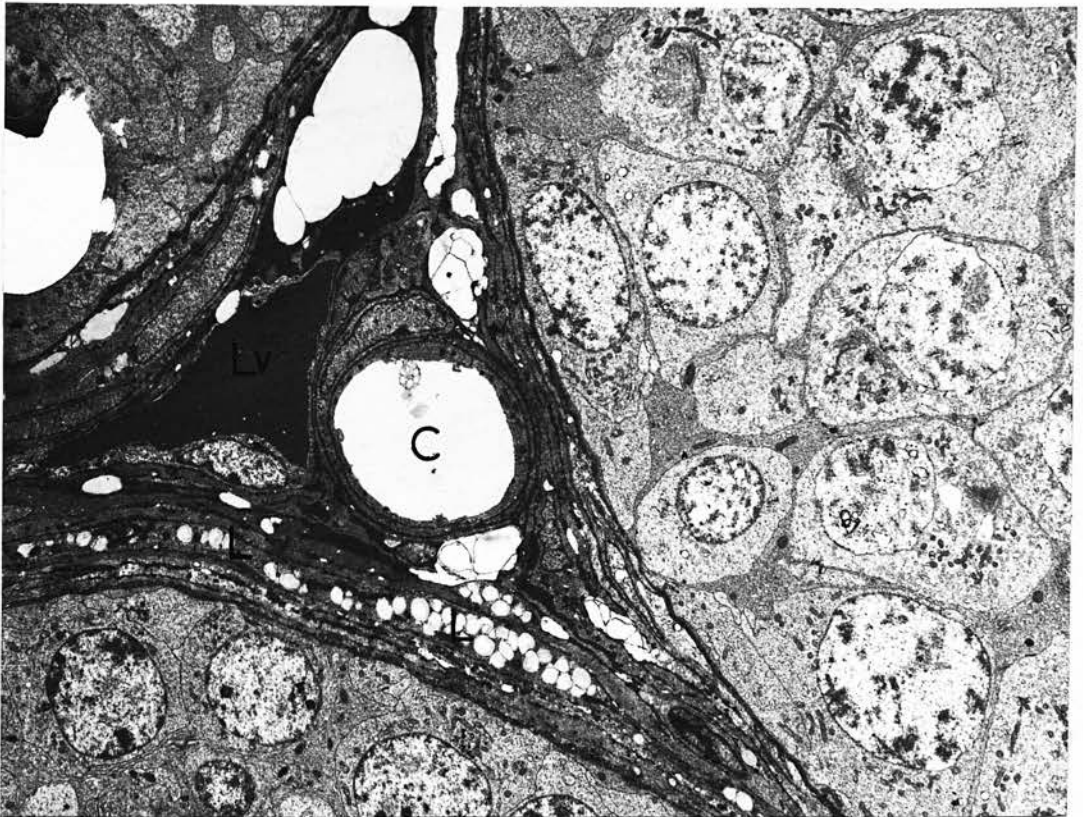
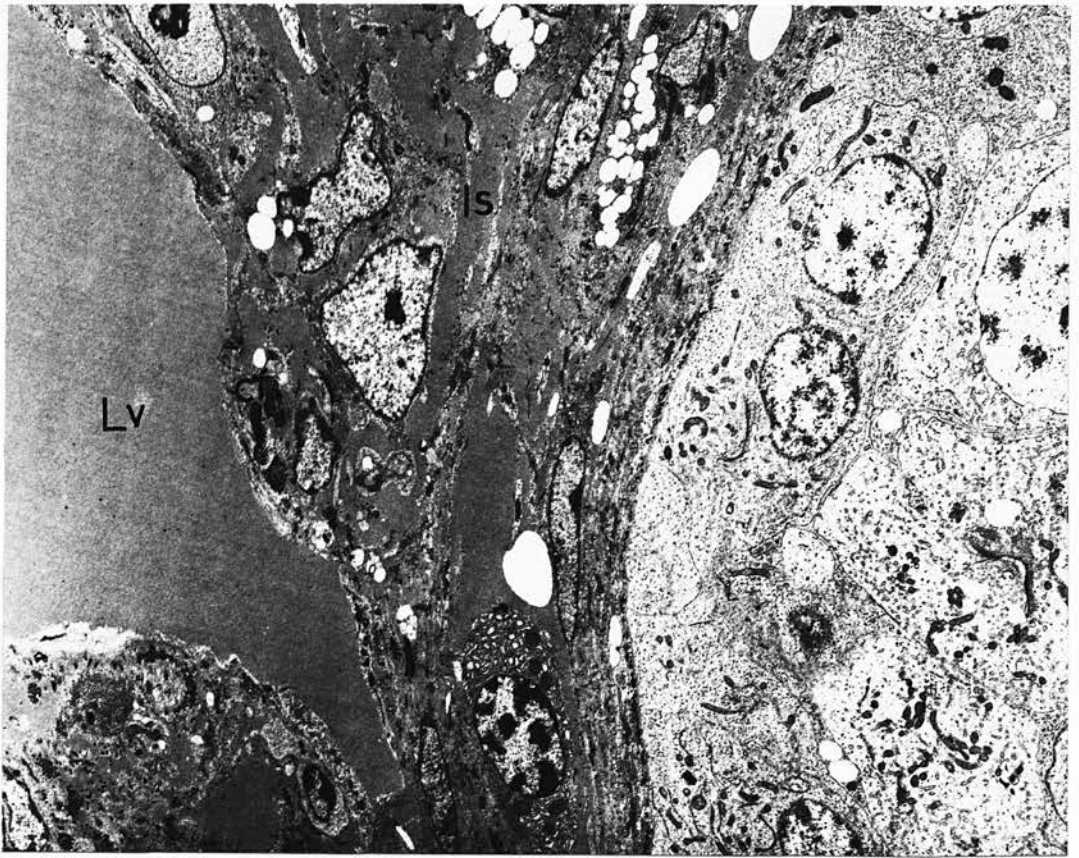


Figure 3.70

20 week old bird. Groups of Leydig cells are seen lying in a loose connective tissue with large interstitial spaces filled with a fine grained precipitate.

X 9,500

Figure 3.71

20 week old bird. Intertubular region containing a blood capillary with closely associated Leydig cells and a lymphatic vessel of irregular shape that corresponds to the angle of the intertubular region.

X 5,100

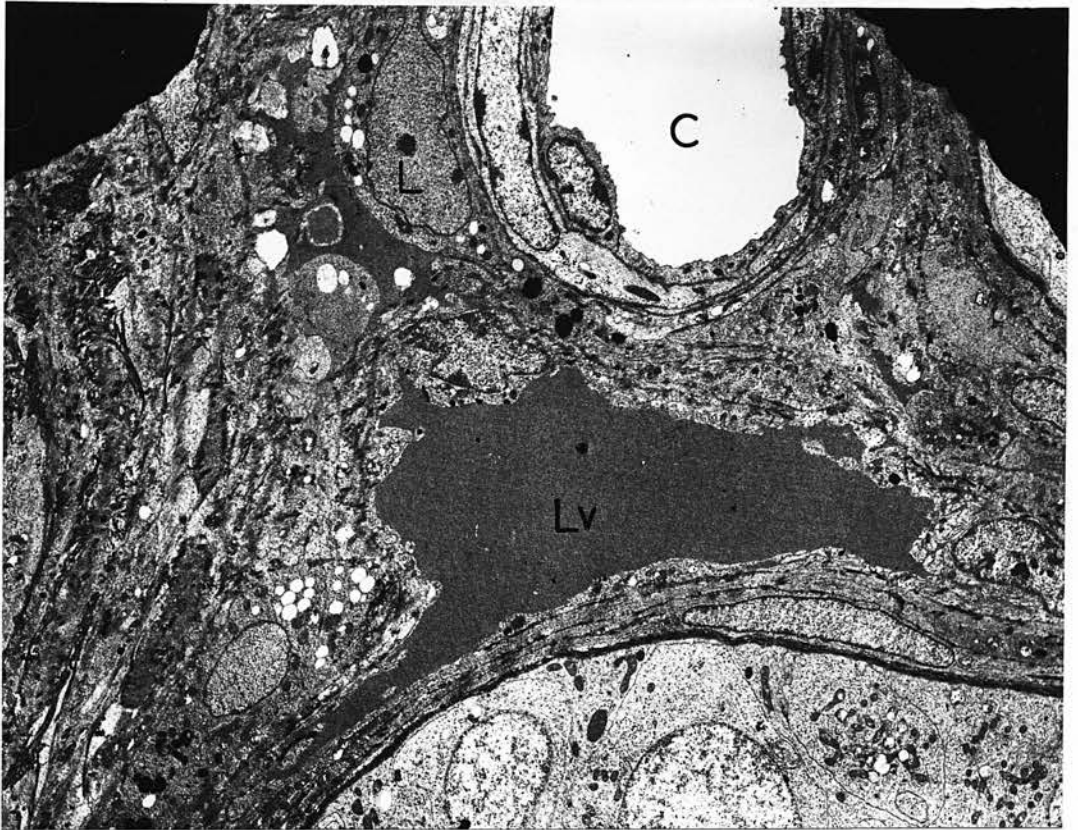
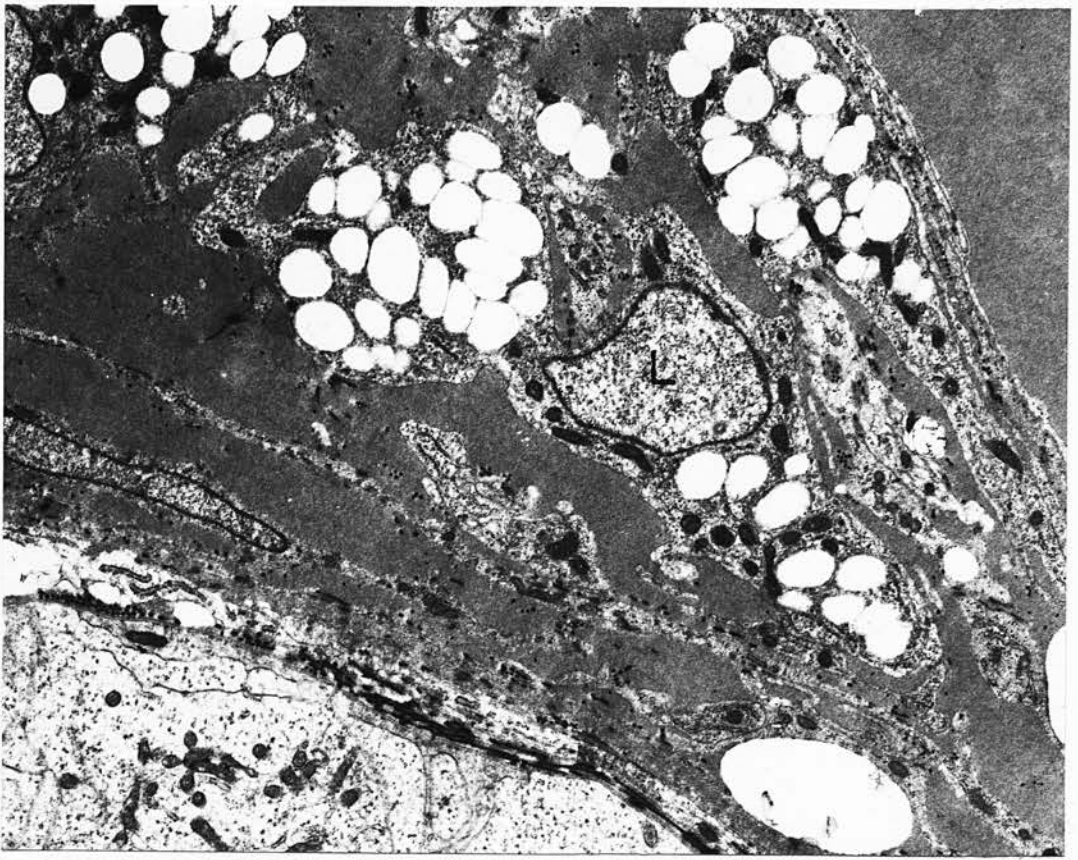




Figure 3.72

3 week old bird. The intertubular tissue consists almost entirely of polygonal Leydig cells. Small central blood capillaries and small interstitial spaces are seen.

X 2,800

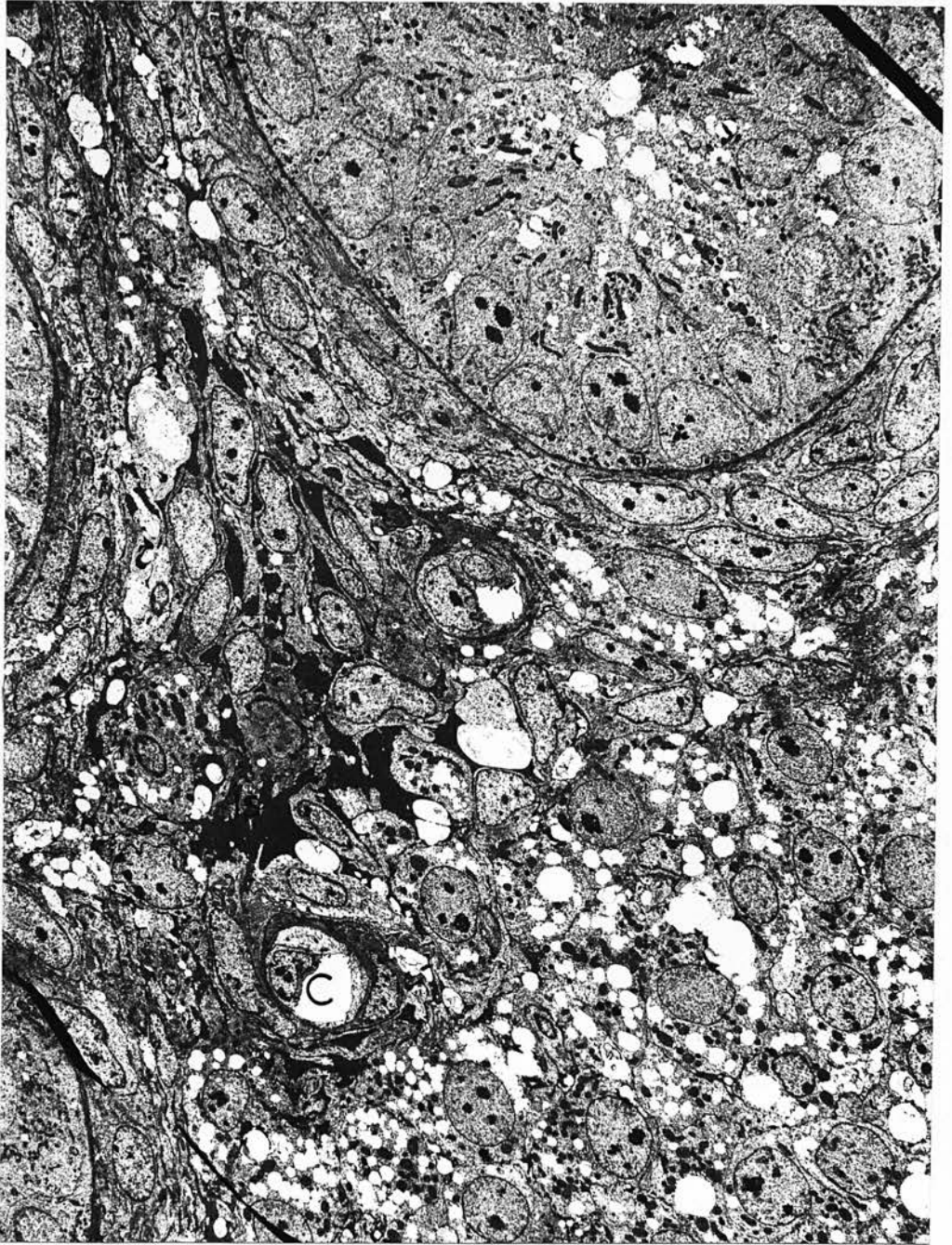


Figure 3.73

6 week old bird. The intertubular tissue consists largely of polygonal Leydig cells with a few central blood capillaries. Small lymphatic vessels are seen.  
X 1,900

Figure 3.74

9 week old bird. The intertubular tissue consists of Leydig cells and blood capillaries in association with the boundary tissue. Large lymphatic vessels are seen in the angle of the tubule.  
X 2,800

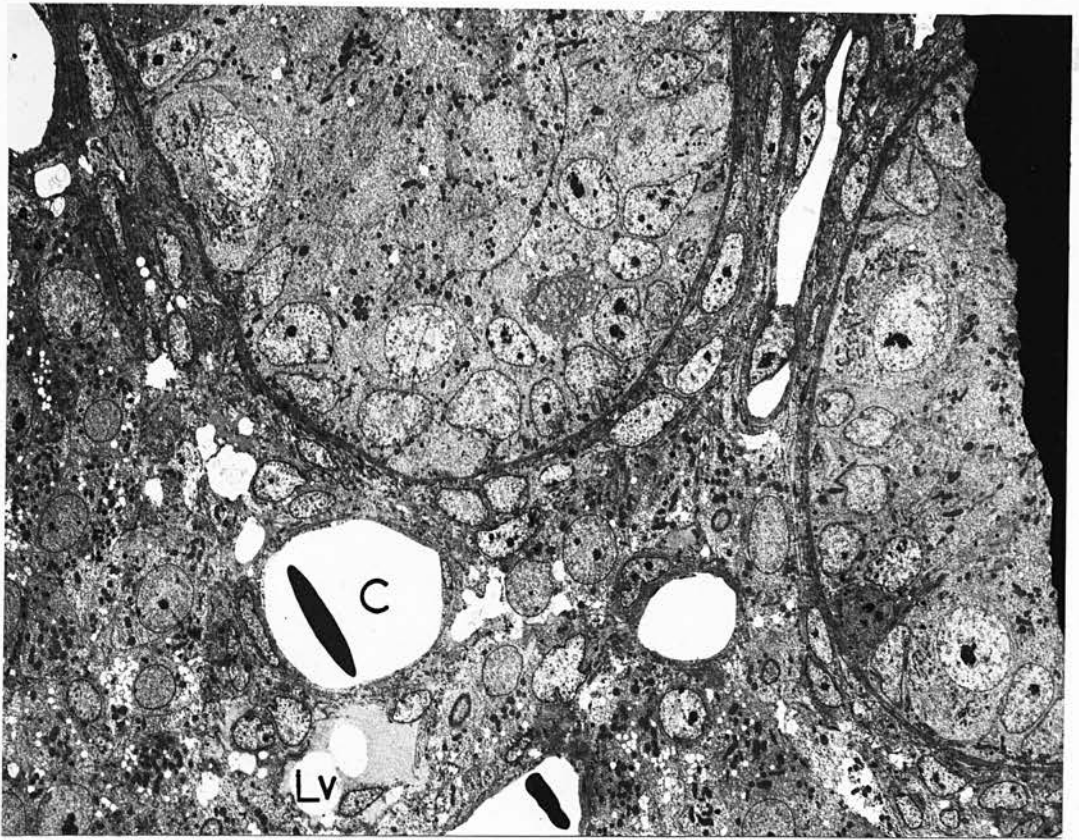


Figure 3.75

9 week old bird. The Leydig cells are polygonal and closely packed. Note the increase in interstitial spaces.

