The Normal Development of the Blood-Testis Barrier and the Effects of Clomiphene and Estrogen Treatment [1]

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ABSTRACT Previous work with electron opaque intercellular markers has indicated that in adults occluding junctions between adjacent Sertoli cells are the principal morphological basis of the blood-testis barrier (Dym and Fawcett, '70). Since the barrier is not present at birth this study was undertaken to determine when it is established during postnatal development of the rat and to correlate its appearance with other developmental events in the seminiferous epithelium. From birth to 16 days of age interstitially injected tracers, such as horseradish peroxidase, freely entered the seminiferous epithelium and permeated the 200 Å intercellular clefts between presumptive Sertoli cells and germ cells, reaching the center of the seminiferous cords and site of the future tubule lumen. Between day 16 and 19 occluding junctions between Sertoli cells appear and thenceforth interstitially injected electron opaque tracers were effectively prevented from reaching the tubule lumen. Therefore, in rats, the blood-testis barrier is established between 16 and 19 days of age.

In an attempt to determine whether the development of the Sertoli cell junctions and the blood-testis barrier is under hormonal control circulating gonadotropins were suppressed using daily injections of clomiphene or estrogens from birth. The appearance of blood-testis barrier was delayed approximately seven days in the absence of gonadotropins. However, by day 26 the Sertoli cell junctions did appear and interstitially injected tracers were prevented from reaching the tubule lumen. Thus the development of the occluding junctions between Sertoli cells that constitute the permeability barrier does not appear to be directly dependent upon gonadotropins.

The failure of the nuclei in the seminiferous tubules to stain after intravenous administration of acridine dyes led Kormano ('67) to postulate a blood-testis permeability barrier comparable to the well documented blood-brain barrier. It was initially suggested that the barrier in the testis, like that in the brain, might reside in the walls of the capillaries. There was conflicting evidence based upon other methods, however, indicating that the capillaries of the interstitial tissue are highly permeable to plasma proteins and other large molecules (Everett and Simmons, '58; Lindner, '63; Mancini et al., '65). In later studies involving cannulation of both the testicular lymphatics and the rete testis of rams, Setchell, Voglmayr and Waites ('69) clearly demonstrated that many substances introduced into the blood appeared rapidly in the lymph but were excluded from the rete testis fluid. It was concluded therefore that the blood-testis barrier was not in the capillary wall but in or around the wall of the seminiferous tubules.

This conclusion has now been substantiated in morphological studies employing electron-opaque extracellular tracers of varying size (Fawcett, Leak and Heidger, '70; Dym and Fawcett, '70; Dym, '73). When the larger particulate markers car-

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bon and thorium dioxide are injected interstitially they are excluded from the seminiferous tubules of rat and guinea pig by the continuous peritubular layer of epithelioid contractile cells. This layer is therefore regarded as a significant component of the barrier in these species. It is not completely effective, however, for when tracers of smaller molecular size such as the protein peroxidase or lanthanum nitrate are injected intravascularly they rapidly traverse the capillary walls and accumulate in the interstitial spaces. They are also able to reach the base of the seminiferous epithelium in some areas by passing through occasional intercellular clefts in the myoid layer that lack occluding junctional specializations. They can penetrate the intercellular clefts around the spermatogonia and preleptotene spermatocytes but are barred from deeper penetration toward the tubule lumen by occluding junctional specializations between adjacent Sertoli cells. These sites of membrane fusion between the supporting cells at or near the base of the seminiferous epithelium are the most effective component of the blood-testis barrier.

The development of the blood-brain barrier to proteins begins early in fetal life (Olsson et al., '68) and is already well established at birth (Dobbing, '68). Light microscopic studies of the distribution of acridine dyes in the testis, on the other hand, (Kormano, '67) indicate that the permeability barrier in this organ is absent at birth and develops gradually over a period of about 20 days. The present electron microscopic study using the protein tracer peroxidase and lanthanum nitrate was undertaken in the hope of defining more precisely the time of appearance of the blood-testis barrier and of determining its ultrastructural and functional correlates.

Results of the present study indicate that the blood-testis barrier in the rat is established between the sixteenth and nineteenth postnatal days in close temporal correlation with the appearance of the junctional complexes between Sertoli cells, the onset of fluid secretion by the seminiferous epithelium, stratification of the germinal epithelium, and the development of a lumen in the seminiferous tubules. Suppression of gonadotropin release by daily administration of clomiphene or estrogen from birth delayed, but did not prevent, the appearance of the barrier. The development of the occluding junctional complexes between Sertoli cells thus does not appear to be under direct control of gonadotropic hormones.

MATERIALS AND METHODS

Normal development series. The littermate offspring of 12 Charles River Sprague-Dawley female rats were sexed at birth and, where possible, six males were left to each mother. All rats were weaned at four weeks. Rats were sacrificed at 4, 10, 15, 17, 18, 20, 30, 45 and 60 days, and the testes were fixed by vascular perfusion. The testes of the four day and ten day old rats were perfused via the thoracic aorta according to the following technique: Under deep ether anesthesia an abdominal incision was made, and the diaphragm was exposed and cut along its origin from the costal margin. A cut was made through the left anterior thoracic wall extending from the inferior costal margin to the clavicle. Two Halsted mosquito forceps were used to retract the borders of the incision. The left lung was reflected anteromedially thus exposing the thoracic aorta. A 25 gauge needle at the end of a Y venoclysis system was attached to a small reservoir of 0.9% saline. While slowly dripping saline the needle was inserted in the direction of the blood flow into the descending aorta near its origin. Saline was allowed to flow briefly and then was followed by the aldehyde fixative.

