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The Ultrastructure of Immature Sertoli Cells. Maturation-Like Changes During Culture and the Maintenance of Mitotic Potentiality

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ABSTRACT

Cell aggregates dissociated from 10-day-old rat testes form epithelial colonies in culture. These epithelial cells have been identified as supporting cells (immature Sertoli cells) by the close similarity between the nuclear and cytoplasmic structures of the cultured cells and those of supporting cells *in vivo*. The cultured supporting cells undergo maturation-like changes in serum-enriched media containing follicle stimulating hormone (FSH). These changes include a change in the pattern of chromatin condensation, the development of large nucleoli and nuclear infoldings, a progressive predominance of the smooth over the rough endoplasmic reticulum and the appearance of luminal (apical) differentiations. The differentiations of the luminal surface consist of finger-like cytoplasmic extensions, a regional accumulation of microfilaments and microtubules and the junctional complexes between neighboring cells near the lumen. In the presence of FSH there is a low but sustained mitotic activity in the population of cultured supporting cells. This FSH-dependent mitotic activity was observed from Day 4–15 in culture. It is suggested that while several structural differentiations of the supporting cells develop under these conditions *in vitro*, the luminal region of these cells does not differentiate in the same way as the apical region of Sertoli cells *in vivo*.

INTRODUCTION

The culture of Sertoli cell-enriched preparations has been recently described by Dorrington et al. (1974, 1975) in this laboratory and by other research groups (Steinberger et al., 1975; Welsh and Wiebe, 1975). Light and electron microscopic observations showed the presence of Sertoli cells in these cultures, although contaminant cell types were not completely excluded (Dorrington et al., 1975; Welsh and Wiebe, 1975). The testicular cell which responds to follicle stimulating hormone (FSH) *in vivo* has been tentatively identified as the Sertoli cell by a variety of criteria (Dorrington and Fritz, 1974; reviewed by Means, 1975; Fritz, 1977). In cultured Sertoli cell-enriched preparations, several specific biochemical responses to FSH have been described (Dorrington et al., 1975; Dorrington and Armstrong, 1975; Tung et al., 1975; Fritz et al., 1976; Griswold et al., 1976). Thus, an ultrastructural study of the long term effects of FSH and culture conditions on Sertoli cells could possibly provide information on the relationships between subcellular structures and the biochemical responses to that hormone.

The purpose of the present paper is to describe the ultrastructure of immature Sertoli cells (supporting cells) and to characterize the ultrastructural changes of these cells during culture in the presence of FSH. In particular, the development of nuclear and cytoplasmic maturation and the maintenance of the mitotic potentiality of some of these cells will be described.

MATERIALS AND METHODS

Preparation of Sertoli Cell-Enriched Aggregates (Dorrington et al., 1975)

Testes removed from 10-day-old Wistar rats (Canadian Breeding Laboratories, Montreal) are placed into Hanks' balanced salt solution (BSS) and after the tunica albuginea is removed the tissue is chopped into

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fragments of about 0.5 mm in diameter. The testicular fragments are incubated in Hanks' BSS containing 0.25% trypsin (Sigma Chemical Co., St. Louis) and 10 μg/ml of deoxyribonuclease (type I, Sigma) for 25 min at 32°C with shaking (60 oscillations/min). After the addition of soybean trypsin inhibitor (type 1S), the tissue suspension is passed through a copper wiregrid (1–2 mm mesh), washed in Hanks' BSS and incubated in Hanks' BSS containing 1 mg/ml collagenase (type I, Sigma) at 32°C for 25 min with shaking. The cell aggregates are then sedimented in polypropylene tubes by centrifugation for 2 min at 400 rpm in an International clinical centrifuge. The loosely packed pellet is resuspended in Hanks' BSS containing 1% bovine serum albumin and the cell aggregates are further dispersed by repeatedly drawing the suspension through a Pasteur pipette. The aggregates are filtered through a double layer of cloth obtained from nylon hose, resedimented and then washed in Hanks' BSS before being resuspended in culture medium for plating.

Culturing Procedure

Aliquots of cell aggregates suspended in medium are plated at intermediate density (approximately 0.5 mg cell protein/5 ml of medium). The basic culture medium (type A) consists of a modified Eagle's minimum essential medium (MEM) supplemented with 0.1 mM of each of seven nonessential amino acids and 4 mM glutamine (Steinberger et al., 1975; Dorrington et al., 1975). Antibiotics are present in the following final concentrations: penicillin (50 units/ml); streptomycin (50 μg/ml); and fungizone (0.51 μg/ml). A second medium (type B) consists of the basal (type A) medium with the addition of 10% calf serum. For culture of cell aggregates prepared from testes of older rats (20 days old), 2 additional media are used. Types C and D media consist of the basal medium enriched with 10% fetal calf serum and 1% rat serum, respectively. FSH (NIH-FSH-S10 and S11), when present, is added to the medium at a final concentration of 1 μg/ml, usually from the beginning of the culture. Cells are cultured on plastic Petri dishes 5 cm in diameter (Falcon Plastics, Oxnard, Canada) and they are kept at 32°C in a humidified incubator in an atmosphere of 95% air and 5% CO_2. The medium is changed every 48 h and the cultures are maintained for various periods of time up to 15 days. For studies on cell polarity, some cultures are grown on Millipore filters (type HA, 0.45 μm of pore size, Millipore Filter Corp., Bedford, Mass.) or on Nucleopore filters (0.2 μm pore size, A. Thomas Co., Philadelphia). The filters are either immersed in the medium, or held on top of a stainless steel grid with the medium level adjusted to touch the surface of the filter.