X 3,400

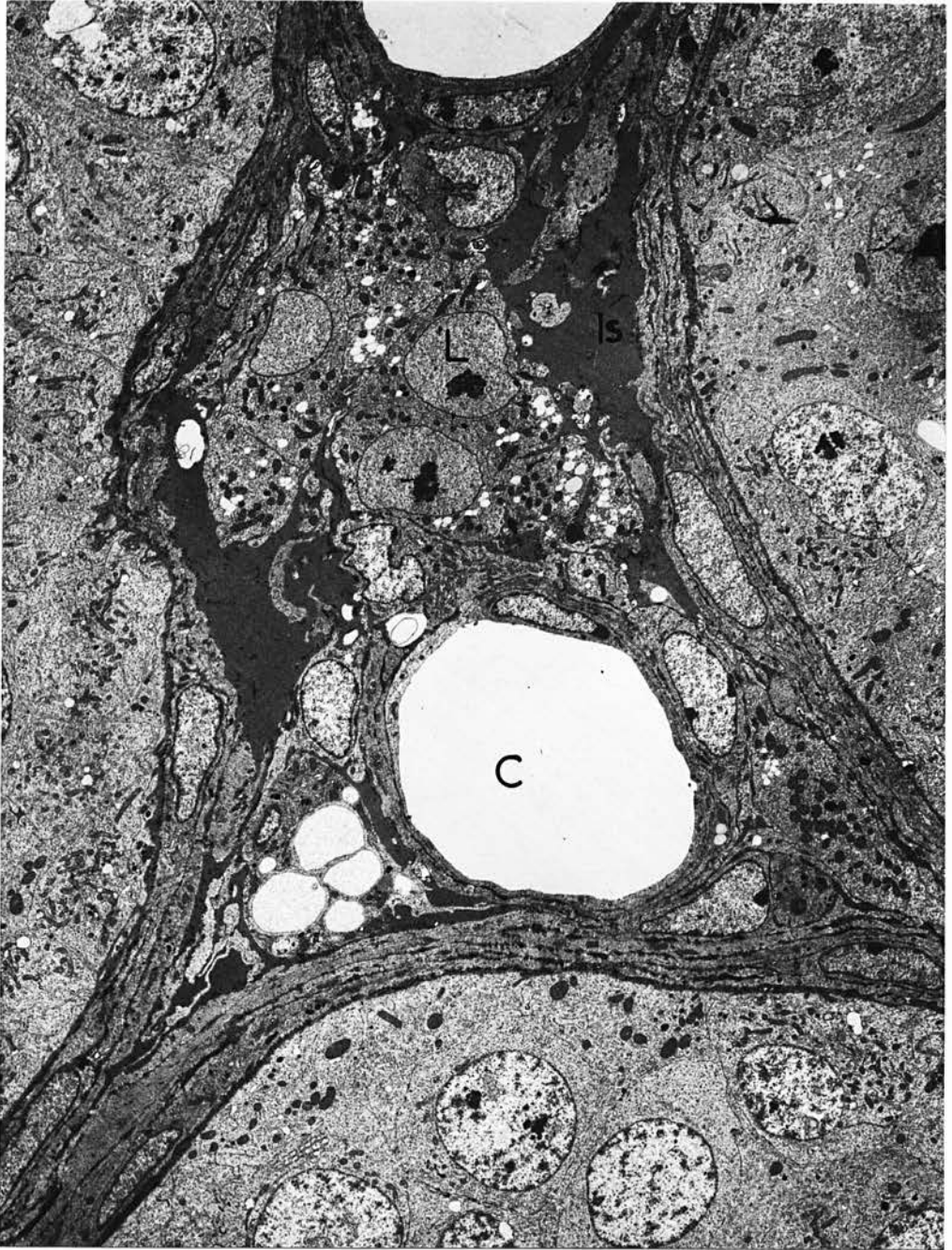


Figure 3.76

12 week old bird. Intertubular tissue contains much interstitial space and the Leydig cells are no longer closely packed.

X 3,800

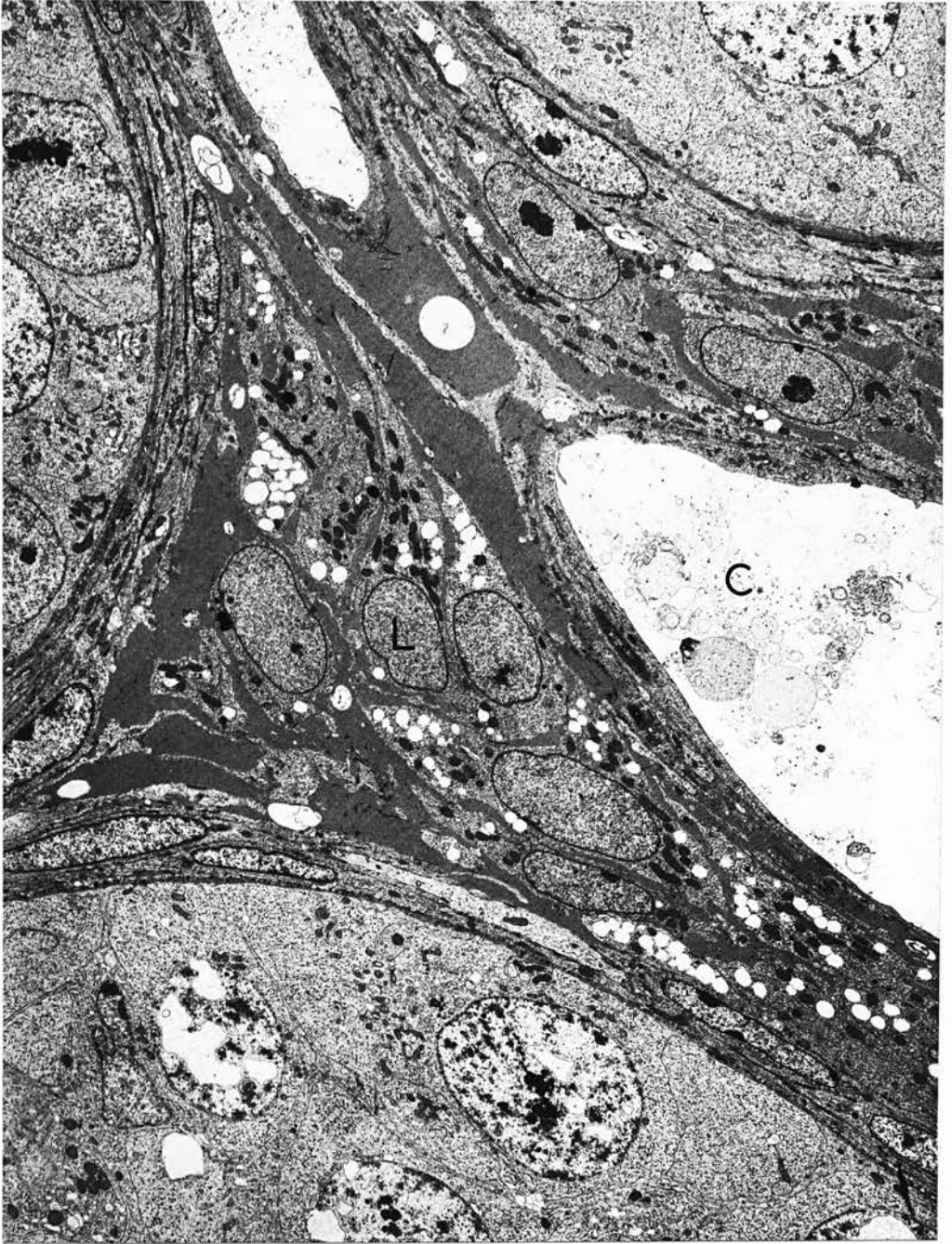




Figure 3.77

12 week old bird. Regions of intertubular tissue that consist almost entirely of polygonal closely packed Leydig cells can be seen in the pubertal bird.

X 2,200

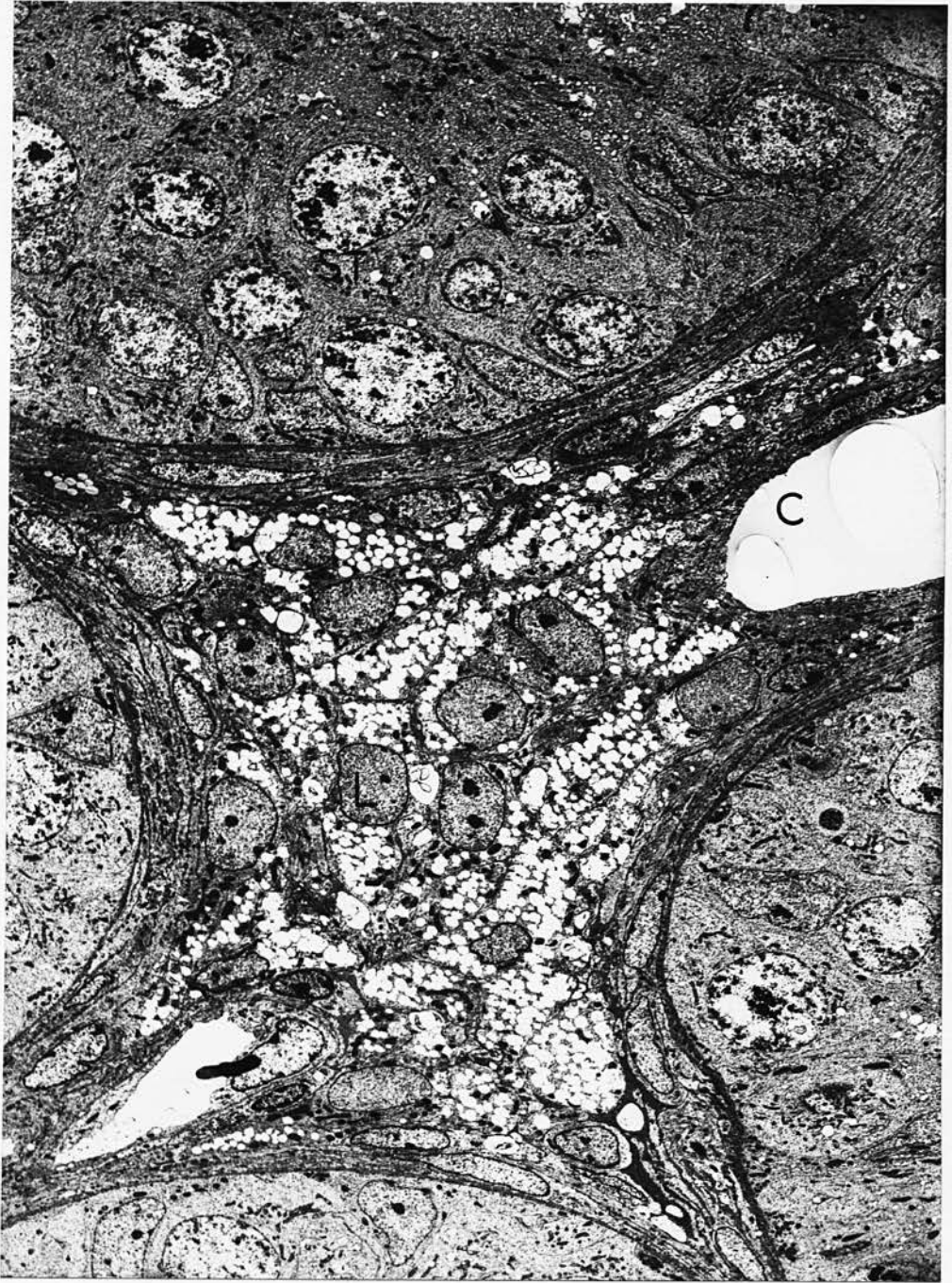


Figure 3.78

12 week old bird. Regions of intertubular tissue that consist largely of lymphatic vessel or interstitial space are found in the pubertal bird.

X 2,300

Figure 3.79

15 week old bird. The intertubular region shows the adult pattern of organisation. A blood capillary is seen in association with the boundary tissue and a lymphatic vessel fills the irregular space between the tubules. Leydig cells are clamped near the blood vessel.

X 5,100

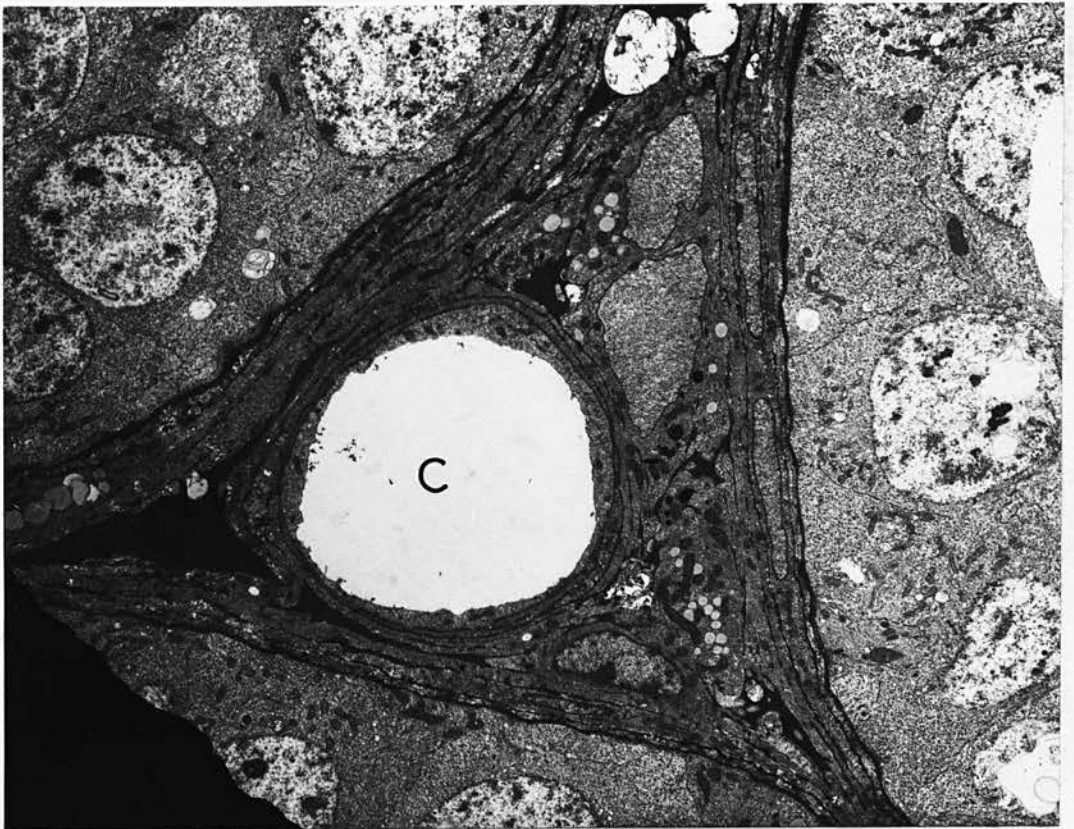
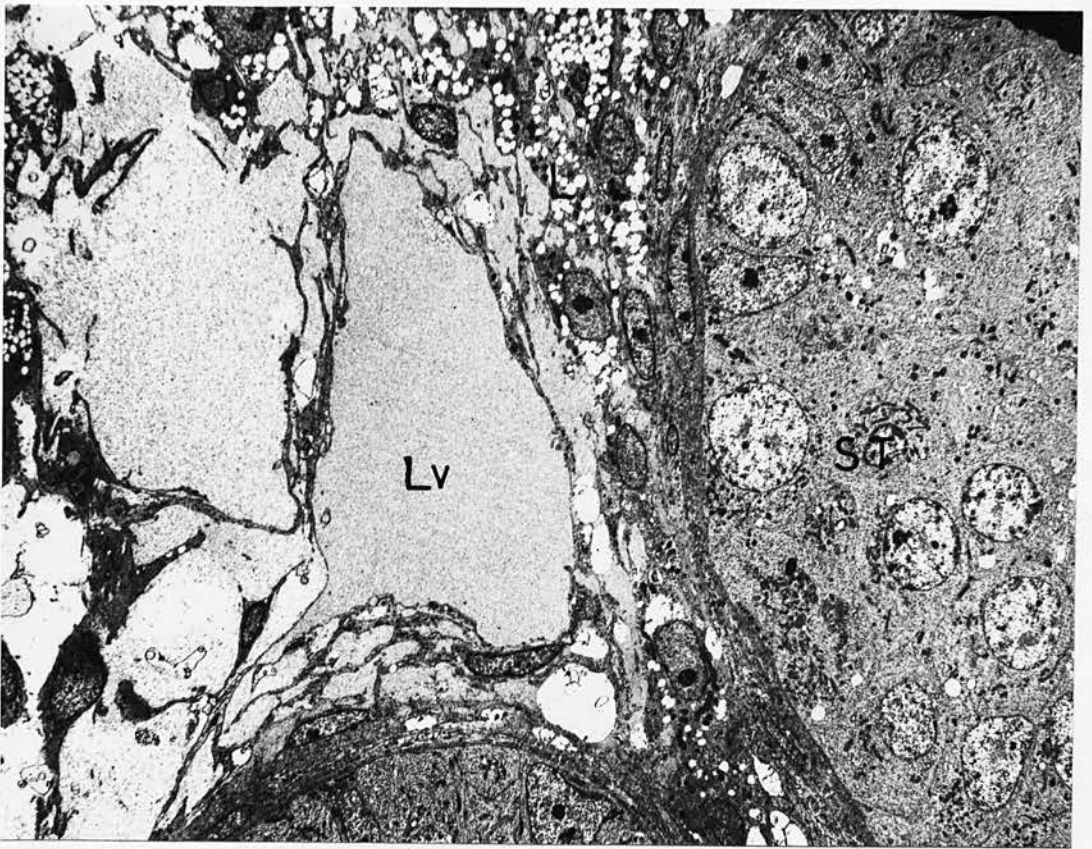


Figure 3.80

1 week old bird. Sertoli cells lie in contact with the basal lamina and their apical cytoplasm reaches the centre of the tubule. Junctional complexes are observed at the lateral apical membranes. Note interdigititation of membranes.

X 9,500

Figure 3.81

3 week old bird. Sertoli cells lie in contact with the basal lamina. Note subsurface cisternal junctions and cell membrane interdigititation.