The testes of animals from 15 days of age to adults were perfused using a retrograde abdominal aorta perfusion technique. The abdominal cavity was opened and the abdominal aorta was carefully exposed and cleared of connective tissue and fat. Care must be exercised in order to avoid touching the inferior vena cava and the testicular vessels which are visible on the posterior abdominal wall. The left kidney was reflected anteromedially and the retroperitoneal space just above the kidney was opened with a curved blunt iris forceps. After identifying the left renal artery and tracing it to its origin a silk suture (000) was placed around the aorta just

above the origin of the left renal artery and left untied. The lowest portion of the aorta was grasped with a curved Halsted forceps and slightly elevated. A needle (ranging from 18 to 25 gauge, according to the size of the aorta) was inserted in a retrograde direction into the abdominal aorta near its bifurcation. Immediately the flow of saline was established at a pressure of 130 cm of H_2O, the suture around the aorta was tied and the inferior vena cava or the left renal vein incised to permit an egress of fluid. As soon as the testicular vessels were cleared of blood, saline was followed with the fixative. The volume of fixative varied with the age of the rat up to 100–150 cm^3 in an adult.

The fixatives used were either 5% glutaraldehyde buffered in s-collidine (Bennett and Luft, '59) or diluted Karnovsky's fixative (Karnovsky, '65) buffered with collidine. Following perfusion, the hardened testes were removed from the animal and 1 mm blocks were cut from thin transverse slices and postfixed by immersion in the same solution for an additional 30 minutes at 4°C. After a brief wash in buffer (15 minutes) the tissue was transferred to buffered osmium tetroxide for two to three hours at 4°C, dehydrated rapidly in graded concentrations of ethanol and embedded in Epon or Epon-Araldite mixture.

When using lanthanum as an intercellular tracer 4% aqueous lanthanum nitrate was slowly adjusted to pH 7 with 0.1 N NaOH (Revel and Karnovsky, '67). An equal volume of this solution was added to the aldehyde fixative so that the final concentration of glutaraldehyde was 5% and the lanthanum 2%; the same lanthanum concentration was maintained in all subsequent solutions up to and including the osmium tetroxide.

Peroxidase studies involved the intratesticular injection of 0.05 to 0.1 milliliter of Sigma type II horseradish peroxidase at a concentration of 20–40 mg per milliliter of Ringer's solution. After 15–20 minutes the testis was removed, 1 mm cubes were prepared and fixed in full strength Karnovsky's ('65) fixative using 0.2 M cacodylate buffer. In some experiments 1 mm blocks of testis tissue was incubated for 15 minutes in 25 mg of peroxidase dissolved in 0.3 ml of Ringers and then fixed. Following a two to four hour fixation and an overnight wash in cacodylate buffer the tissue was reacted in diaminobenzidine tetrachloride and hydrogen peroxide using the method of Graham and Karnovsky ('66). Subsequently the tissue was osmicated in 1.3% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon.

Experimental series. Littermates were sexed at birth leaving six males per mother. One half of each litter was injected intraperitoneally from birth with 1 mg/kg/day of clomiphene citrate [2] suspended in distilled water. Animals were sacrificed at three day intervals after day 20 of treatment. The other half of each litter was sacrificed at the same intervals and used as controls. A second group of littermates was injected subcutaneously daily from birth to 60 days with 100 μg/day of β-estradiol-3-benzoate suspended in corn oil. Animals from this group were sacrificed at the same intervals, again using the other half of each litter as controls. Lanthanum nitrate and horseradish peroxidase were used as described above to determine the presence of a diffusion barrier in the seminiferous tubules. Control groups were processed routinely for electron microscopic examinations.

OBSERVATIONS

Normal postnatal development of the blood-testis barrier

In the four-day rat testis, the seminiferous tubules are represented by solid cords consisting of gonocytes and relatively undifferentiated supporting cells (fig. 1). The gonocytes tend to occupy the central portion of the seminiferous cords and are surrounded by the Sertoli cell precursors. The germ cells are large pale cells with a centrally placed nucleus and a cytoplasm poor in organelles. The immature Sertoli cells are smaller and more deeply staining. Their nuclei are somewhat irregular in outline and the cytoplasm contains a number of mitochondria, elements of the smooth and rough endoplasmic reticulum, small Golgi complex, and numerous free ribosomes. The lateral surfaces of the supporting cells are relatively straight and sepa-

[2] Clomiphene citrate (Lot C-74374) was generously supplied by W. S. Merrell, Co., Cincinnati, Ohio.

rated by an intercellular cleft about 200 Å wide. No desmosomes or other specializations for cell-to-cell attachment are noted at this time. The ends of the Sertoli cell precursors directed toward the center of the cord, where a lumen will later form, are thrown up into elaborate folds and irregular cell processes that interdigitate with those on the apex of opposing cells of the same type.

The interstitial tissue is quite cellular and consists of vascular elements and plump spindle-shaped cells resembling fibroblasts. These are arranged circumferentially around the seminiferous cords but do not form a continuous epithelioid layer as in adult testis (fig. 1). No junctional complexes or areas of membrane apposition are observed at sites of close approximation between adjacent cells.

Peroxidase administered interstitially in vivo or lanthanum perfused with the fixative passes readily between the cells lining the capillaries and accumulates in the interstitial tissue. It also penetrates freely through the adventitia of the seminiferous cords and permeates the intercellular clefts among the primitive supporting cells and between these and the gonocytes (fig. 2). Thus on the fourth postnatal day, there is no demonstrable blood-testis permeability barrier.