Processing of the Cells for Light Microscopy

The usual treatment with Giemsa's strain (Fisher Scientific Co., New Jersey) provides scarce evidence for cell identification in the cultures. For total mitotic counts, the monolayers are fixed with Carnoy's fixative (1 part acetic acid:3 parts of ethanol, v/v) for 15 min, dried and lightly stained with Giemsa's stain. Thick (1 μm) sections from epoxy-embedded cultures are most useful. Horizontal sections, cut parallel to the base of the dish or vertical thick sections are cut in a Porter-Blum ultramicrotome, spread with xylene vapour and attached to glass slides. The sections are stained with warm toluidine blue at alkaline pH.

Mitotic indices are obtained from the screening of 8000–10000 nuclei of supporting cells in thick sections (horizontal) of randomly selected regions of the cultures.

Procedures for Electron Microscopy

The cultures are fixed in 2.5% glutaraldehyde (Biological grade, Fisher) in 0.1 M Clark's phosphate buffer, pH 6.9 for 2 h at room temperature. After being washed in buffer, the cultures are postfixed in 1% OsO_4 in 0.1 M phosphate buffer for 1 h. In some cases the cultures are then washed in 0.1 M cacodylate buffer, pH 7.2, washed 2 min in 0.1 M sodium acetate, stained in a saturated solution of uranyl acetate in water for 20 min and washed again in sodium acetate solution before dehydration (Terzakis, 1968). The cultures are dehydrated with ethanol and embedded with Maraglas 655 without any passage through intermediate solvents. The embeddings are partially cured at 55°C overnight. The Maraglas resin sheet carrying the culture is then manually detached from the plastic dish and left for 24 h at 55°C to complete polymerization. Small pieces of the epoxy sheey are cut and glued to the top of flattened Maraglas blocks either lying flat (for horizontal sections) or perpendicular to the upper surface of the block (for vertical sections). Thin sections (silver or pale-gold interference colour) are cut with a Porter-Blum ultramicrotome in series of 5–20 or more for vertical sections. Horizontal thin sections are obtained as short series or single sections. Sections are picked on single-hole grids, transferred to Formvar membranes (Sjostrand, 1967) and dried. The sections attached to the membrane are stained with a saturated solution of uranyl acetate in methanol for 5 min, washed with methanol and stained with lead citrate (Reynolds, 1963). The sections are examined in a Philips-300 electron microscope.

RESULTS

Structure of the Seminiferous Epithelium in Testes from 10-Day-Old Rats

In 10-day-old rats the seminiferous epithelium is predominantly composed of supporting cells (Fig. 1). These cells have elongated nuclei with the major axis radially oriented towards the center of the seminiferous cords. These nuclei contain many small and dense chromatin flakes (Fig. 1), and are distinctly different from the round and homogeneously pale nuclei of primitive spermatogonia (Fig. 1). The supporting cells have not yet developed a conspicuous nucleolus. In contrast, spermatogonia have large nucleoli. The oval nuclei of the supporting cells have a regular outline except at the pole facing the basement membrane (Fig. 1), in which place nuclear infoldings may be present.