X 12,500

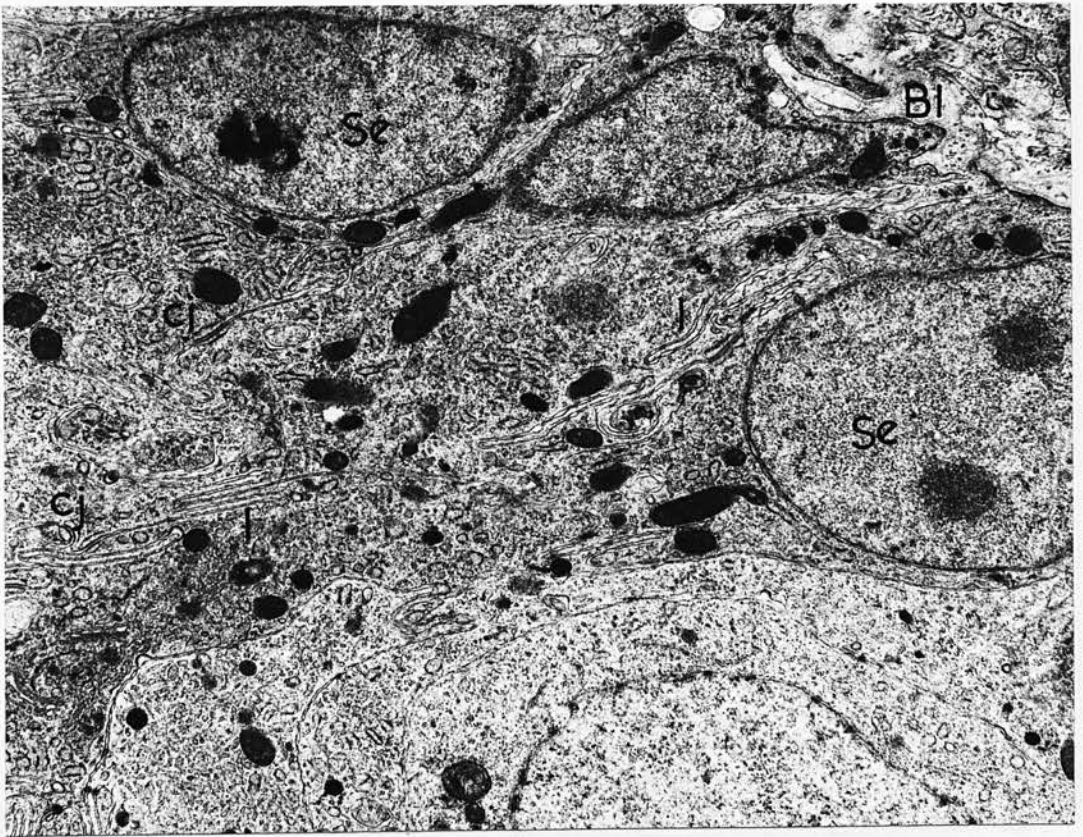
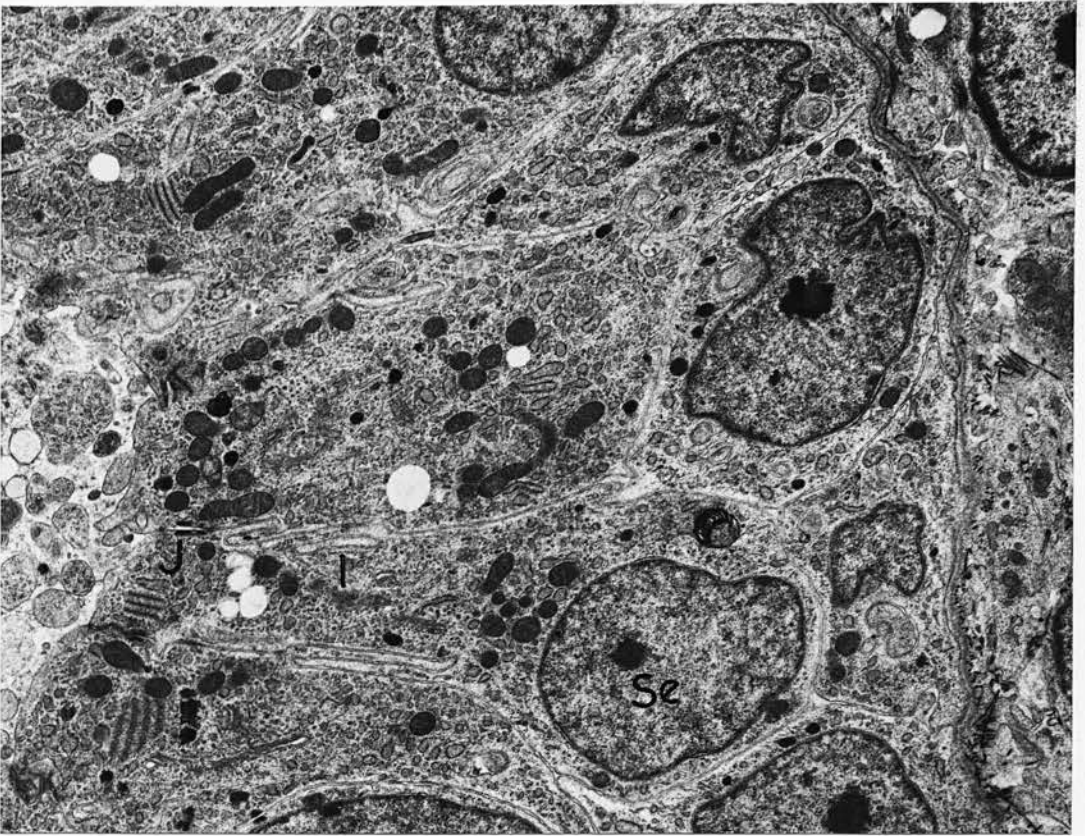


Figure 3.82

1 week old bird. High magnification of the junctional complex between Sertoli cells at the centre of the tubule. A series of alternating zonula occludens and zonula adherens are followed by a macula adherens. Note fibrils adjacent to zonula adherens junction.

X ~~1,5,800~~ 15,800

Figure 3.83

1 week old bird. Membranes within the zonula occludens fuse to give a pentalaminar appearance (arrows). The macula adherens has electron dense material in the subjacent cytoplasm and striations of moderately dense material within the junction.

X 175,500

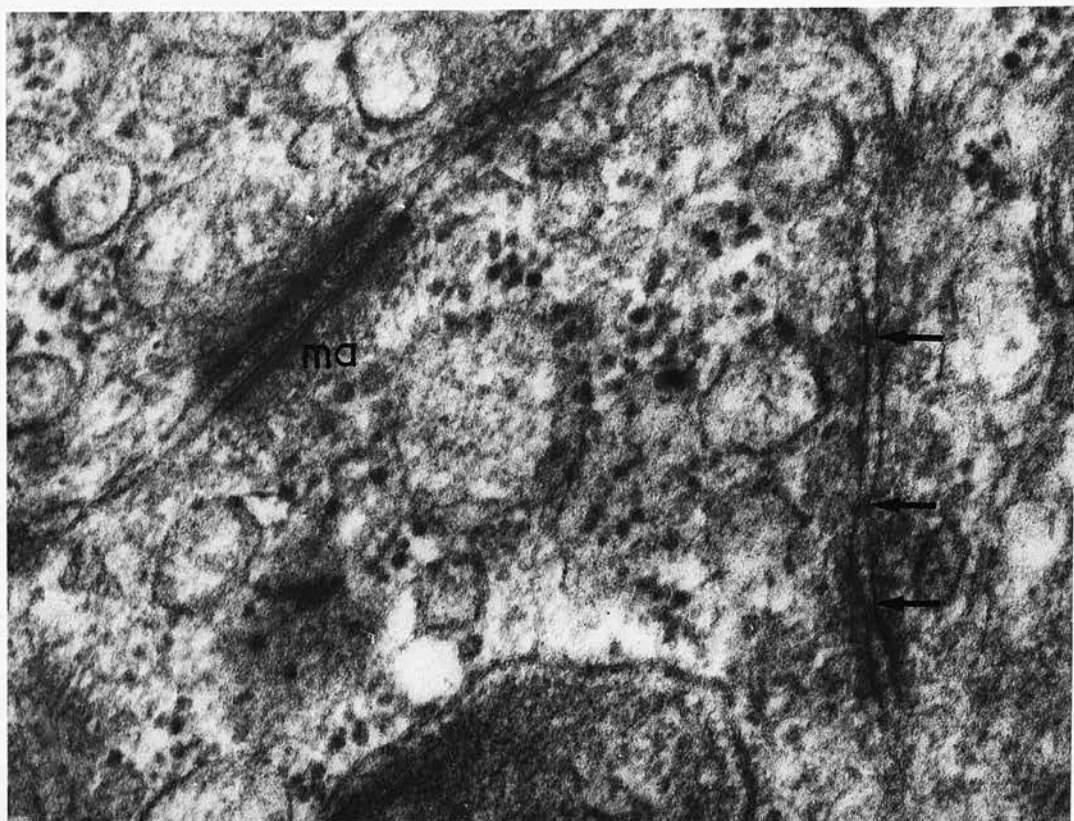
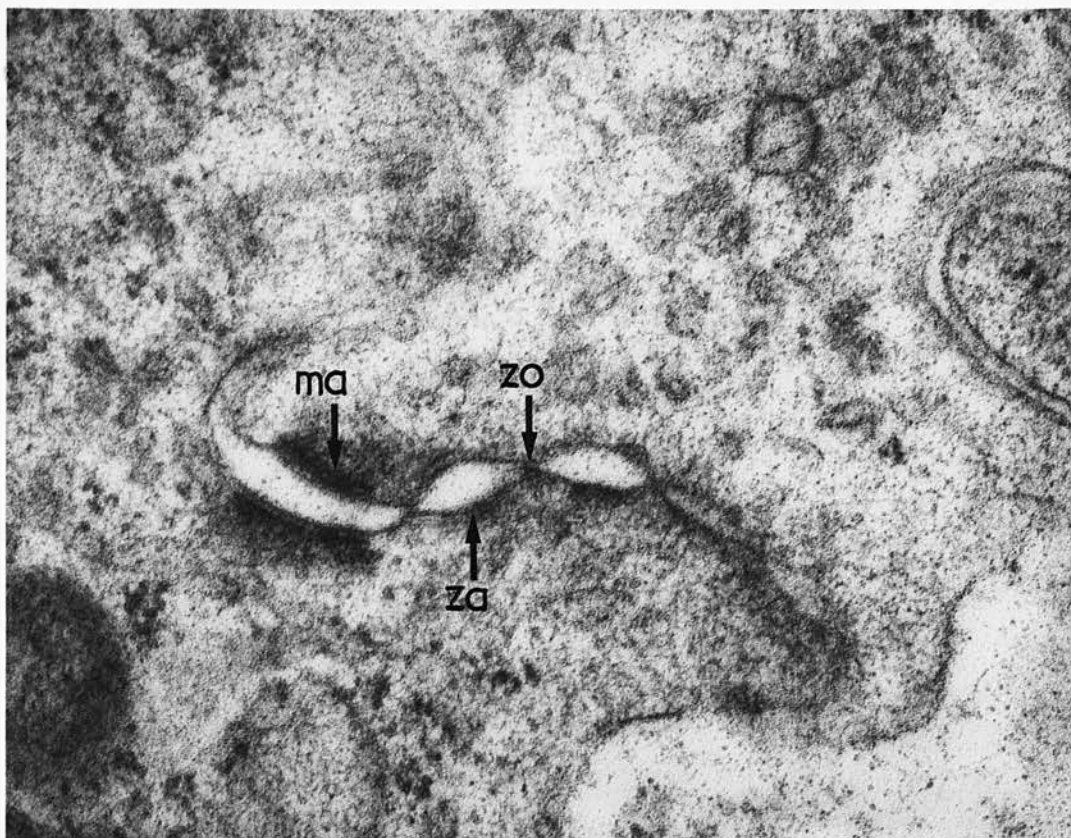




Figure 3.86

1 week old bird. Interdigitating Sertoli cell membranes. Note inner cell surface at the base of the undulations has electron dense accumulations of material.  
X 20,500

Figure 3.87

6 week old bird. Subsurface cisternal junction. Note the ribosomes on the inner cell surface of the hemispherical cisternae. There is no narrowing of the intercellular space throughout the junction.  
X 43,200

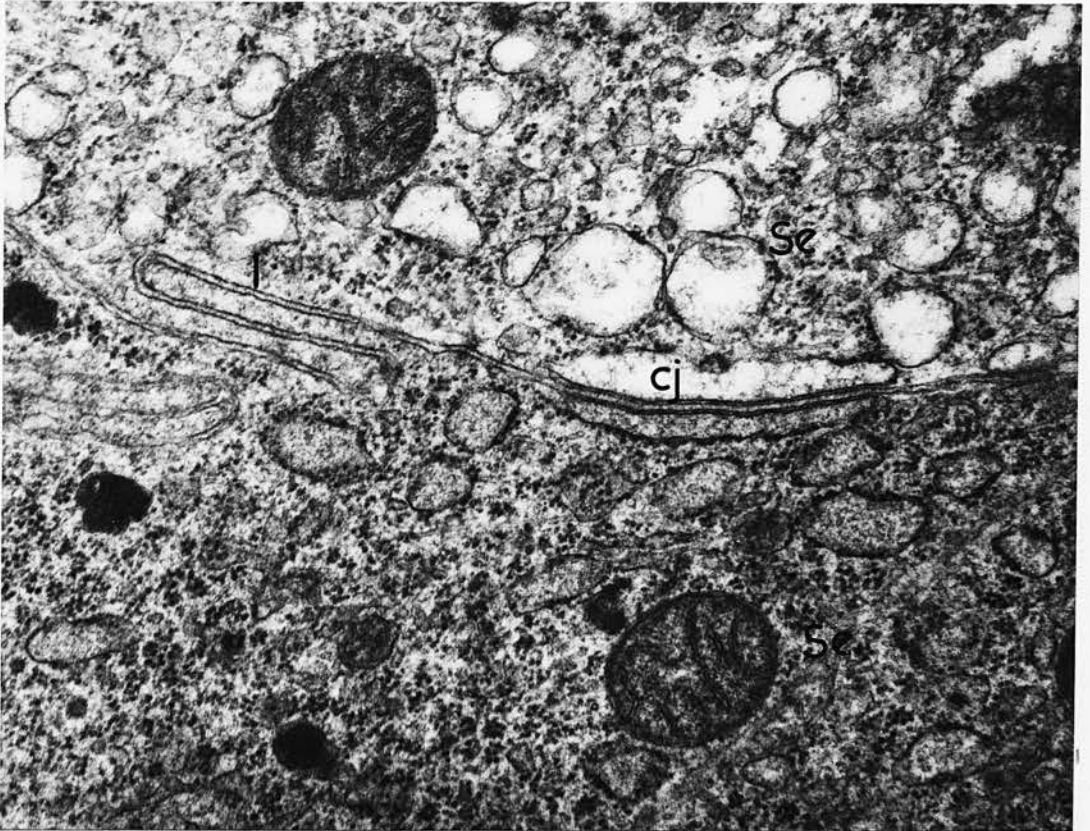
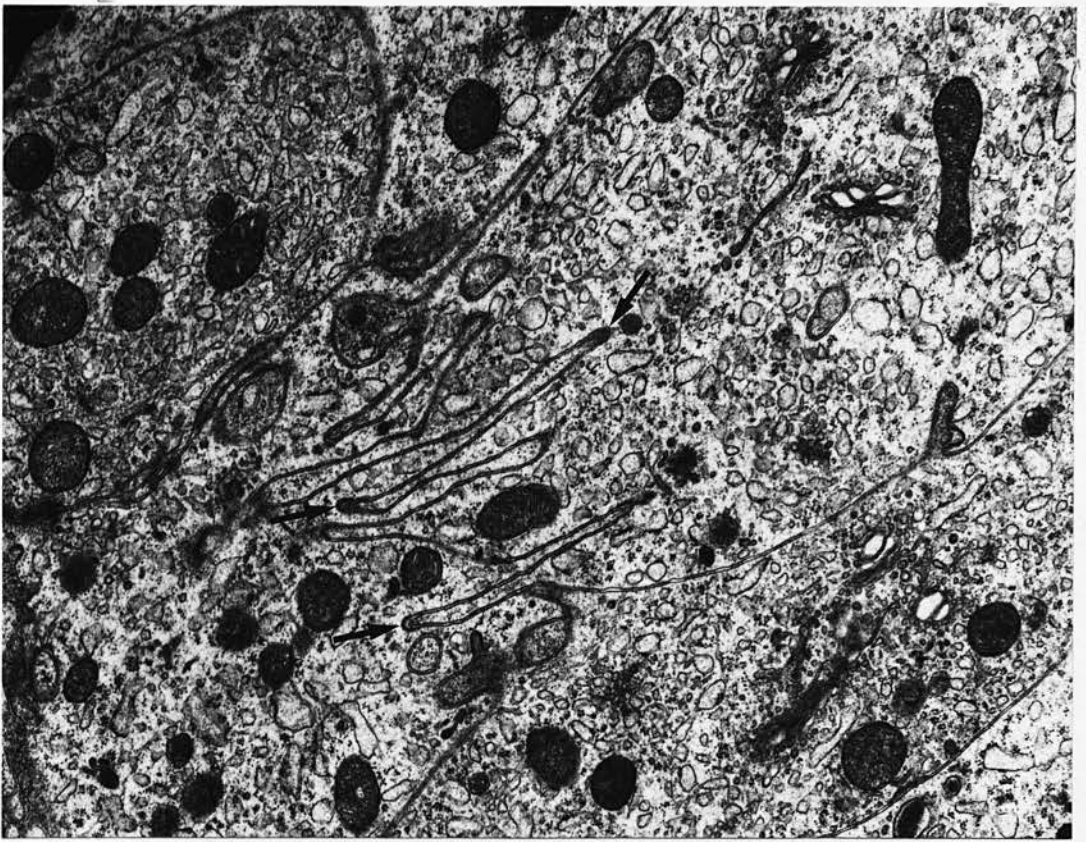


Figure 3.88

1 week old bird. No tight junction is observed where two Sertoli cells meet above a spermatogonium (arrow). Occluding junctions are observed at the apical junctional complex.

X 21,000

Figure 3.89

3 week old bird. Electron dense material is observed in the subplasmalemmal cytoplasm of two Sertoli cells where they adjoin above a spermatogonium. There is no constriction of the intercellular space at this point.