In the continuing postnatal development of the cords, the gonocytes move to the periphery where they undergo a number of divisions and differentiate into typical spermatogonia (fig. 3). Type A spermatogonia are identifiable by the eighth postnatal day, Type B spermatogonia are present by day 9 and preleptotene spermatocytes at day 15 to 16 (Clermont and Perey, '57). By the twelfth day, the lamina propria of the seminiferous cords has become recognized so that the cells form a single layer with their margins in close apposition. Occluding junctions are occasionally seen on these cell boundaries. The cells already contain cytoplasmic filaments like those of smooth muscle cells. From this time onward, the peritubular layer of myoid cells is similar to those of mature seminiferous tubules.

During the period from the fourth to the fifteenth day, the intercellular clefts of the seminiferous cords remain freely accessible to the electron opaque tracers, lanthanum and peroxidase. Up to day 15, tracers penetrate easily to the center of most of the cords (figs. 2, 4) and outline all of the germ cells and supporting cells.

During days 15 to 18, electron micrographs begin to show typical junctional complexes between Sertoli cells near the base of the epithelium. Their development is heralded by the appearance of small subsurface cisternae, near the apposed cell membranes. These profiles elongate and neighboring cisternae coalesce to form more extensive cisternae. Bundles of filaments are formed between the cisternae and the cell membrane. The intercellular space in these specialized areas becomes narrowed to about 70 Å and at irregular intervals the opposing membranes appear to be in contact. These junctional specializations between Sertoli cells are often located near the basal lamina but they also frequently develop immediately above spermatogonia or preleptotene spermatocytes. Concurrently with the development of these junctions, a lumen begins to open in the center of the seminiferous cords (fig. 5).

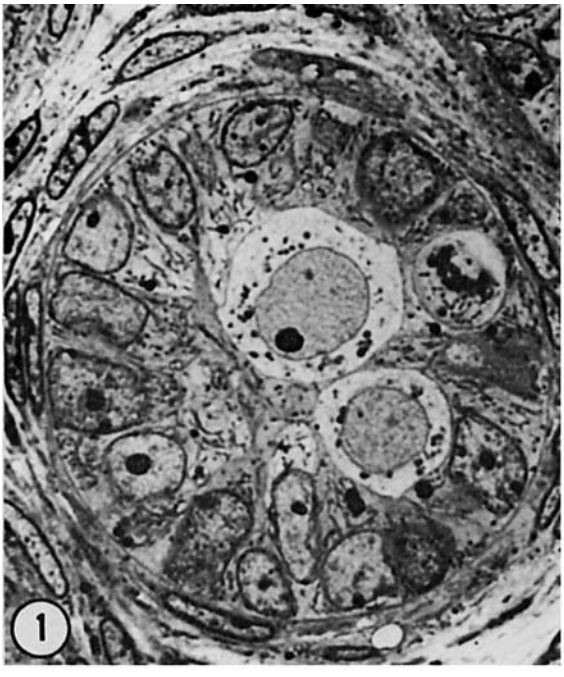
From day 18 onward, the electron opaque tracers penetrate only into the base of the seminiferous epithelium where they outline the spermatogonia and preleptotene

Fig. 1 Light micrograph of a seminiferous cord of a four-day-old rat as it appears in a toluidine blue stained plastic section. The large germ cells possess a pale staining cytoplasm and tend to be situated toward the middle of the cord. The Sertoli cell precursors are more numerous and line the periphery of the cord. × 500.

Fig. 2 Light micrograph of a seminiferous cord of a four-day-old rat. In this otherwise unstained section, the distribution of the black product of the histochemical reaction for peroxidase reveals that the tracer has penetrated the intercellular spaces throughout the seminiferous cord. There is thus no "blood-testis" barrier at this stage of development. × 500.

Fig. 3 Light micrograph of a seminiferous cord of a 15-day-old rat. Spermatogonia are now localized on the basal lamina of the seminiferous epithelium and spermatogenesis has progressed to pachytene spermatocytes. × 1400.