X 155,500

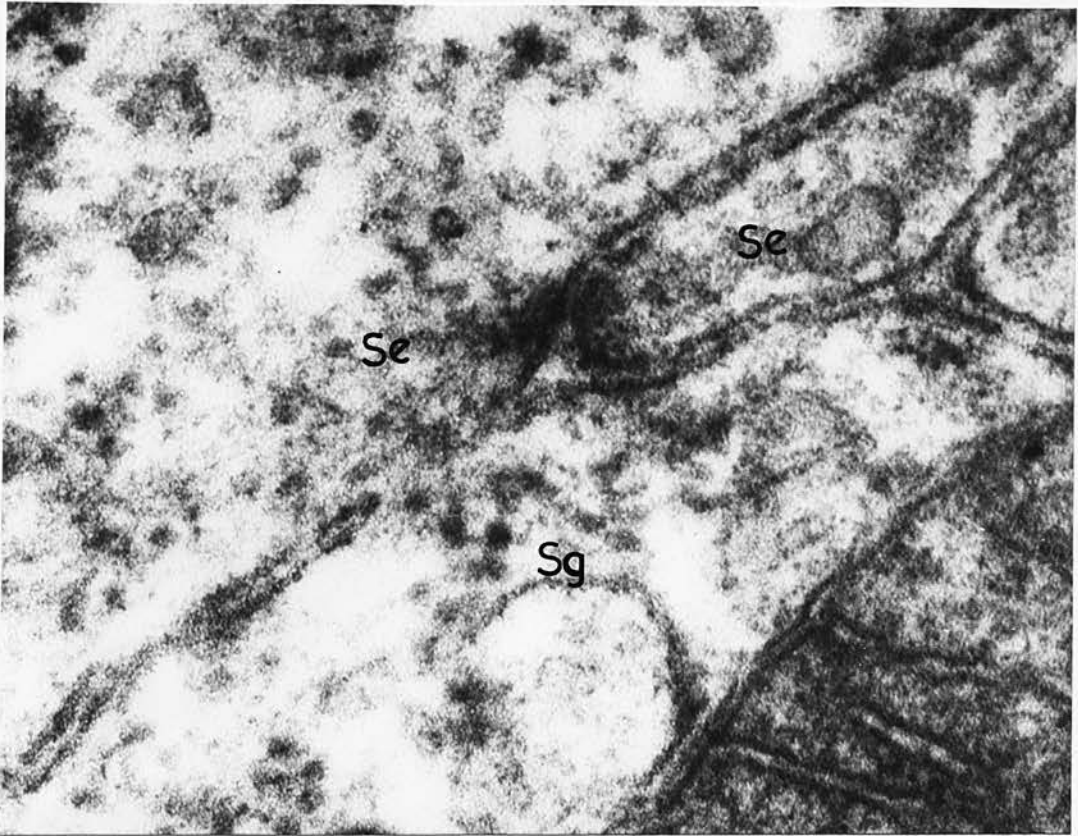
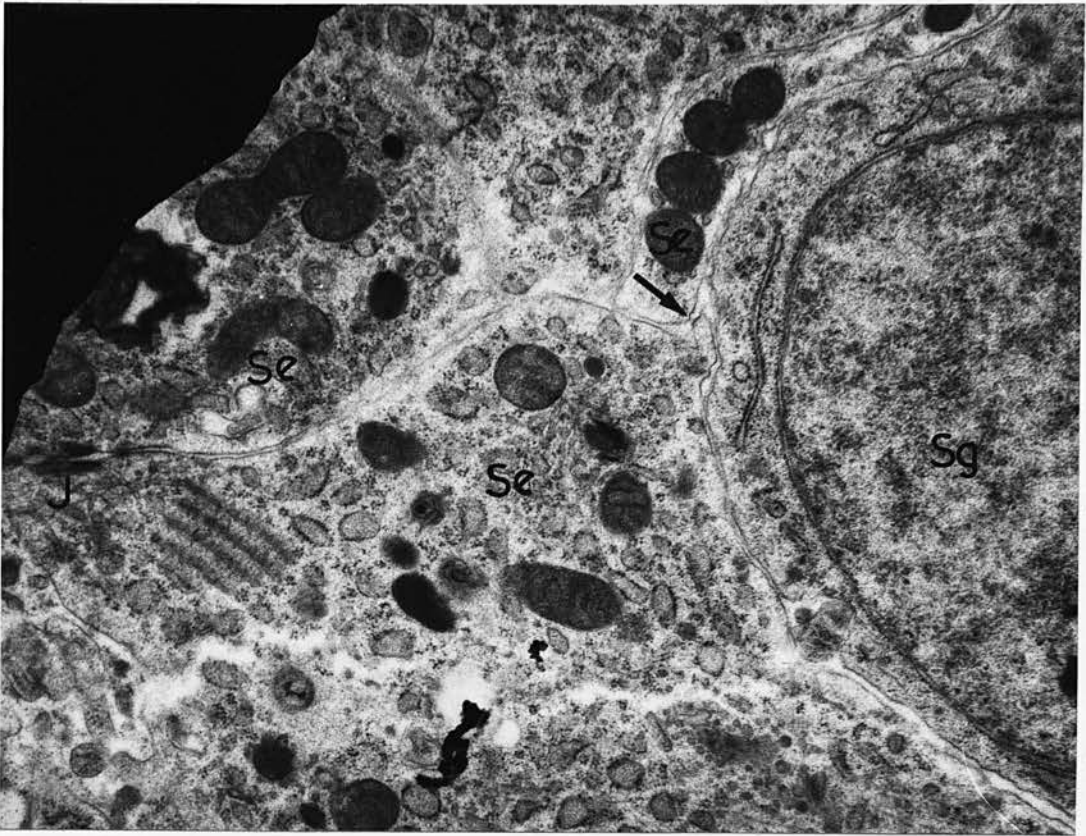


Figure 3.90

12 week old bird. (Once pachytene) spermatocytes are present in the tubule) punctate tight junctions with a pentalaminar structure (arrow) are observed where two Sertoli cells meet above a spermatogonium.

X 129,000

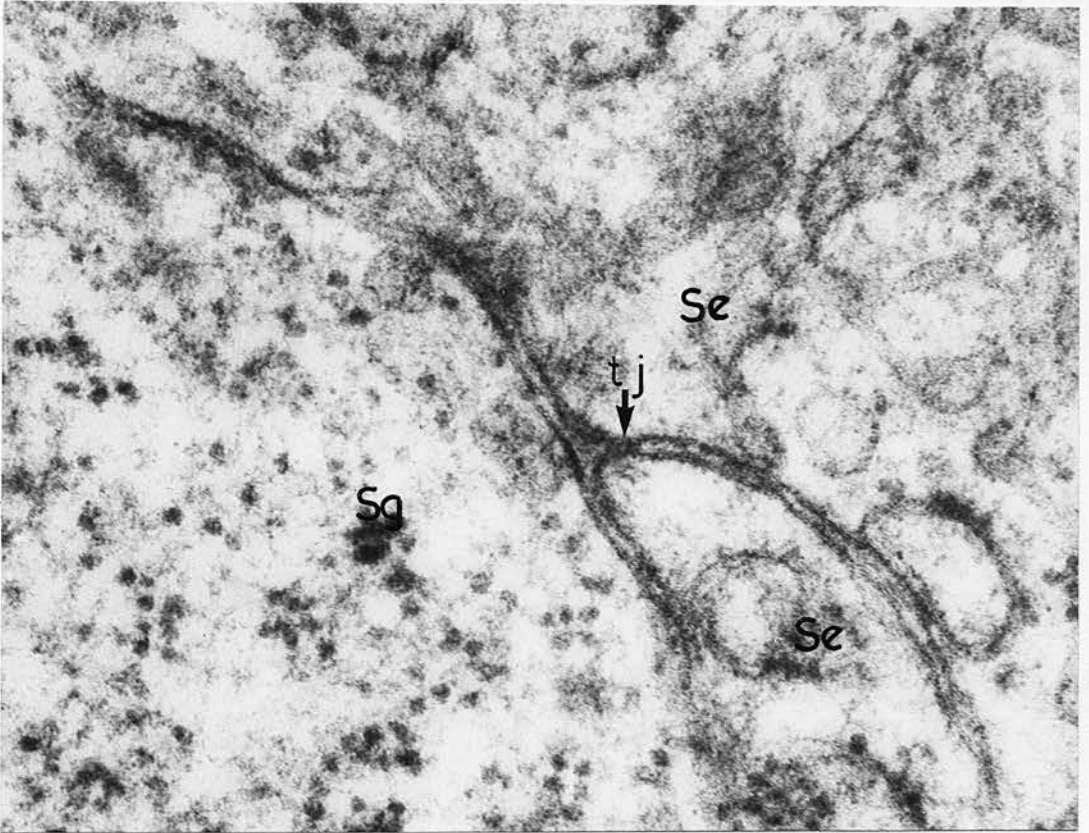


Figure 3.91

16 week old bird. Punctate tight junctions (arrow) are observed between two Sertoli cells where they meet between two early pachytene spermatocytes.

X 15,100

Figure 3.92

16 week old bird. Higher magnification of figure 3.91 demonstrating pentalaminar structure of the Sertoli-Sertoli tight junctions (arrow).

X 178,000

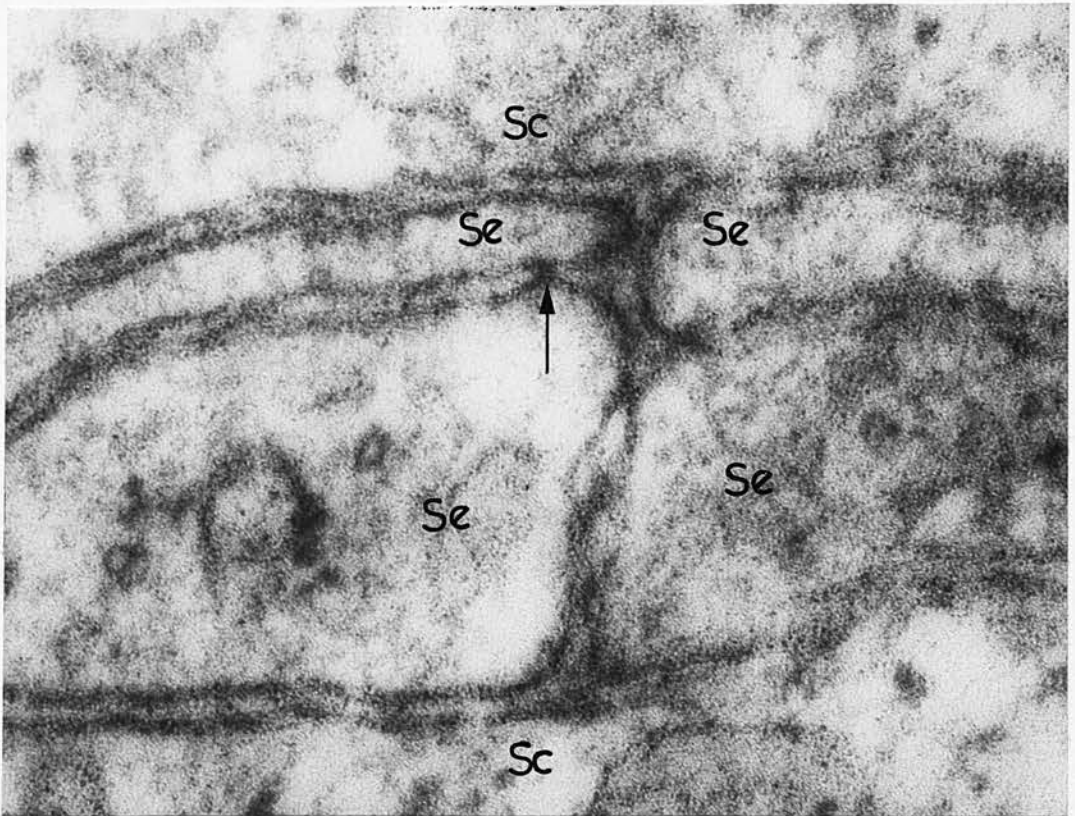
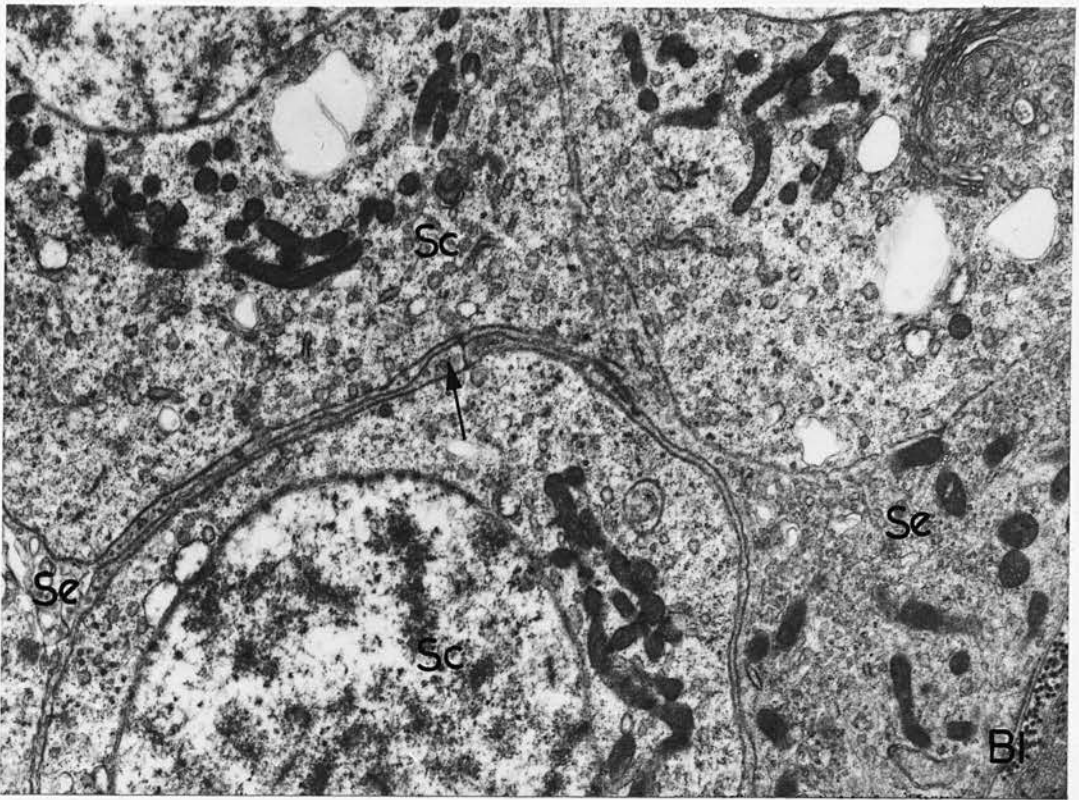




Figure 3.93

12 week old bird. Sertoli-Sertoli tight junction and subsurface cisternal junction (arrow) of two Sertoli cells located above the level of the resting spermatocyte nuclei.

X 7,500

Figure 3.94

12 week old bird. Subsurface cisternal junction between two Sertoli cells. There is no narrowing of the intercellular space within the junction. A tight junction showing clearly the pentalaminar structure is seen in close proximity to the cisternal junction. The tight junction is situated towards the basal position portion of the tubule.

X 129,600

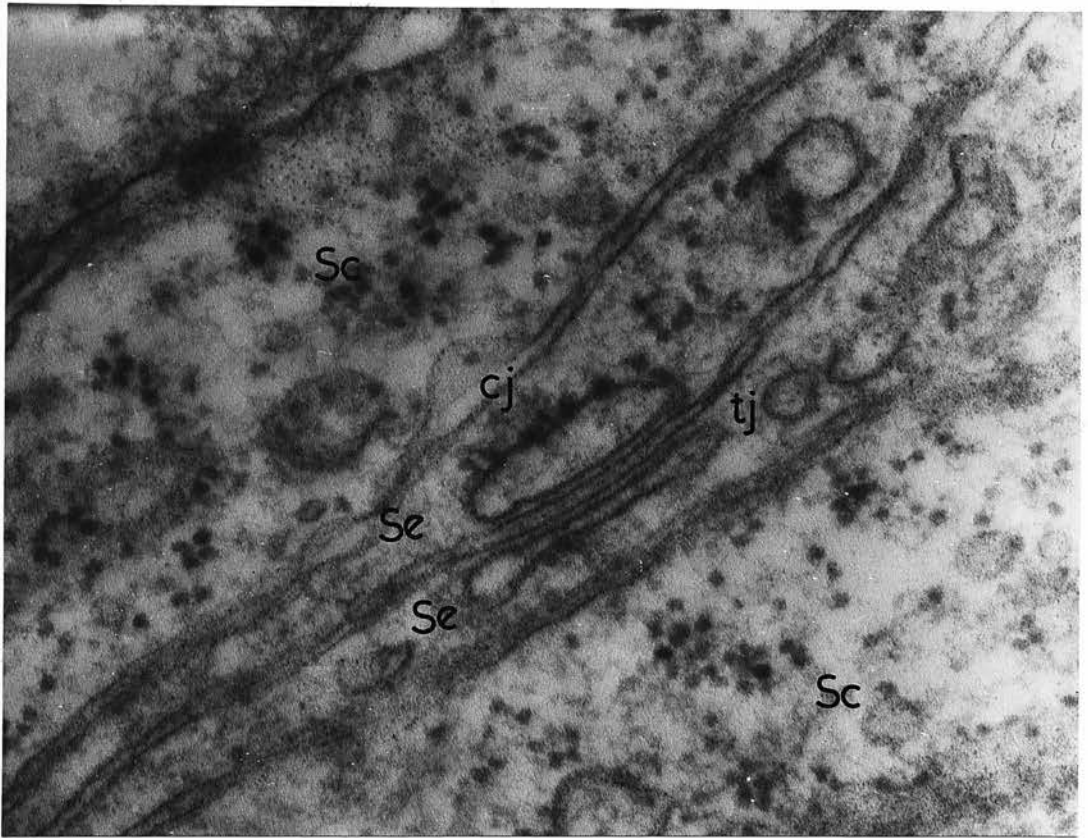
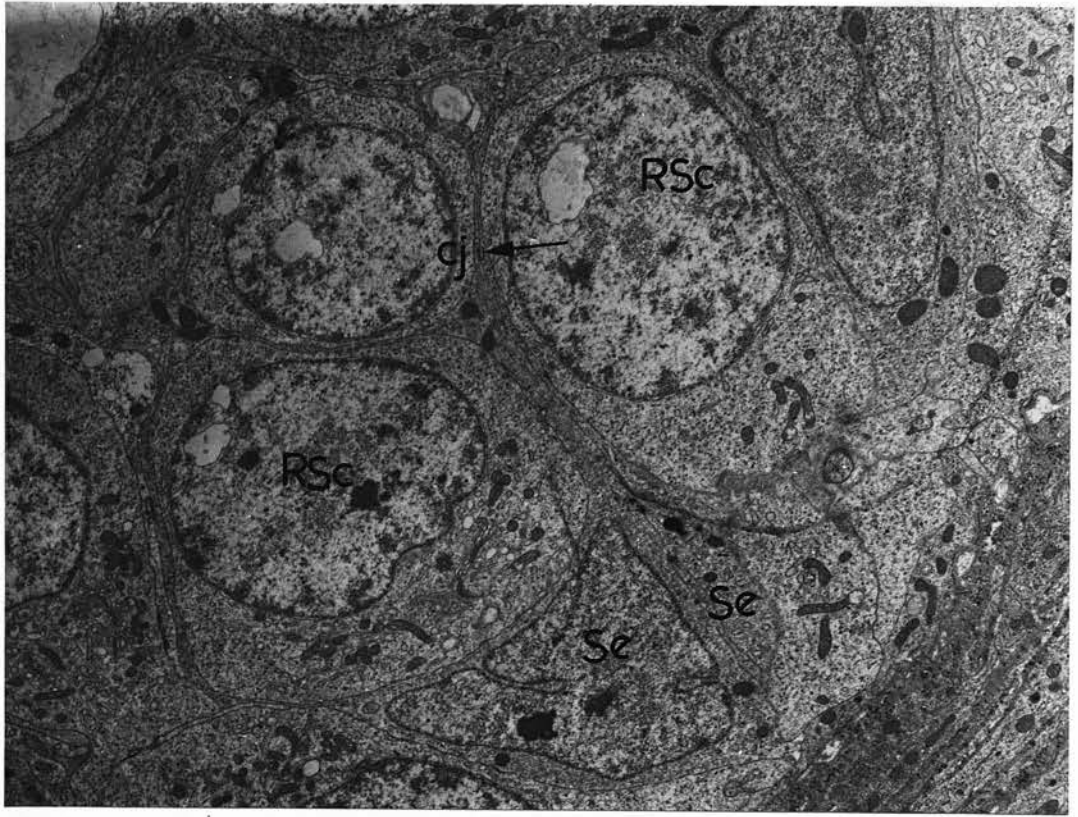


Figure 4.1

Prewash : 0.075M Na cacodylate, 4% Dextran

Initial fixative : 4% GTA/0.075M Na cacodylate, 2% Dextran, 1%  $\text{La}(\text{NO}_3)_3$

Post-fixative : 1%  $\text{OsO}_4$ /modified Dalton's buffer

Lanthanum is precipitated on the endothelial cell membranes and is not observed outside the capillary.

X 7,750

Figure 4.2

Prewash : 4% GTA/0.075M Na cacodylate

Initial fixative : 4% GTA/0.075M Na cacodylate, 1%  $\text{La}(\text{NO}_3)_3$

Post-fixative : 1%  $\text{OsO}_4$ /0.1M  $\text{PO}_4$

Lanthanum is observed in the Sertoli-Sertoli inter-cellular clefts and surrounding the resting spermatocytes.