Fig. 4 Light micrograph of a peroxidase preparation of a 15-day-old rat demonstrating that an effective blood-testis barrier has still not been established. This is an oblique longitudinal section. The peroxidase freely permeated all the intercellular spaces including the apical elaborations of the Sertoli cells at the site of the future tubule lumen (left central area). × 1100.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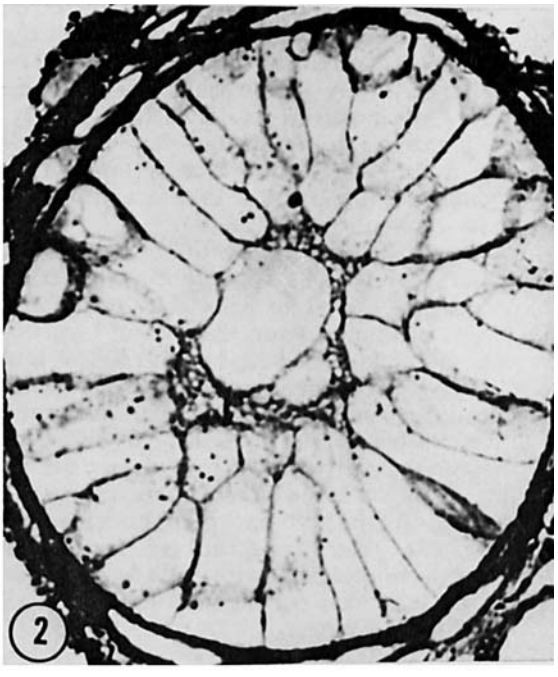
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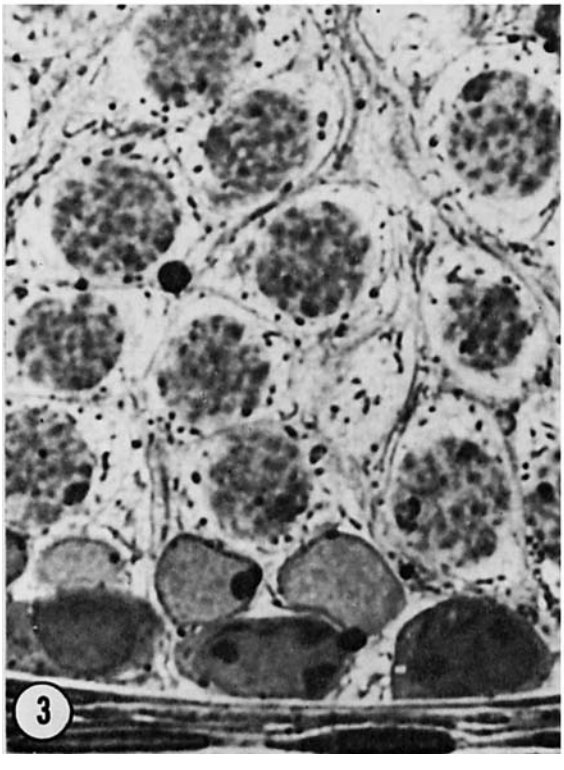
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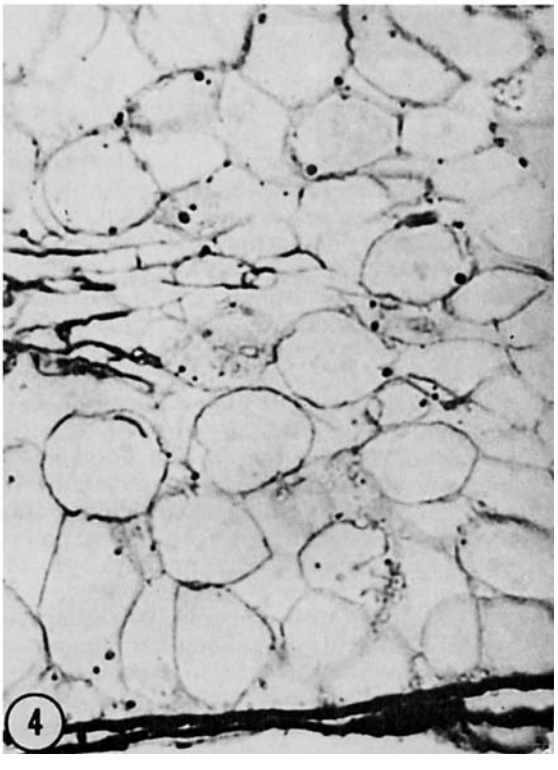
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spermatocytes (figs. 6, 9). Deeper penetration seems to be prevented by the recently developed junctional specializations between adjacent Sertoli cells. The blood-testis barrier is thus already established by the eighteenth to nineteenth day and electron micrographs show tracer confined to the basal compartment of the epithelium as in the adult (figs. 7, 8, 9).

In the adult, the Sertoli-Sertoli junctional complexes constitute a barrier separating the germ cells that rest upon the basal lamina from the second layer of cells closer to the tubule lumen (figs. 7, 8). During development prior to 14 days of age the germinal component of the seminiferous epithelium consists only of spermatogonia and early spermatocytes forming a single layer of cells on the basal lamina. As pachytene spermatocytes differentiate they move off of the basal lamina. Coinciding with this event, the Sertoli junctions develop and establish the blood-testis barrier.

Effect of gonadotropin suppression on development of blood-testis barrier

The coincidence in the time of appearance of (1) the junctional complexes between Sertoli cells, (2) the establishment of a permeability barrier, (3) the onset of fluid secretion, and (4) the appearance of pachytene spermatocytes, suggested that physiological compartmentation of the epithelium may be a necessary precondition for the normal completion of meiosis and initiation of spermiogenesis. Since differentiation of large numbers of germ cells beyond pachytene spermatocytes does not occur in the absence of hormones, it was of interest to investigate whether the differentiation of the Sertoli cell junctions is hormone dependent. Hypophysectomy is not technically feasible in newborn rats, therefore, we undertook to suppress gonadotropin release by administration of exogenous estrogen or of clomiphene.

The ability of exogenous estrogens to produce infertility in the male by suppressing gonadotropin secretion is well established (Emmens and Parkes, '47; Ladosky and Kesikowski, '69). This effect can be overcome by simultaneous administration of gonadotropin (Steinberger and Duckett, '65; Perklev and Groning, '69). Thus the results of estrogen administration in the male mimic hypophysectomy and seem to provide a useful experimental approach to the assessment of possible direct effects of gonadotropin differentiation of Sertoli cell junctions. An alternative is administration of clomiphene, a non-steroidal congener of the synthetic estrogen chlorotrianicene. Clomiphene has been extensively used in clinical gynecology to induce ovulation. Its action in the rat appears to be quite different from that in man. It has been shown that in the male rat it inhibits the release of gonadotropin. Administration of 1 mg/Kg to adult rats for 30 days produces results on the testis and accessory glands that are comparable to hypophysectomy (Nelson and Patanelli, '65).

In the normal animals, the testis descended into the scrotum at about 20 days of age. The testes of estrogen and clomiphene-treated newborn rats remained intra-abdominal until about 40 days and were softer and about half the size of those in control animals.