X 7,750

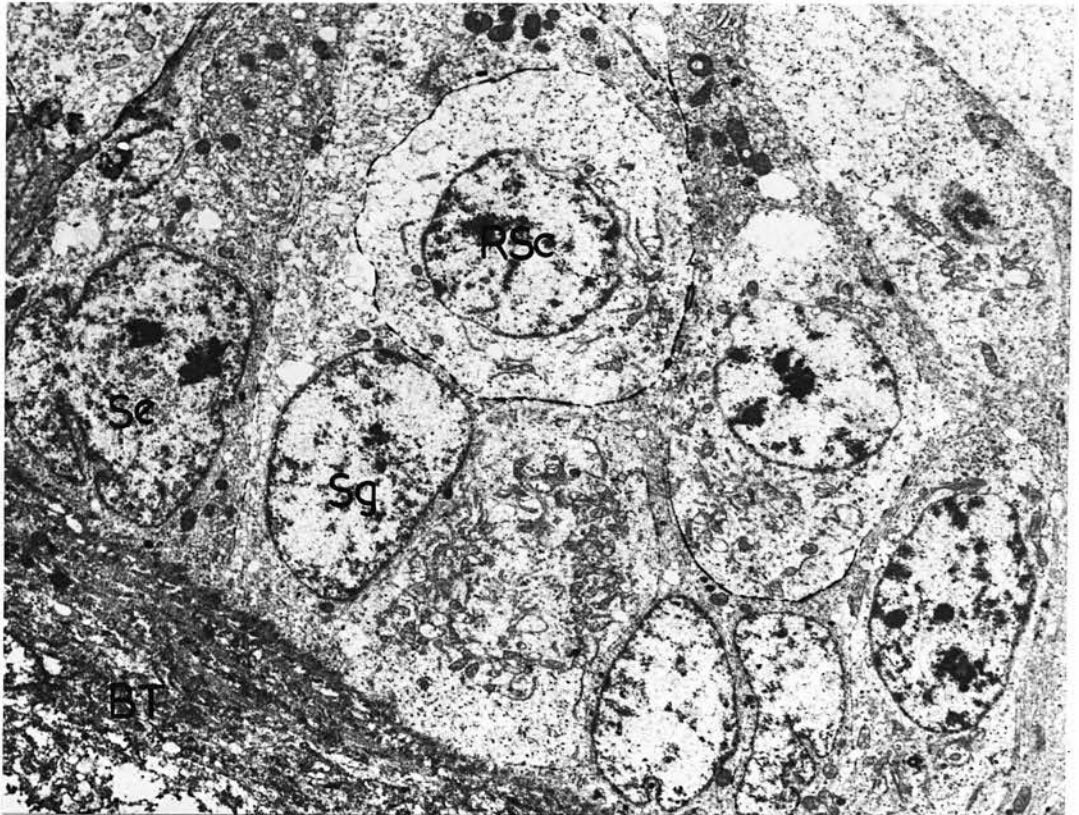
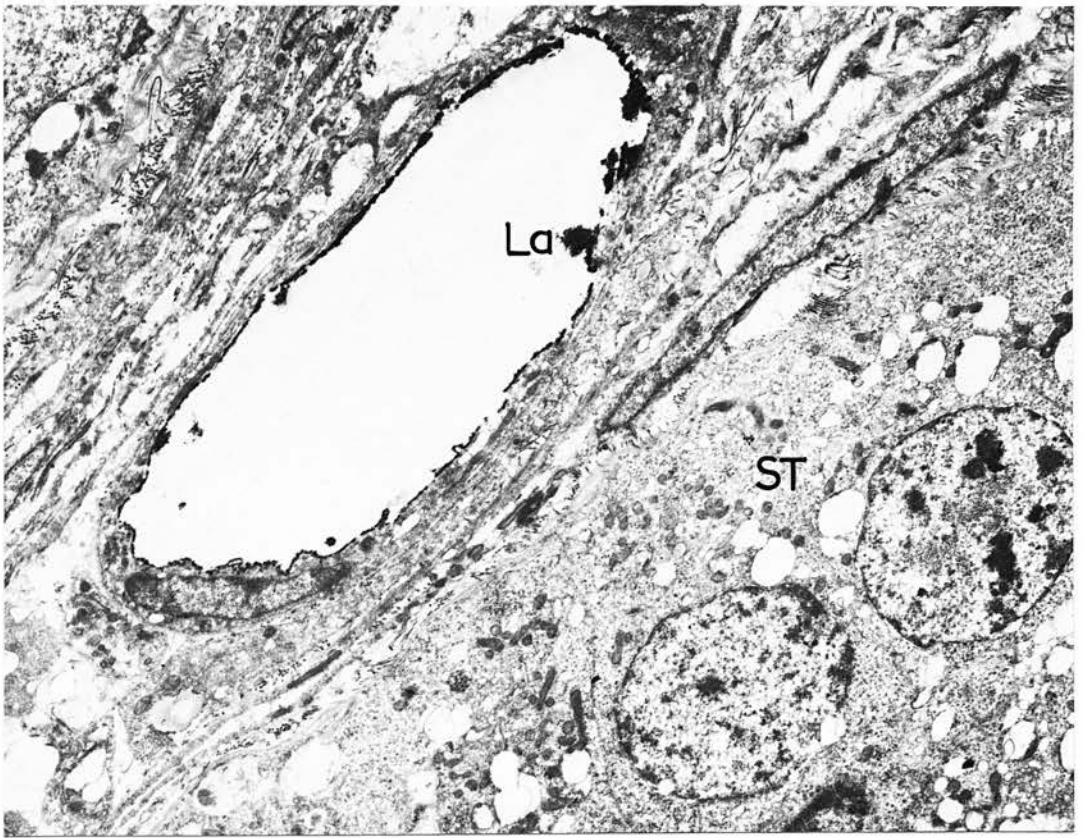


Figure 4.3

Prewash : 1% saline

Initial fixative : 4% GTA/0.075M Na cacodylate, 1%  $\text{La}(\text{NO}_3)_3$

Post-fixative : 1%  $\text{OsO}_4$ /0.1M  $\text{PO}_4$

Lanthanum is observed in the intercellular spaces of the peritubular myofibroblast cells and between the spermatogonia and Sertoli cells. Note disrupted leached appearance of the cytoplasm and the tendency for lanthanum to block.

X 6,000

Figure 4.4

Prewash : Avian Ringer

Initial fixative : 4% GTA/0.075M Na cacodylate, 1%  $\text{La}(\text{NO}_3)_3$

Post-fixative : 1%  $\text{OsO}_4$ /0.1M  $\text{PO}_4$

Lanthanum is observed in the intercellular clefts around the spermatogonia. Note disrupted fixation, large nuclear vesicles and separated boundary tissue

X 5,800

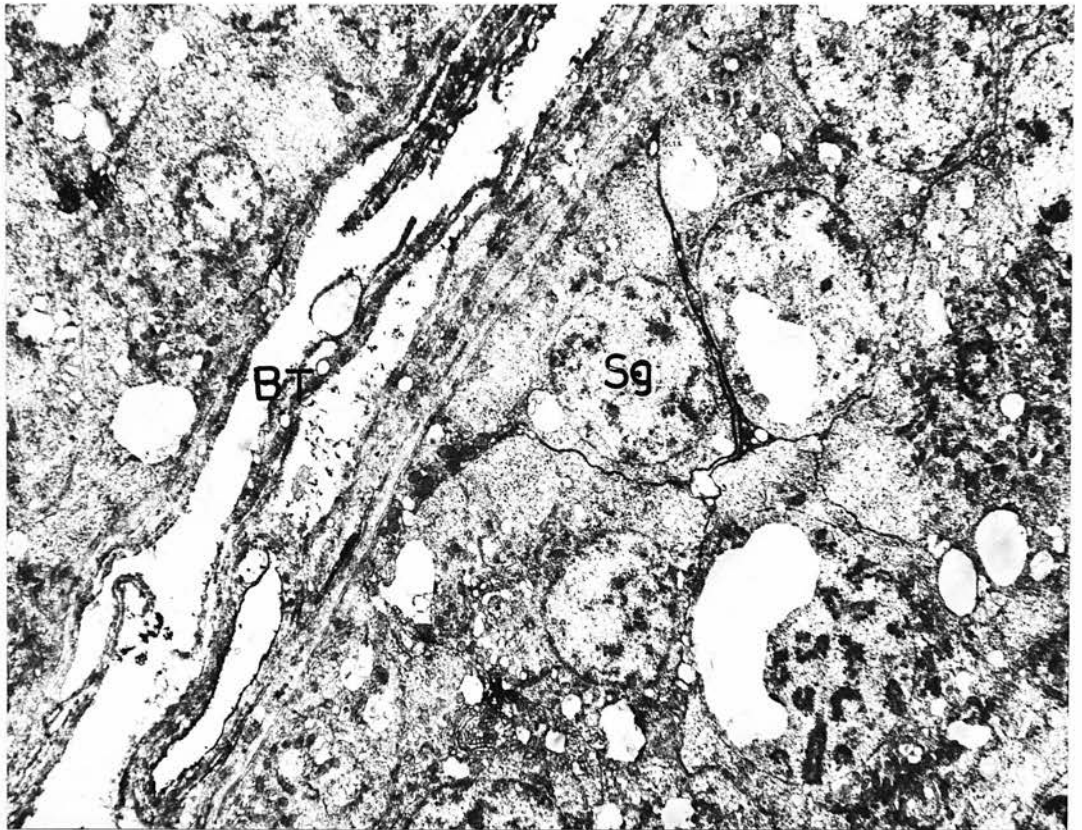
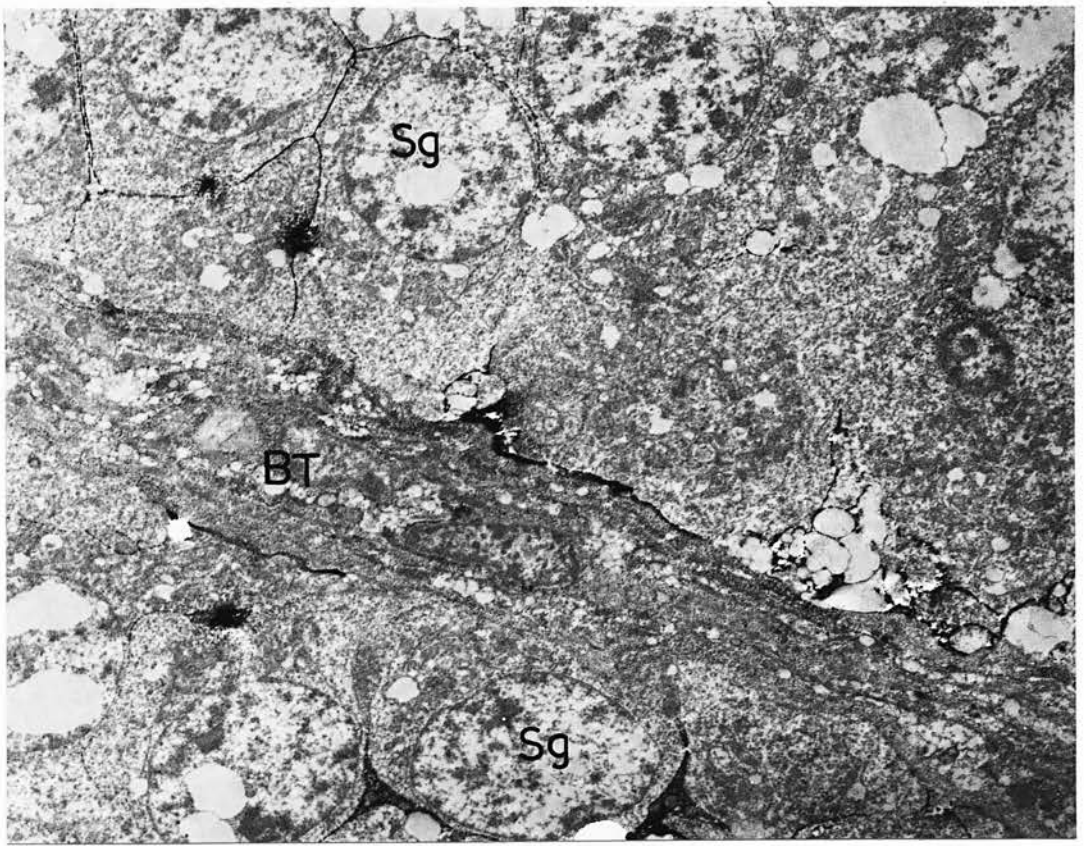


Figure 4.5

Prewash : 4% GTA/0.1M s-collidine

Initial fixative : 4% GTA/0.1M s-collidine, 1%  $\text{La}(\text{NO}_3)_3$

Post-fixative : 1%  $\text{OsO}_4$ /0.1M s-collidine, 1%  $\text{La}(\text{NO}_3)_3$

Lanthanum is precipitated on the endothelial cell membranes and is not seen outside the capillary.

X 5,000

Figure 4.6

4% GTA/0.075M Na cacodylate, 1%  $\text{La}(\text{NO}_3)_3$  hand perfused with a syringe. Lanthanum is observed in the intercellular clefts of the spermatogonia. The intertubular tissue is completely destroyed and the boundary tissue very disrupted.

X 6,700

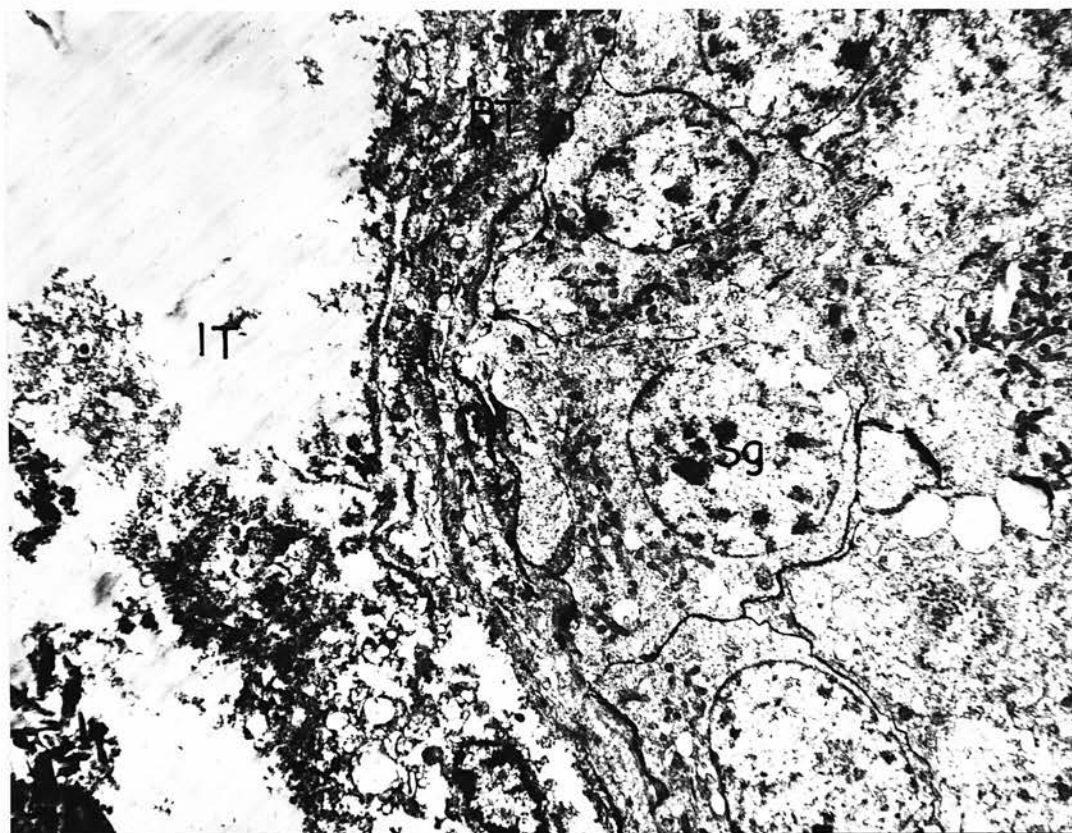
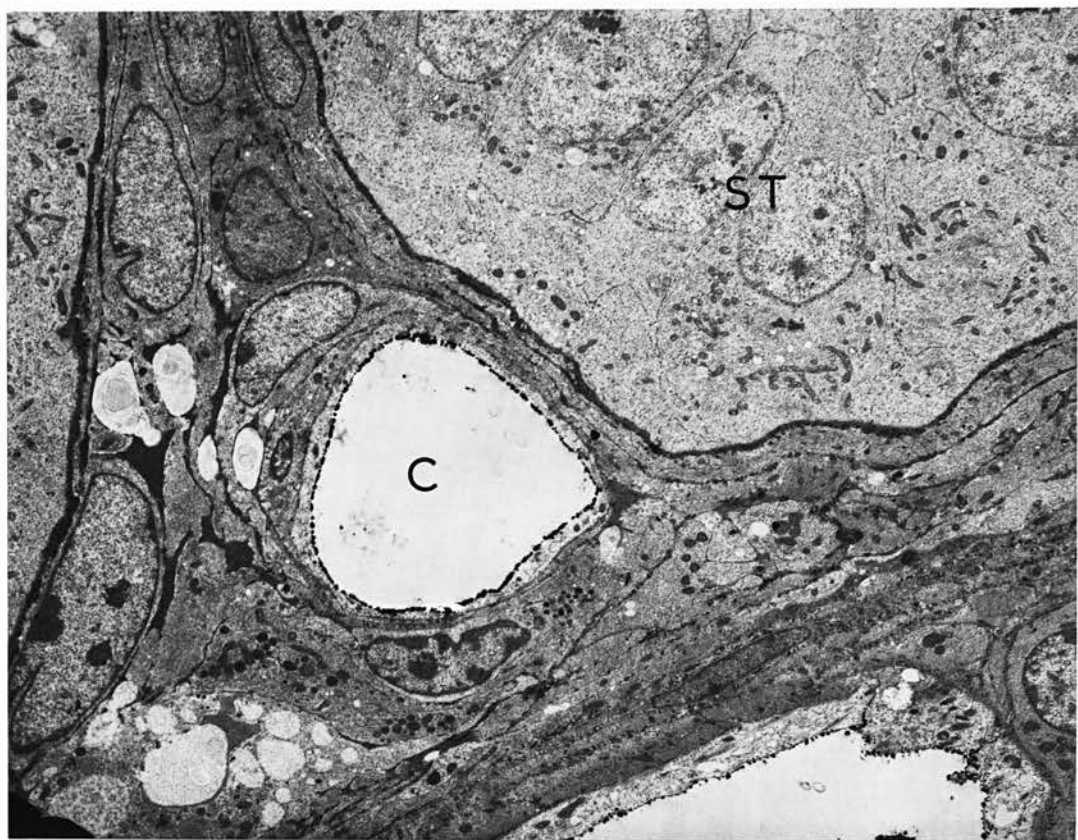




Figure 4.7

4% GTA/0.075M Na cacodylate, 1%  $\text{La}(\text{NO}_3)_3$  immersion fixation 15 week old bird. Arrows show where lanthanum stops in Sertoli-Sertoli intercellular space. Note the good preservation of the tubule cells but the heavy lanthanum deposits in the boundary tissue masking the ultrastructure.

X 6,000

Figure 4.8

Initial fixative : 4% GTA/0.075M Na cacodylate, 1%  $\text{La}(\text{NO}_3)_3$

Post-fixative : 1%  $\text{OsO}_4$ /1.5%  $\text{k}_4\text{Fe}(\text{CN})_6$

21 week old bird. Lanthanum is observed in the intercellular spaces around the spermatogonia and resting spermatocytes. Arrow shows where the point beyond which the lanthanum does not penetrate the Sertoli-Sertoli interspace.

X 7,500.