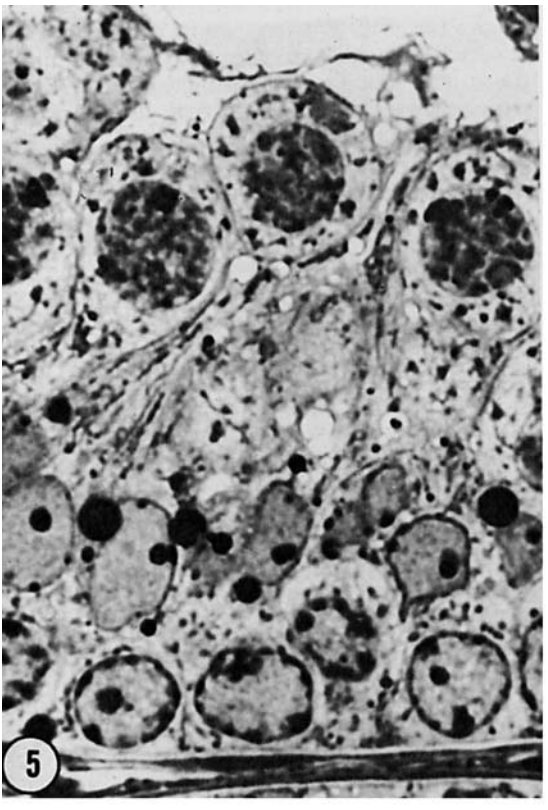
Whereas the appearance of junctional complexes between Sertoli cells and the establishment of a blood-testis barrier normally occur on the sixteenth to eighteenth day of postnatal life (fig. 11), interstitially injected peroxidase penetrates into all parts of the seminiferous cords of clomiphene or estrogen-treated animals up to about day 24 with some individual variation (fig. 12). The junctional complexes then appear and a permeability barrier to peroxidase is first demonstrable between

Fig. 5 Light micrograph of a section of a seminiferous tubule of an 18-day-old rat stained with toluidine blue. Spermatogonia are aligned on the basal lamina while pachytene spermatocytes border on the recently developed lumen of the tubule. × 1400.

Fig. 6 Light micrograph of peroxidase reaction in the seminiferous tubule of an 18-day rat. The blood-testis barrier has now been established. The peroxidase traverses the tunica propria of the tubule but only penetrates the epithelium far enough to outline the spermatogonia. × 1400.

Fig. 7 Light micrograph of a section of a seminiferous tubule of an adult rat stained with toluidine blue. × 1600.

Fig. 8 Light micrograph of a peroxidase reaction in an adult seminiferous tubule. The blood-testis barrier is essentially unchanged over the condition attained by the 18th to 20th postnatal day. × 1600.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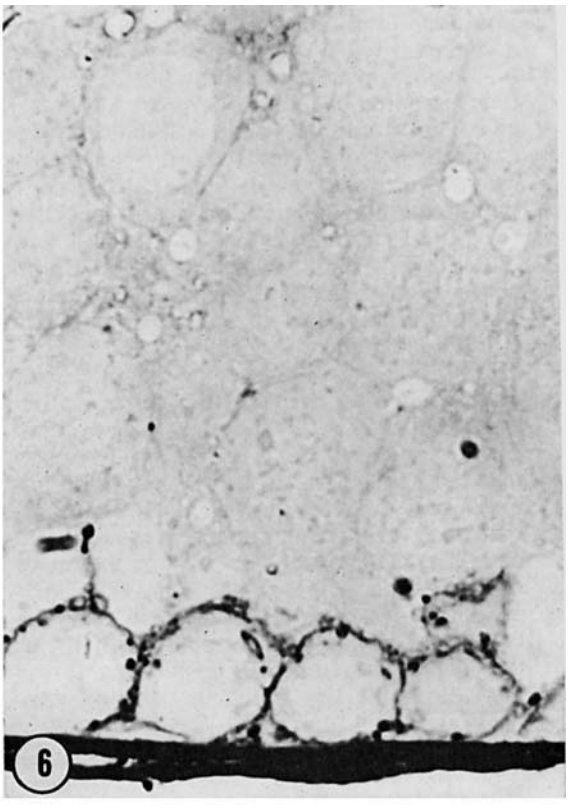
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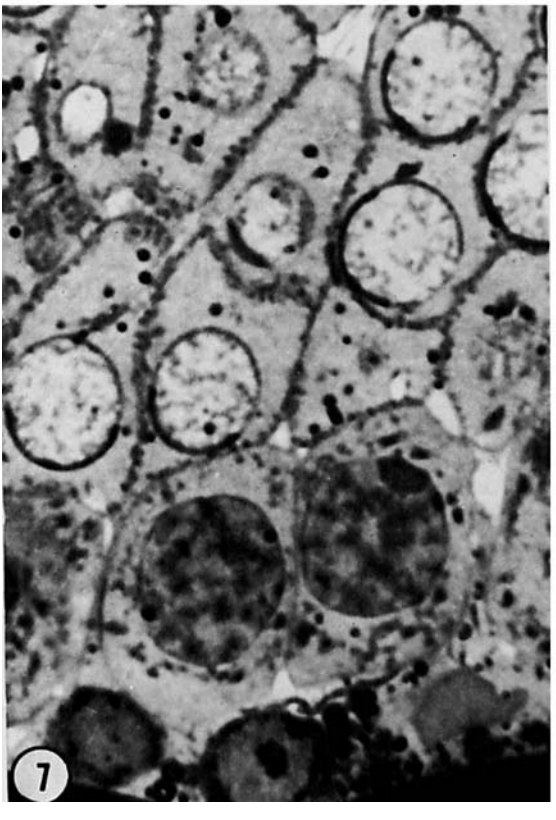
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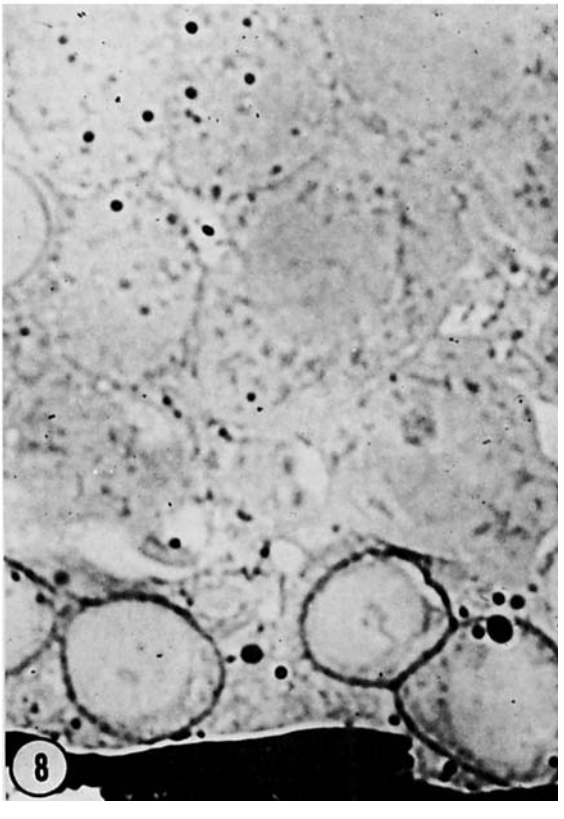
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days 25 and 28. The development of the junctions and appearance of lumen are associated with pachytene spermatocytes in normal postnatal animals. In clomiphene-treated animals, the most advanced germ cell type is rather difficult to determine because preleptotene spermatocytes seem to develop under these conditions into abnormally large and abortive pachytene spermatocytes.

Following 30 days of clomiphene treatment, the lumen of the tubules is unusually large and most of the germ cells are exfoliated and floating free in the lumen. The epithelium at this stage is essentially cuboidal with relatively few degenerating germ cells in it. This appearance continues up to 60 days of treatment when observations were discontinued. The sequence of events following estrogen treatment is similar but nuclear pycnosis and cellular desquamation are even more marked than after clomiphene. The suppression of gonadotropin release thus delays but does not prevent establishment of the blood-testis barrier despite profound alterations in the cytology of the seminiferous epithelium.

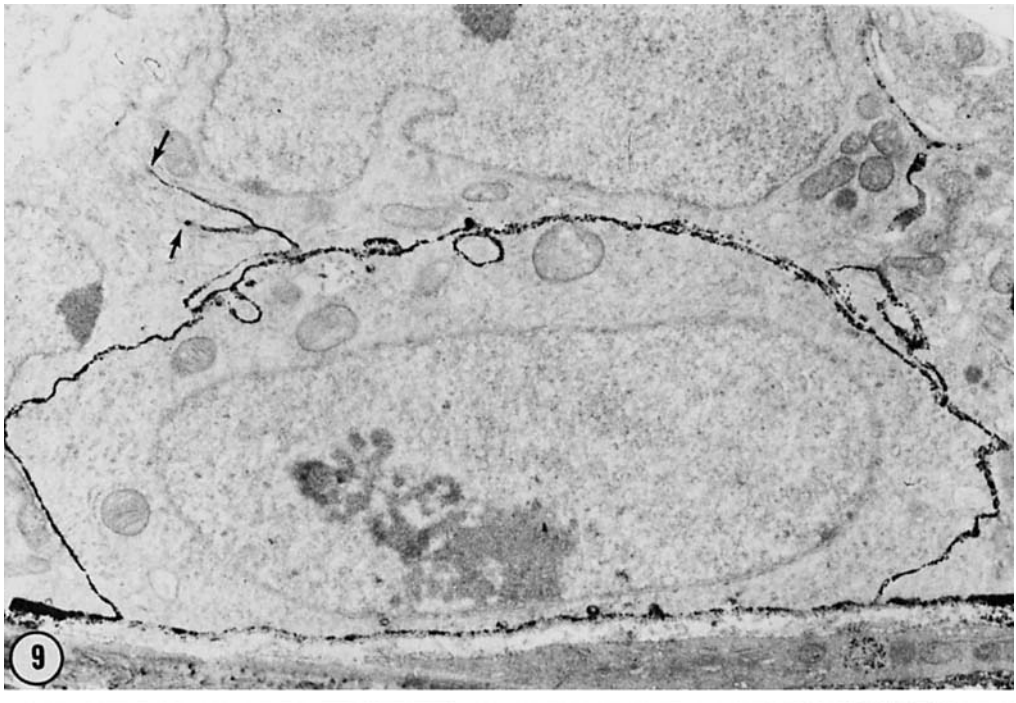
DISCUSSION

It is widely accepted that the vascular bed of the testicular interstitial tissue is highly permeable to large molecules, despite the fact that capillaries are not of the fenestrated type found in most endocrine glands. Morphological evidence for their unusual permeability comes from various sources including radioautographic observations on the distribution and on the rate of extravascular appearance of ^{125}I-labelled serum albumen after intravenous injection (Everett and Simmons, '58); light microscopic observations on the distribution of fluorescein labeled serum proteins (Mancini et al., '65); the rapid detection of interstitial fluorescence after injection of acriflavine (Kormano, '67); and electron microscopic observations on the rapid appearance of intravascularly injected peroxidase in the interstitial spaces of the testis. These observations are strongly reinforced by physiological studies demonstrating that a variety of substances injected intravenously appear rapidly in the testicular lymph but are excluded from the rete testis fluid (Setchell, '67; Setchell, Voglmayr and Waites, '69). These latter observations led to the conclusion that there is a blood-testis permeability barrier which is located in or around the wall of the seminiferous tubules. This assumption was substantiated by electron microscopic studies which employed particulate tracers and showed that these were excluded from the tubules by occluding cell-to-cell junctions between peritubular myoid cells and between the bases of the Sertoli cells in the seminiferous epithelium (Fawcett, Leak and Heidger, '70; Dym and Fawcett, '70; Dym, '73).