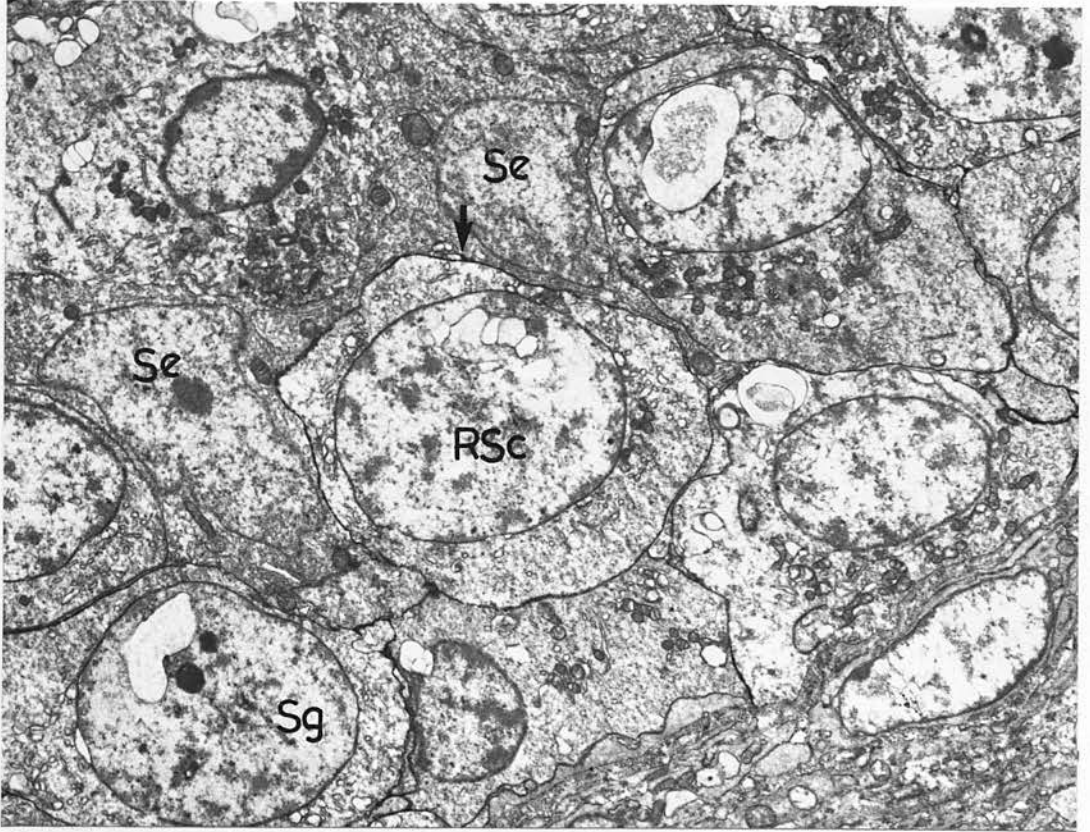
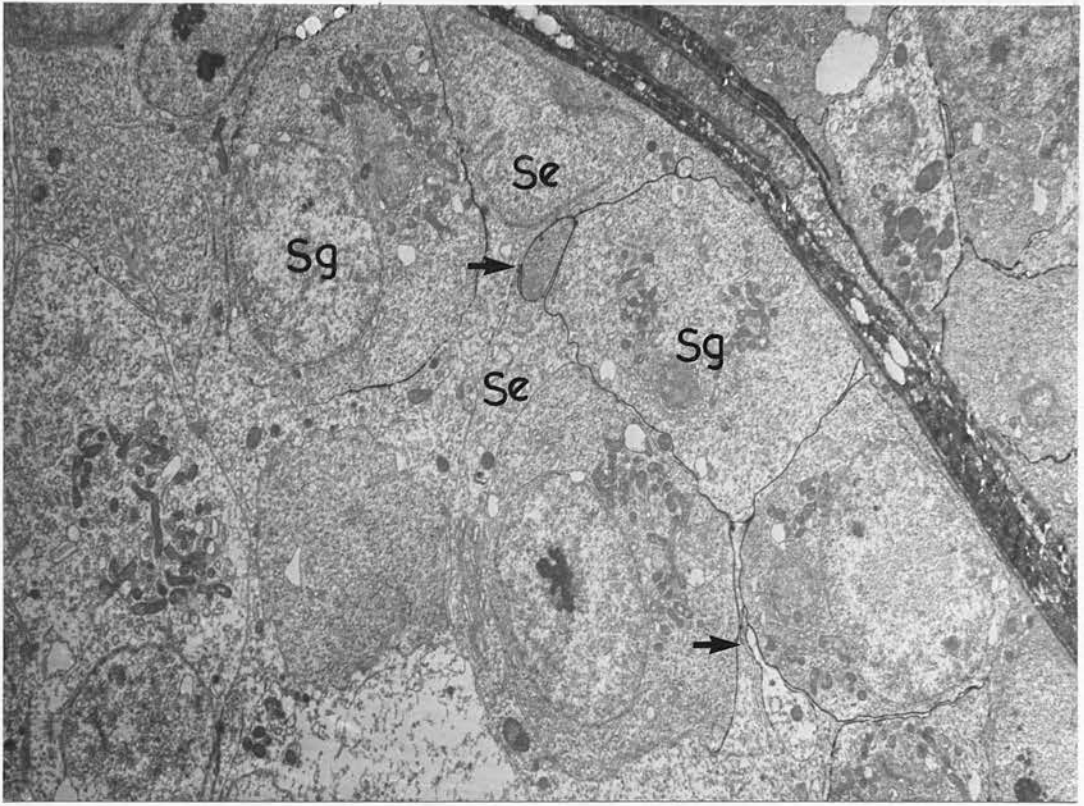


Figure 4.9

Higher magnification of figure 4.8 at the point of the arrow. The lanthanum does not penetrate more than  $0.3\mu$  in the Sertoli-Sertoli intercellular cleft. Note the unit membrane structure of the Sertoli cell membranes.

X 82,000

Figure 4.10

21 week old control bird injected with water and the testis incubated for peroxidase. Endogenous peroxidase is only present in the red blood cell. Unstained.

X 7,500

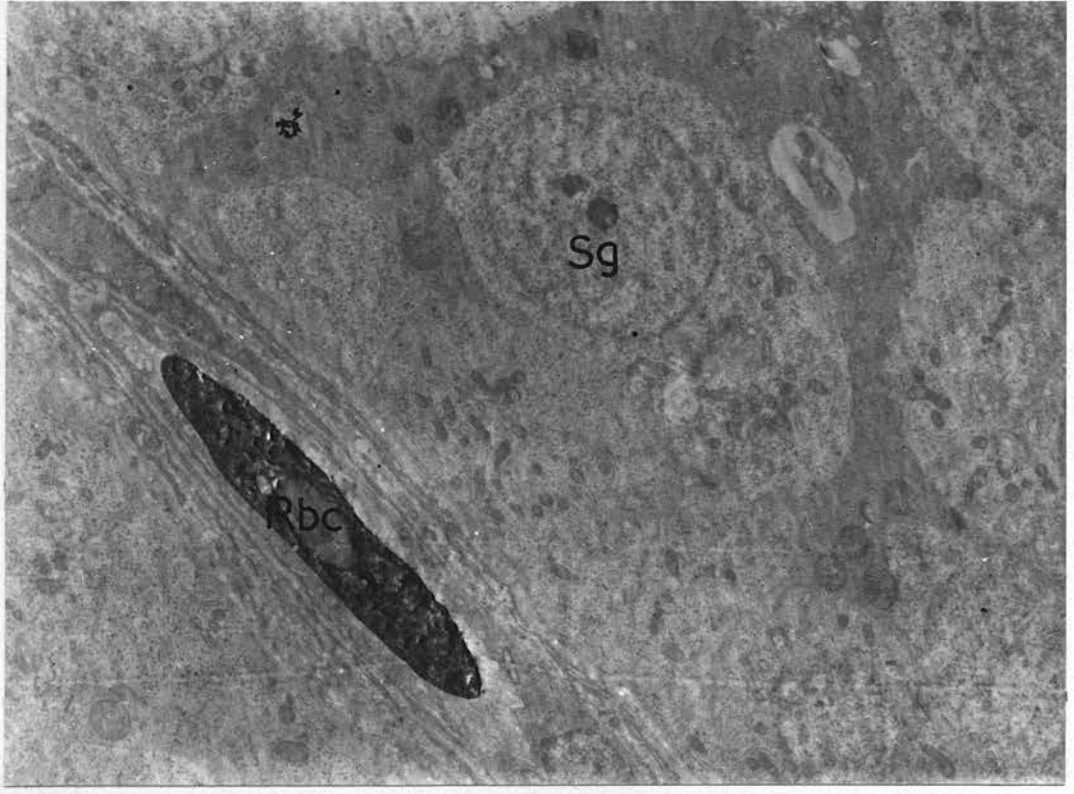
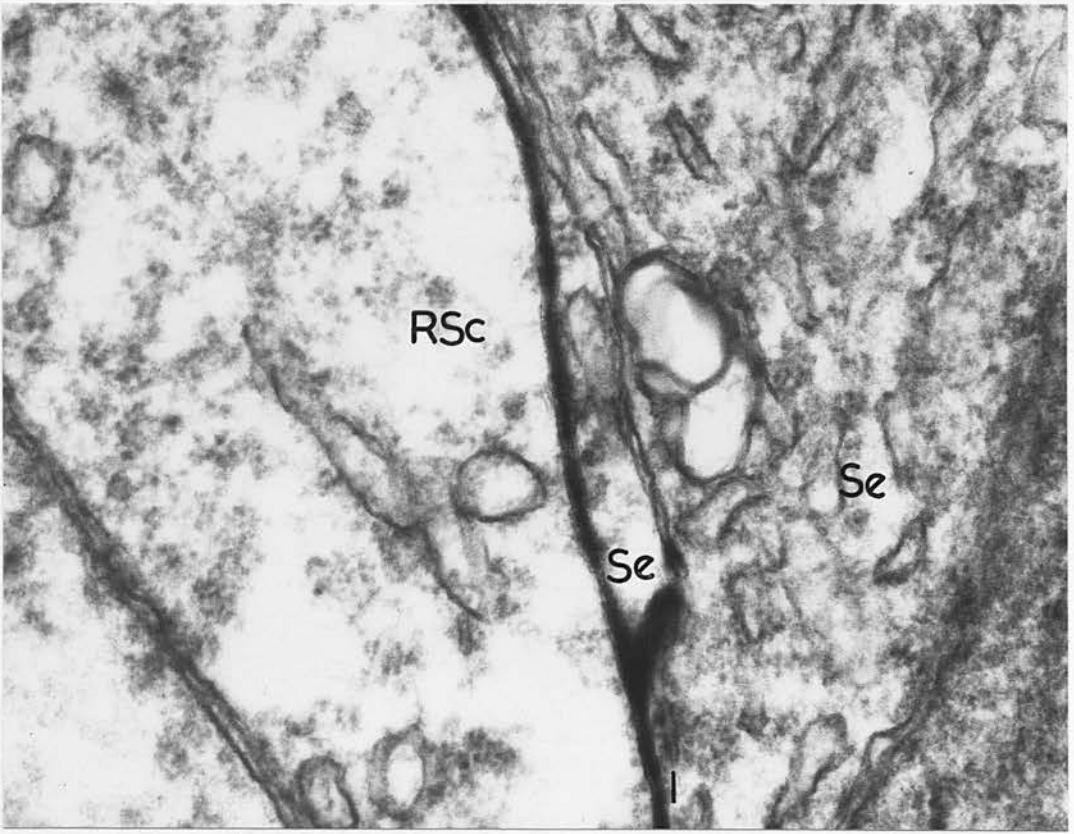


Figure 4.11

9 week old bird. Lanthanum is observed in the capillary endothelial intercellular clefts (arrows) and the intercellular spaces of the boundary tissue.

X 6,000

Figure 4.12

Higher magnification of arrowed portion of figure 4.11. Lanthanum is observed on either side of the capillary wall and within the intercellular cleft of the endothelial cells.

X 21,600

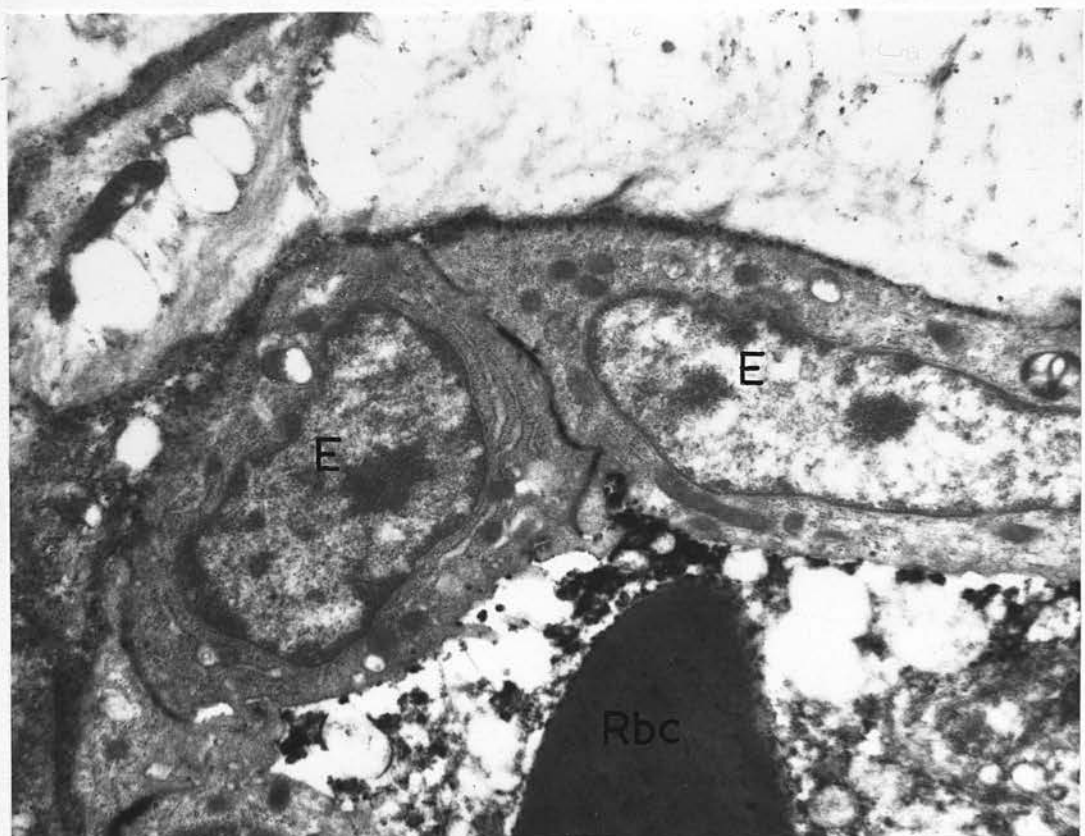
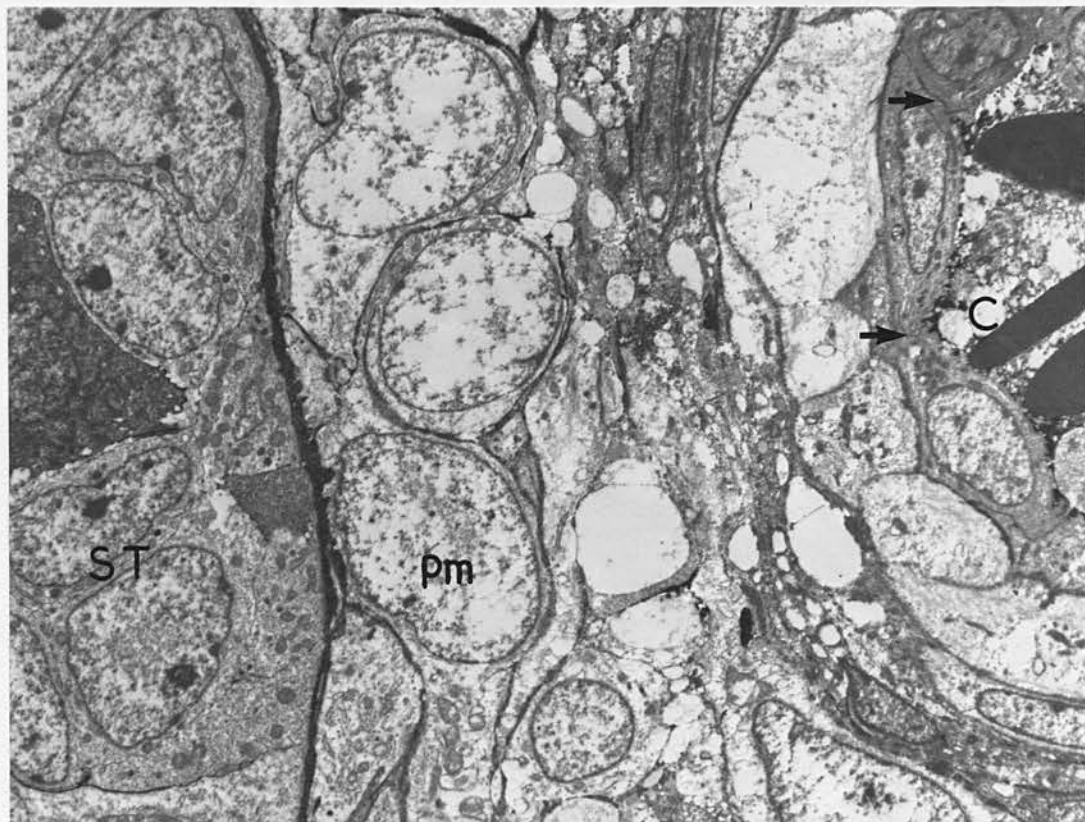


Figure 4.13

21 week old bird. Lanthanum is seen within the inter-cellular spaces of the peritubular myofibroblast cells of the boundary tissue and between the basal cells of the seminiferous tubule.

X 15,500

Figure 4.14

9 week old bird. Lanthanum is seen within the inter-cellular spaces of the peritubular myofibroblast cells. Note the cell junctions with no associated narrowing of the plasma membranes.

X 34,000

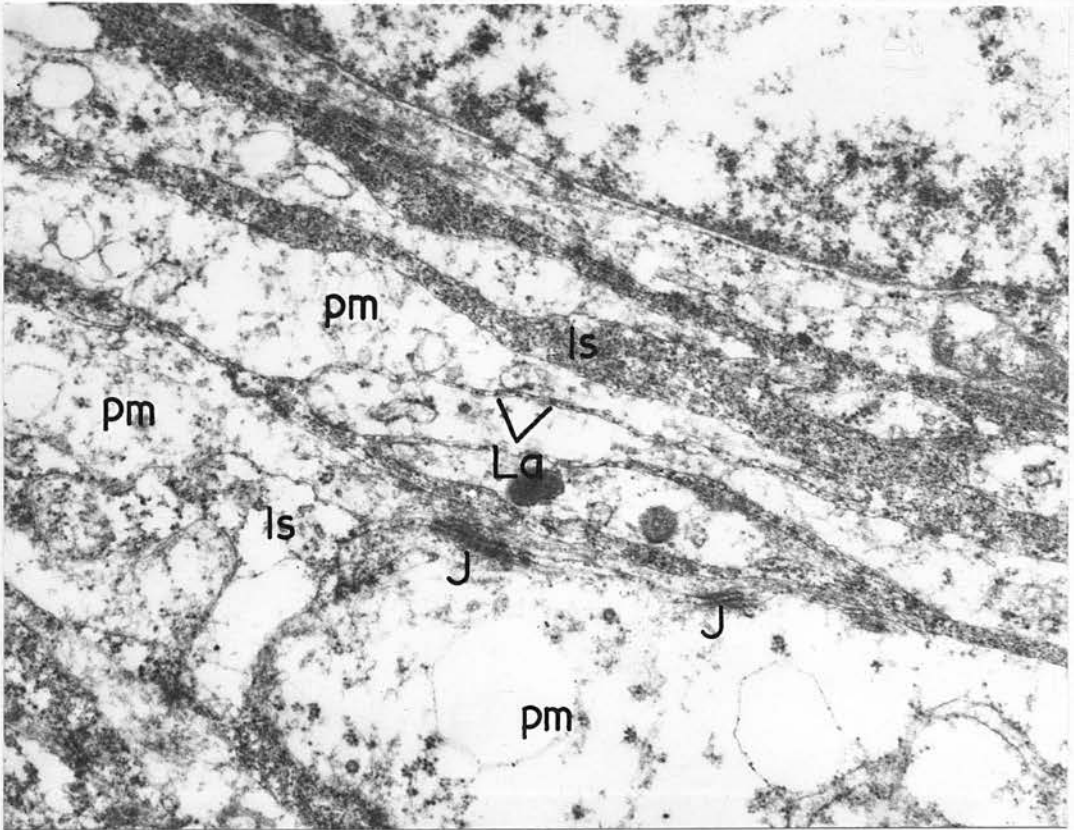
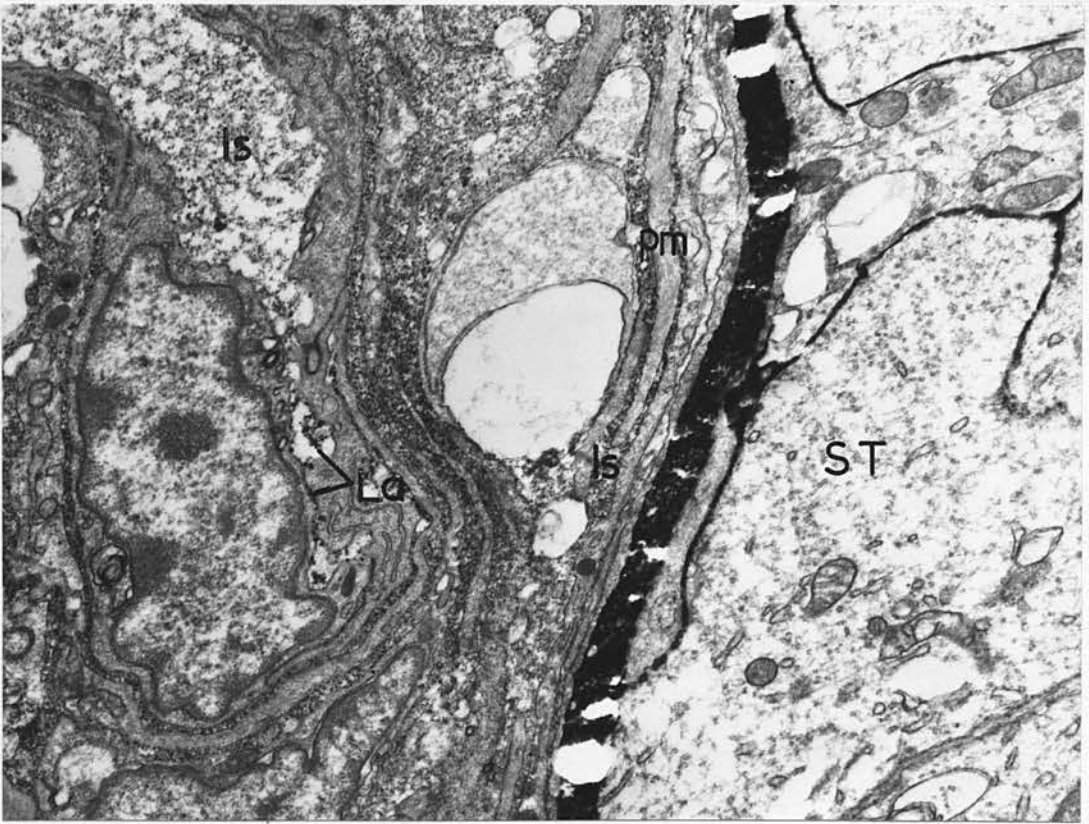




Figure 4.15

21 week old bird, Lanthanum is seen surrounding the spermatogonia and penetrating the Sertoli-Sertoli intercellular space to the point of the occluding tight junctions (Arrow).

X 8,000

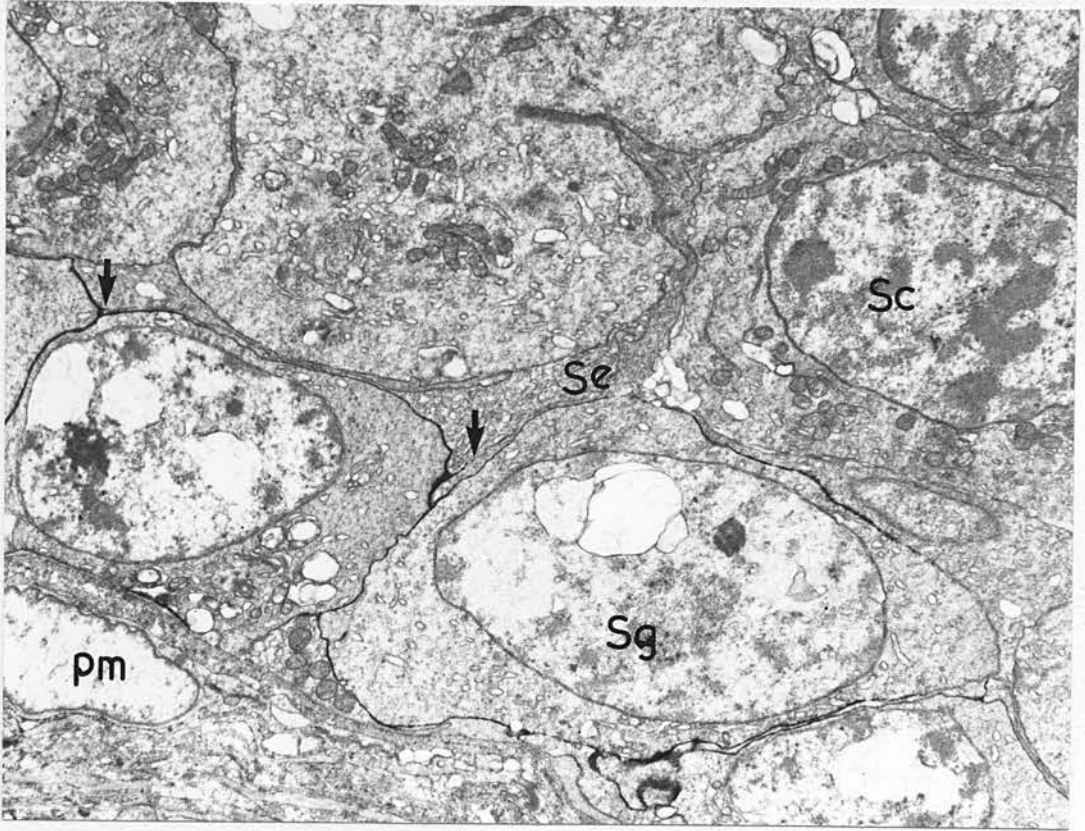


Figure 4.16

Higher magnification of arrowed portion of figure 4.15. Lanthanum is seen penetrating the Sertoli-Sertoli intercellular space to a point prior to two tight junctions (arrows) and a cisternal junction. Note the pentalaminar structure of the tight junctions.

X 96,000

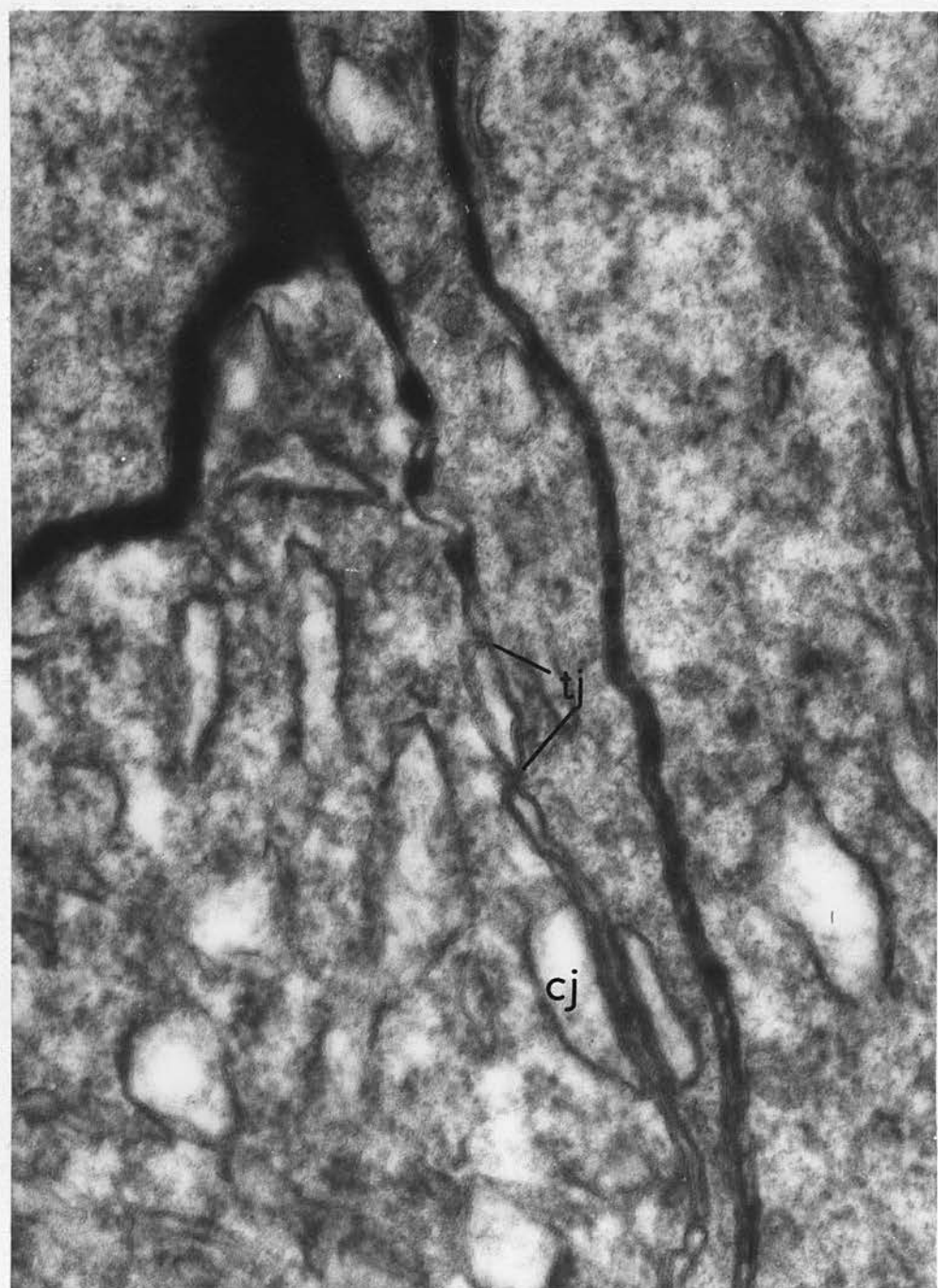


Figure 4.17

21 week old bird. Lanthanum is seen surrounding the spermatogonia and penetrating the Sertoli-Sertoli inter-cellular cleft for a short distance (arrow).

X 12,000

Figure 4.18

Higher magnification of arrowed area of figure 4.17. Lanthanum is seen within the spermatogonia - Sertoli intercellular space and penetrates the Sertoli-Sertoli cleft to a point prior to the tight junction.

X 69,000

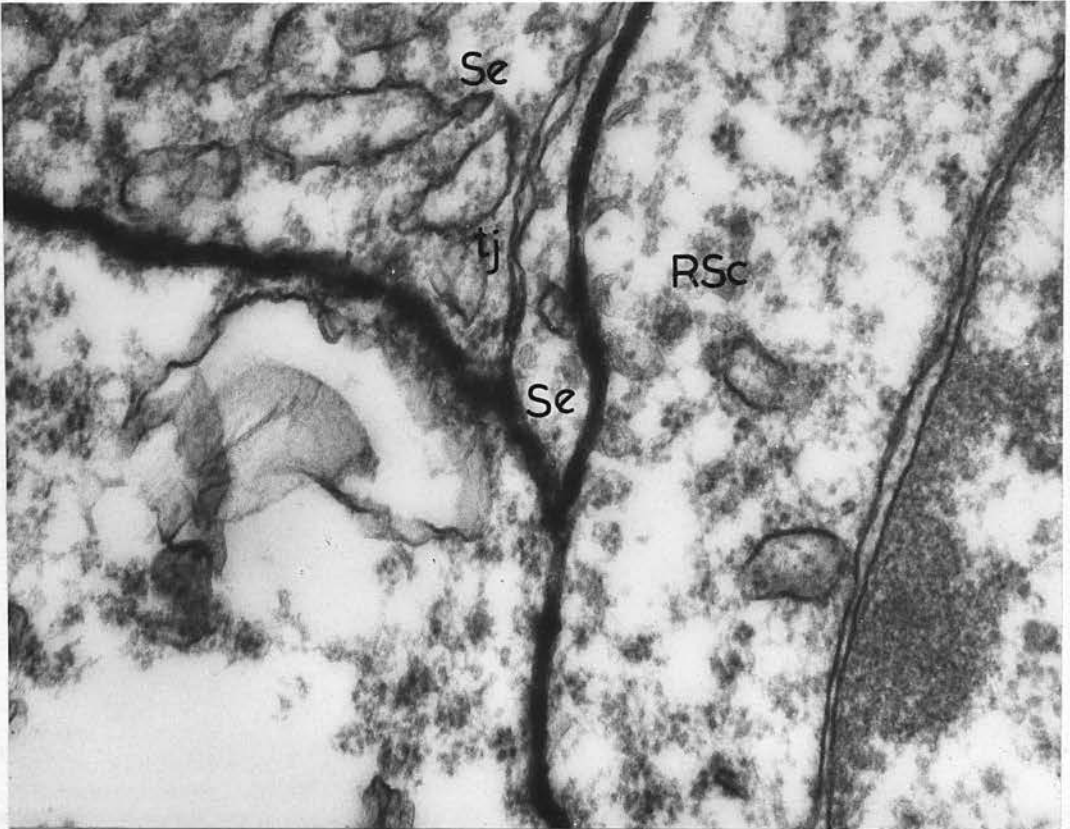
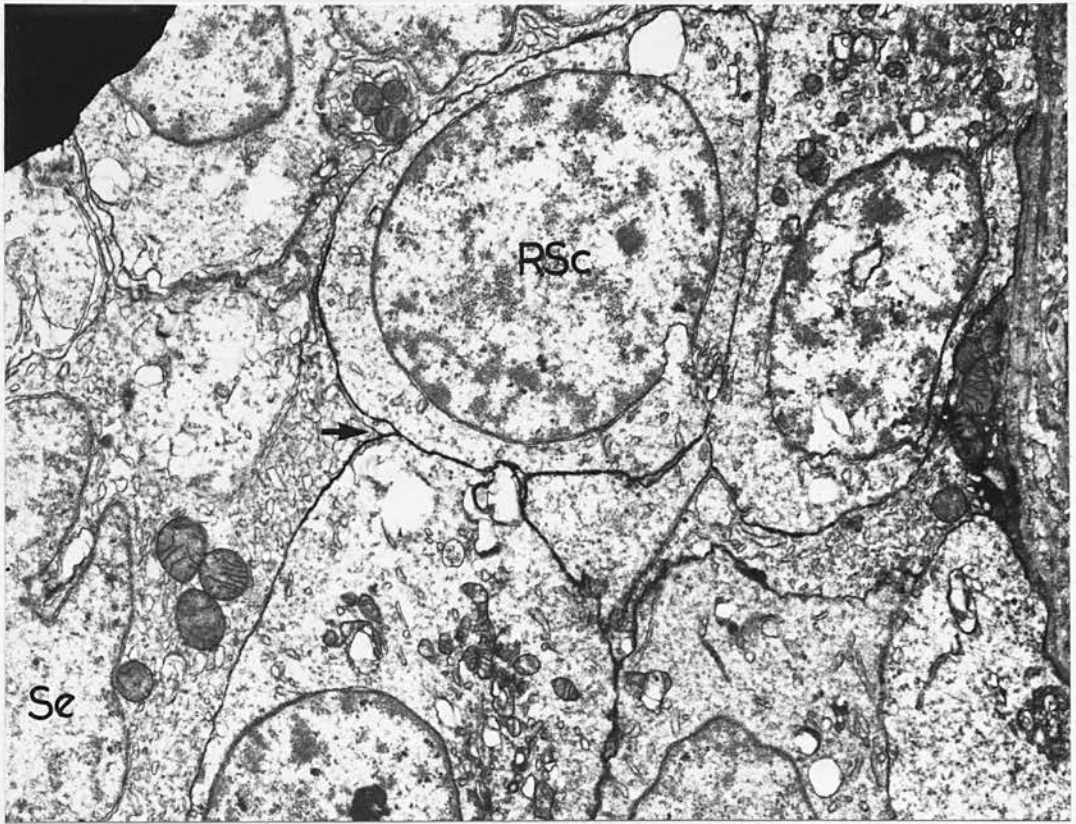


Figure 4.19

9 week old bird. Lanthanum is observed in the inter-cellular spaces between peritubular myofibroblast cells, spermatogonia and Sertoli cells. It penetrates between Sertoli cells to the zonula occludens junctions in the centre of the tubule.

X 6,000

Figure 4.20

9 week old bird. Lanthanum is seen within the Sertoli-Sertoli clefts at the centre of the tubule. No lanthanum penetrates the zonulae occludentes (see inset).