Whether glycoprotein hormones and other biologically active large molecules can gain access to the advanced male germ cells and to the tubule lumen in the adult remains a subject of controversy. Physiological studies have shown a very low concentration of protein in the rete testis fluid (Johnson and Setchell, '68) and have demonstrated exclusion of a wide range of substances injected into the blood (Setchell et al., '69). On the other hand, some morphological studies reported in the literature are in disagreement. Mancini et al. ('65) presented immunohistochemical evidence for diffusion or transport of homologous serum proteins into the epithelium and into the lumen of the tubules. There are other reports that ferritin-conjugated follicle stimulating hormone is incorporated into Sertoli cells and diffuses toward the lumen (Castro et al., '70) and also that in isolated tubules immersed in 20% sodium ferrocyanide, the ferrocyanide could be demonstrated by the Prussian blue reaction in the cytoplasm of the Sertoli cells and ultimately in the lumen (Vilar and Mancini, '70). Similarly ferritin-labeled homologous albumin is said to be taken into Sertoli cells by pinocytosis and after sev-

Fig. 9 Electron micrograph of the base of the seminiferous epithelium of an 18-day postnatal rat testis perfused with lanthanum nitrate. As in the case of peroxidase, the tracer outlines the spermatogonia, starts out into the intercellular cleft between the overarching Sertoli cells, and there it is stopped abruptly (at the arrow) by a specialized occluding junction. × 9000.

Fig. 10 Electron micrograph of a testis prepared similarly to that of figure 9 but from an adult rat. There is no essential difference in the barrier in the 18-day and the adult rat. × 9000.



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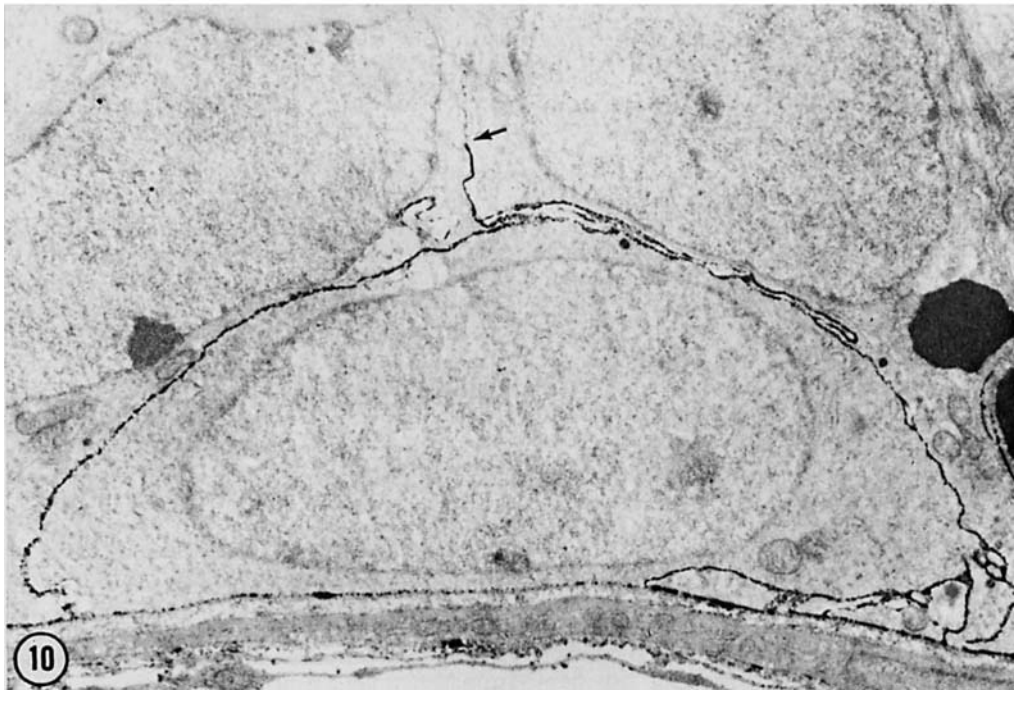

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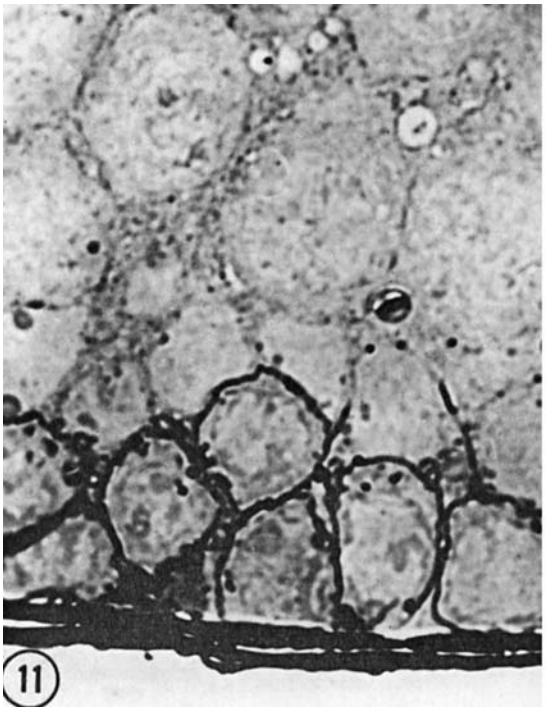