X 22,000

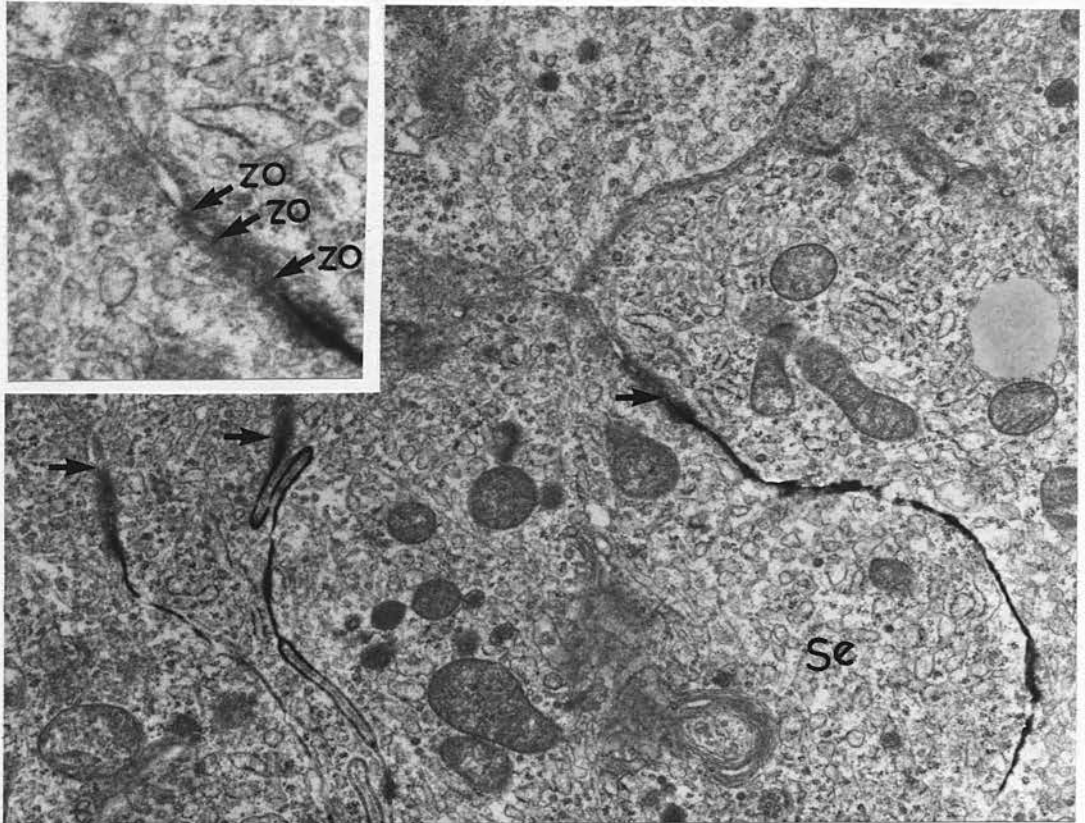
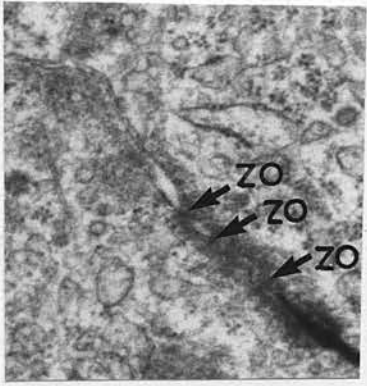
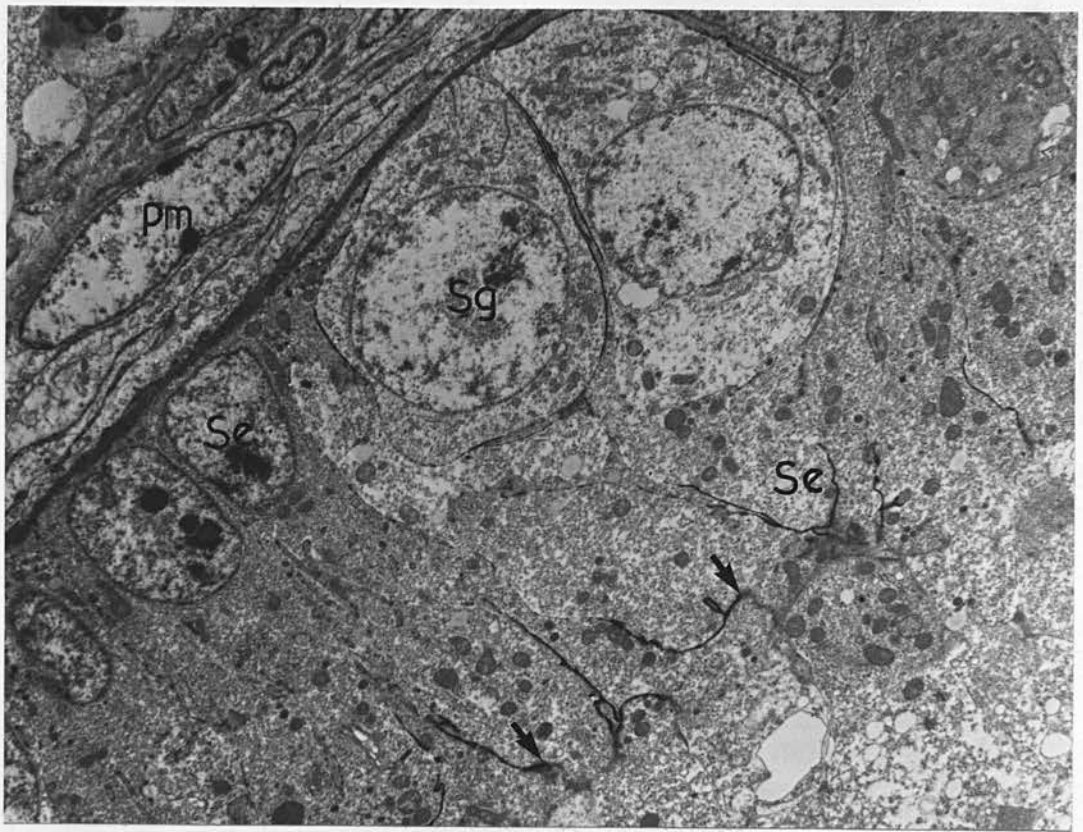




Figure 4.21

9 week old bird. Lanthanum is not observed within the zonulae occludentes (see inset) at the centre of the tubule.

X 26,000

Figure 4.22

9 week old bird. Lanthanum is observed within the Sertoli-Sertoli intercellular space, penetrating between a subsurface cisternal junction.

X 64,000

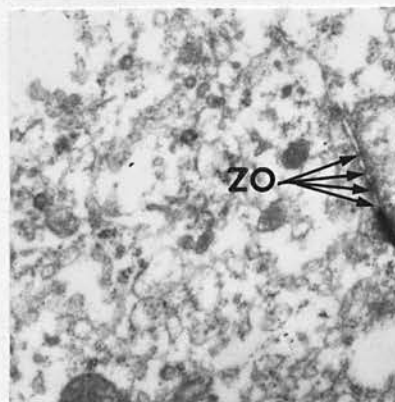
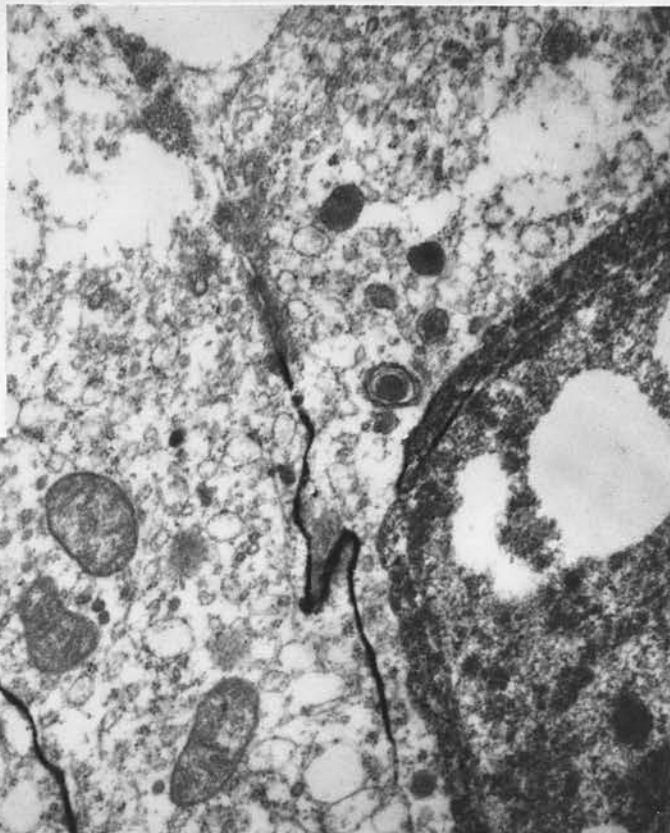
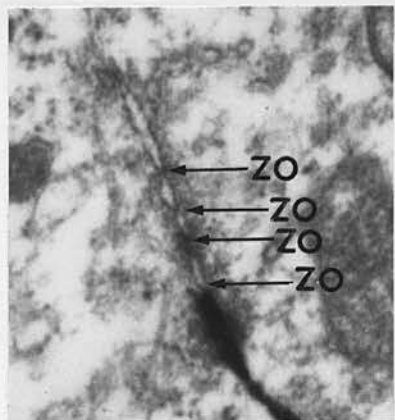


Figure 4.23

21 week old bird. Peroxidase reaction product is observed in the intercellular cleft between endothelial cells and peritubular myofibroblast cells. Note the pinocytotic uptake of peroxidase reaction product. Unstained  
X 50,000

Figure 4.24

21 week old bird. A spermatogonium is outlined by reaction product in the intercellular space. Arrows indicate point beyond which peroxidase is not seen in the Sertoli-Sertoli clefts.  
X 10,000

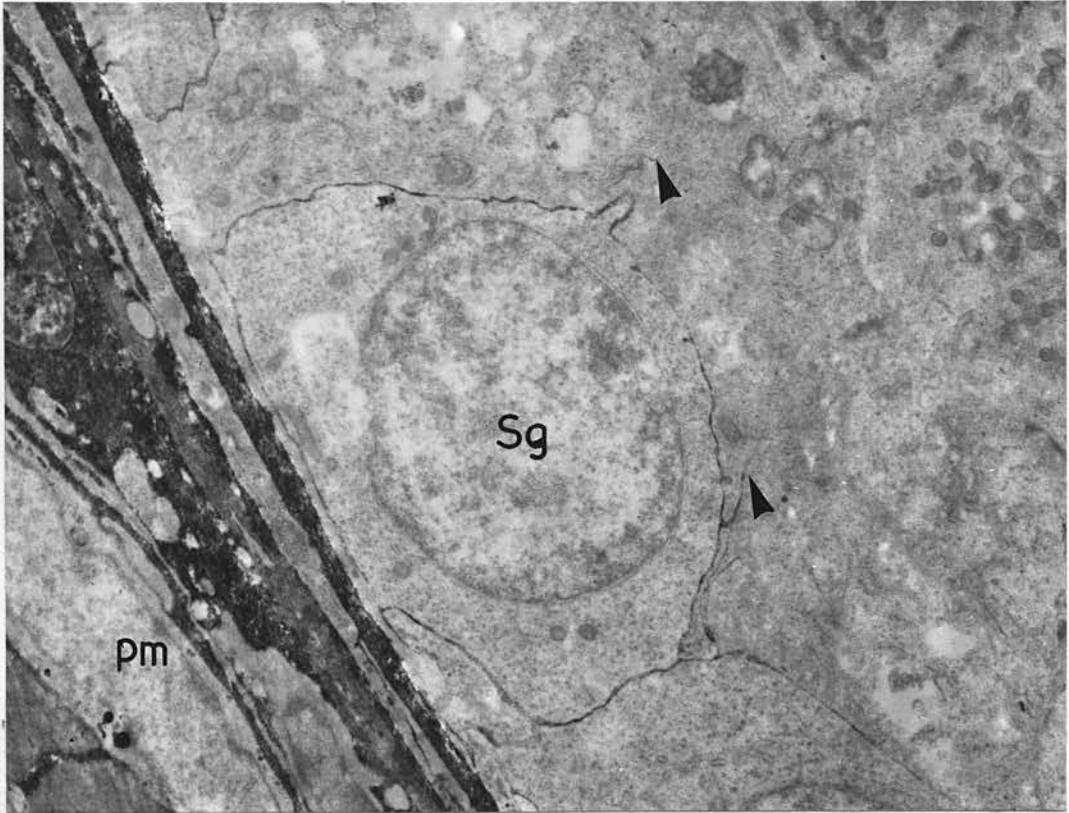
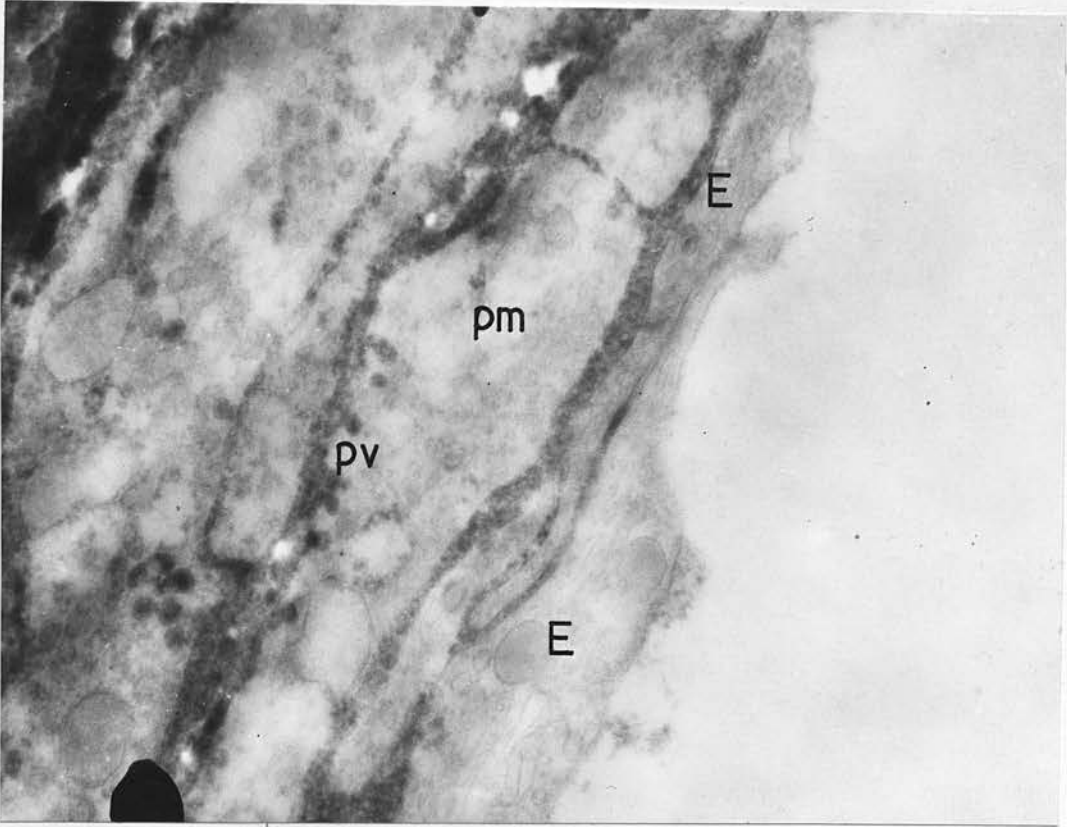


Figure 4.27

21 week old bird. Higher magnification of arrowed portion of figure 4.26. Shows peroxidase reaction product within intercellular clefts between resting spermatocytes and Sertoli cells. Arrow shows point beyond which no reaction product is seen in the Sertoli-Sertoli cleft.

Unstained.

X 39,000.

Figure 4.28

21 week old bird. Peroxidase reaction product is observed in the intercellular cleft between a spermatogonium and a Sertoli cell. It does not penetrate the space between two Sertoli cells. Unstained.

X 84,000

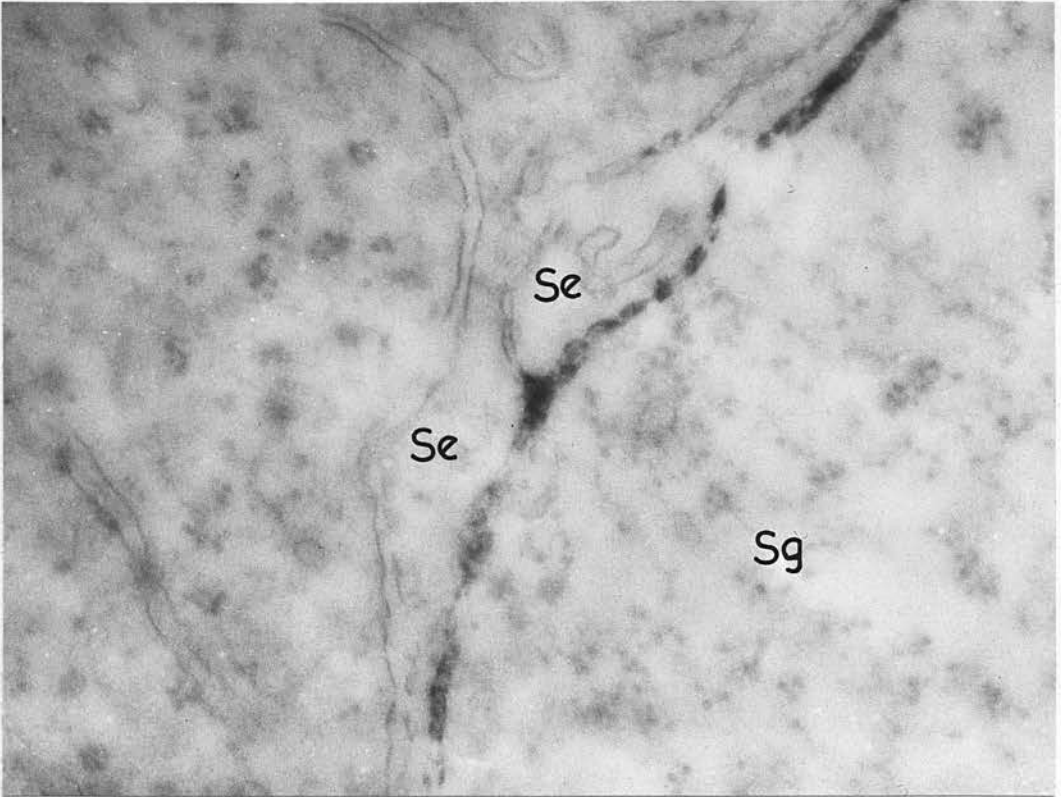
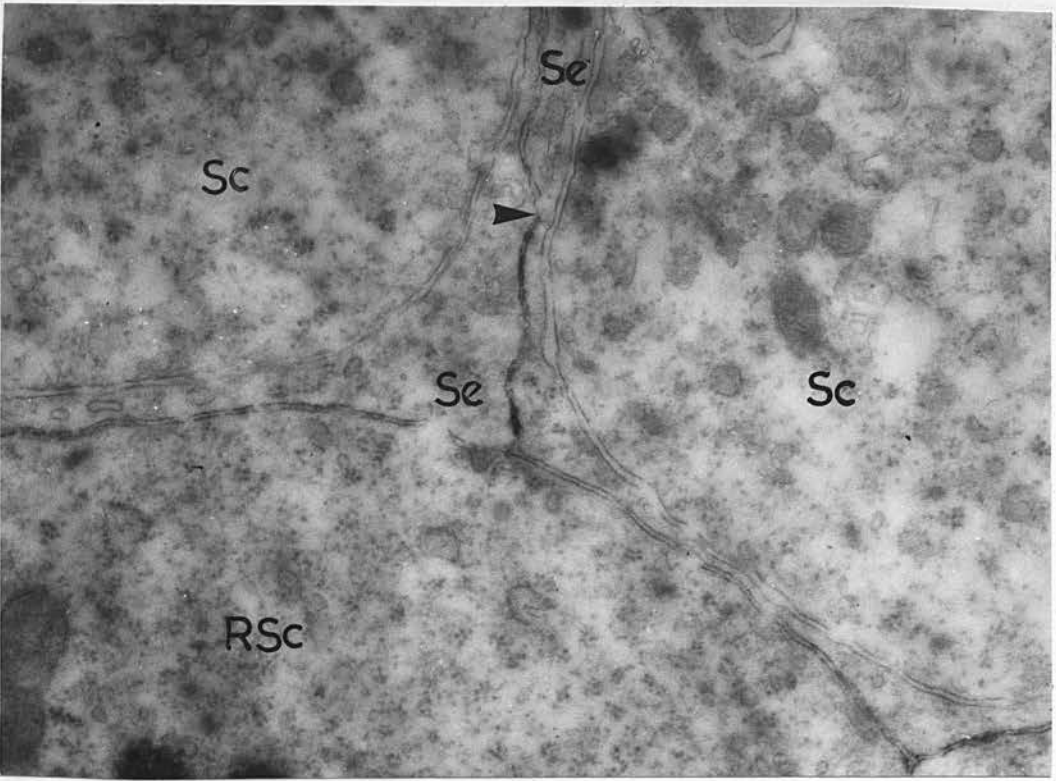


Figure 4.29

9 week old bird. Peroxidase reaction product is observed surrounding the spermatogonia and penetrating the Sertoli intercellular spaces to the centre of the tubule. Unstained.

X 6,000

Figure 4.30

9 week old bird. Peroxidase reaction product outlines a spermatogonium and penetrates the Sertoli intercellular spaces to the centre of the tubule. Unstained.

X 6,000,