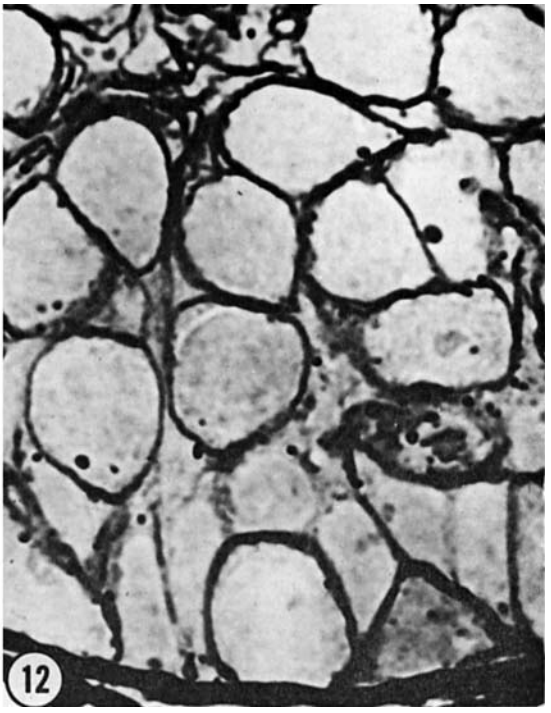

Fig. 11 Photomicrograph of a 25 day control rat with a well established barrier limiting penetration of peroxidase to the basal third of the epithelium. × 1600.

Fig. 12 Photomicrograph of a 23 day estrogen-treated animal showing penetration of peroxidase throughout the epithelium. A barrier has not yet been established a week or more beyond the normal time of appearance of occluding Sertoli junctions. × 1600.

eral hours is visible in the apical cytoplasm and in the lumen (Vilar and Mancini, '70). It is also claimed that the Sertoli cell is capable of taking up peroxidase from blood and transporting it to the lumen (Reddy and Svoboda, '65). The observational basis for this latter claim is so contrary to our own experience using peroxidase that it seems likely there must have been some element of artifact in the results of these authors or that cytotoxicity of the enzyme preparation at the concentrations used caused damage to the Sertoli cell membrane. In our hands, penetration of peroxidase into the rat tubules is retarded by the peritubular contractile layer and, in some areas, the tracer is excluded by occluding junctions between these cells. In other areas, the junctions in this layer are permeable and the marker enzyme reaches the base of the epithelium. It then enters the intercellular clefts between the spermatogonia and the overarching Sertoli cells, outlining the former in electron micrographs with a thin black line of peroxidase reaction product. At longer time intervals, very small amounts of peroxidase are taken up by spermatogonia and Sertoli cells in micropinocytotic vesicles. We have never observed uptake of large amounts of the marker enzyme or its transport to the apical portion of the epithelium and into the lumen such as reported by Reddy and Svoboda ('65) and by Vilar and Mancini ('70).

In our experience, the barrier is remarkably effective in excluding horseradish peroxidase, a protein of molecular weight 40,000. It seems entirely possible that it is equally effective in excluding glycoprotein gonadotropic hormone of molecular weight 30,000. These hormones would have relatively free access to the base of the Sertoli cells and to the spermatogonia, but

it would appear to us that any effects of gonadotropins on germ cells more advanced than spermatogonia and preleptotene spermatocytes would have to be secondary to the action of these hormones on the Sertoli cells.

Gonadotropins are evidently not necessary for spermatogenesis in the adult male rat for sperm production can be maintained in hypophysectomized rats by systemic (Boccabella, '63) or local administration (Ahmad, Haltmeyer and Eik-Nes, '72) of androgen alone. There are indications, however, that gonadotropins are essential for initiation of the process in the immature animal. Shortly before the establishment of the blood-testis barrier, there is a steep rise in the level of gonadotropin in the blood, reaching adult levels by day 25 (Swerdloff et al., '71). It has recently been shown that a marked testosterone peak occurs between 17 and 20 days of age in male rats (Yukitaka et al., '73). This is correlated closely with the time of appearance of the blood-testis barrier, i.e. between 16–19 days.

It may be significant that the absence of a permeability barrier in the postnatal period would make it possible for glycoprotein hormones to penetrate throughout the seminiferous cords. The establishment of the barrier after the onset of spermatogenesis suggested the possibility that the development of the junctional specializations of the membranes that close off the adluminal two thirds of the seminiferous epithelium might be induced by direct action of gonadotropins or androgens on the Sertoli cells. This has not been substantiated in the present study. The junctional specializations creating the barrier developed despite the suppression of gonadotropin release. The delay in their appearance would seem to be correlated with the delay in testis descent and general retardation of testicular growth in the estrogen and in the clomiphene treated animals. Thus the development of the junctions is not specifically dependent upon gonadotropins.

This conclusion is in accord with the findings of Johnson ('70) who compared the permeability barrier to acriflavine in the testis of hypophysectomized and sham-operated mature male rats. The acriflavine staining pattern in the two groups remained the same up to 45 days after hypophysectomy. Similarly in experiments of Setchell et al. ('69) the rubidium rejecting compartment of rat testis was still present five month after hypophysectomy. The small but significant delay in development of the barrier reported here in estrogen treated neonatal rats was not found in similar, but unpublished experiments by Johnson.

The observation that the development of junctional complexes immediately precedes the onset of fluid secretion and appearance of a lumen strongly suggests that creation of a barrier to extracellular movement of fluid through the base of the epithelium may be an essential precondition of these events. There is need for further study of the possible dependence of the secretory activity of the tubules on the presence of these occluding junctions between Sertoli cells. Equally important will be a study of their other possible functions. In addition to the linear fusions of the outer leaflets of opposing membranes that close the intercellular spaces, there are also localized specializations resembling the gap junctions of nexuses which are known to provide for cell-to-cell communication and coordination of activities in other epithelia. The possible role of these in regulating the cyclic activities of the seminiferous epithelium remains to be explored. This and other morphological aspects of the membrane biology of the seminiferous tubules can best be explored in replicas of freeze-cleaved preparations which provide extended views of the internal organization of cell membranes. Some preliminary observations of this kind will be presented in a subsequent paper (Fawcett and Gilula, '73).

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