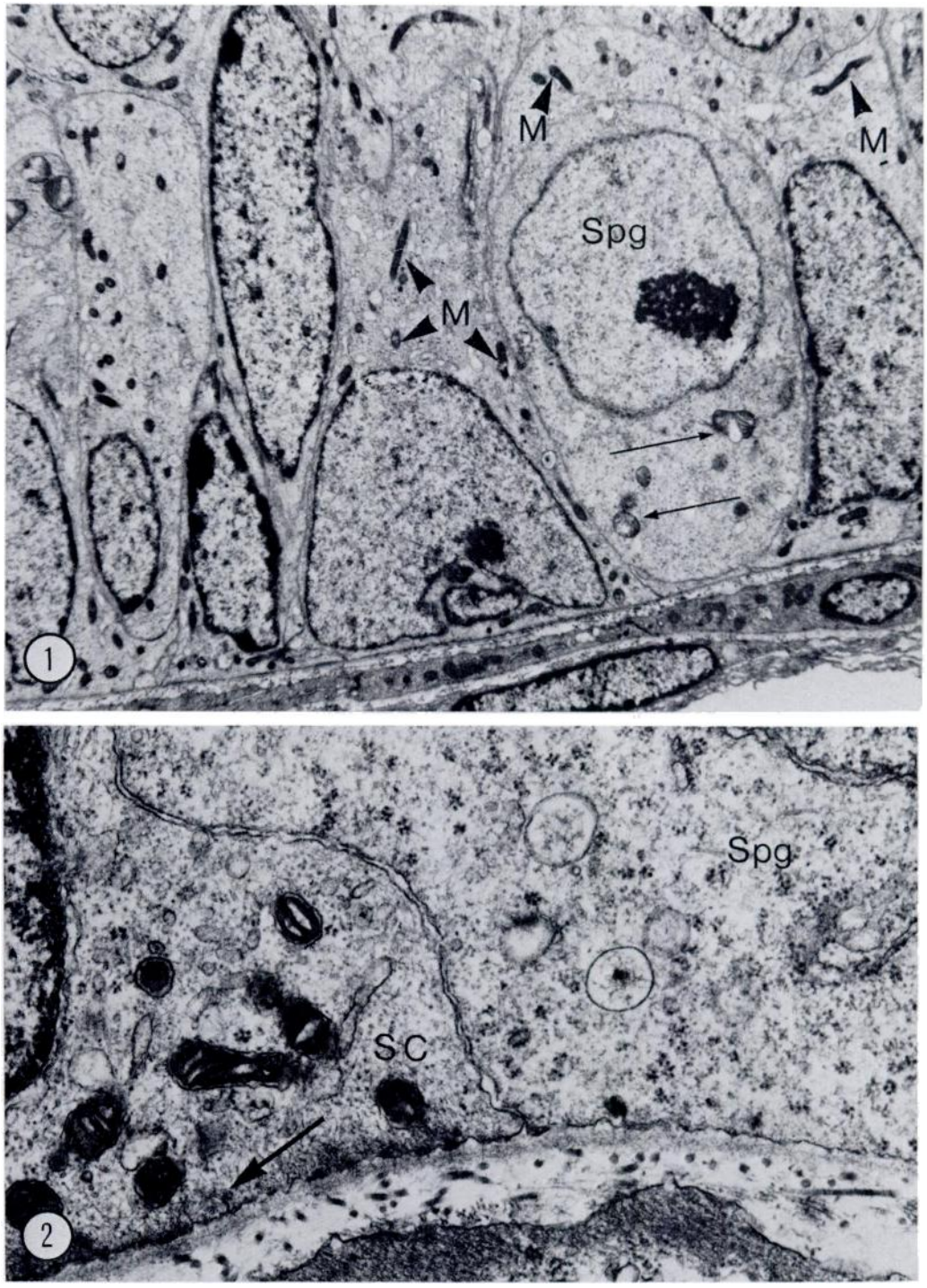


FIG. 1. Seminiferous epithelium of a 10-day-old rat. The large and round mitochondria (arrows) of the spermatogonium (Spg) are different from the mitochondria of the adjacent supporting cells (M, arrowheads). ×7,700.

FIG. 2. *In vivo*, the basal region of supporting cells (SC) shows a dense layer of microfilaments (arrow), which is absent in neighboring spermatogonia (Spg). ×42,000.

The cytoplasm of the supporting cells is bounded by distinct epithelial-like borders without specialized junctional complexes. The cytoplasm of the supporting cells contains a relatively greater number of organelles, microfilaments and microtubules than does the cytoplasm of germinal cells. The supporting cells have large numbers of long, slender (about 0.2 μm wide) mitochondria which contain a very dense matrix and irregularly arranged cristae (Fig. 2). In contrast, germ cells (mainly primitive type A spermatogonia) have wider (0.6–1 μm) and more round mitochondria with a lighter matrix and more regularly arranged cristae.

In the basal region of supporting cells, a dense layer about 50 nm thick is formed by packed, 6–8 nm wide microfilaments (Fig. 2). This dense layer, which also contains some microtubules and small vesicles, lies just beneath the cytoplasmic membrane associated with the basal lamina. On the other hand, germ cells do not show this dense layer. Furthermore, the cytoplasm of germ cells contains scarce quantities of endoplasmic reticulum and microfilaments, although they have large amounts of clustered ribosomes. The seminiferous epithelium makes contact with a thin (about 30 nm) amorphous basal lamina. Peripheral to this lamina, there is a layer of collagen fibers mixed with thinner fibrils and 1 or 2 layers of flattened peritubular cells. The peritubular cells are rich in filament bundles. They do not have epithelial-like borders between cells at this stage, but appear more fibroblast-like.

Ultrastructure of the Cell Aggregates at the Time of Plating

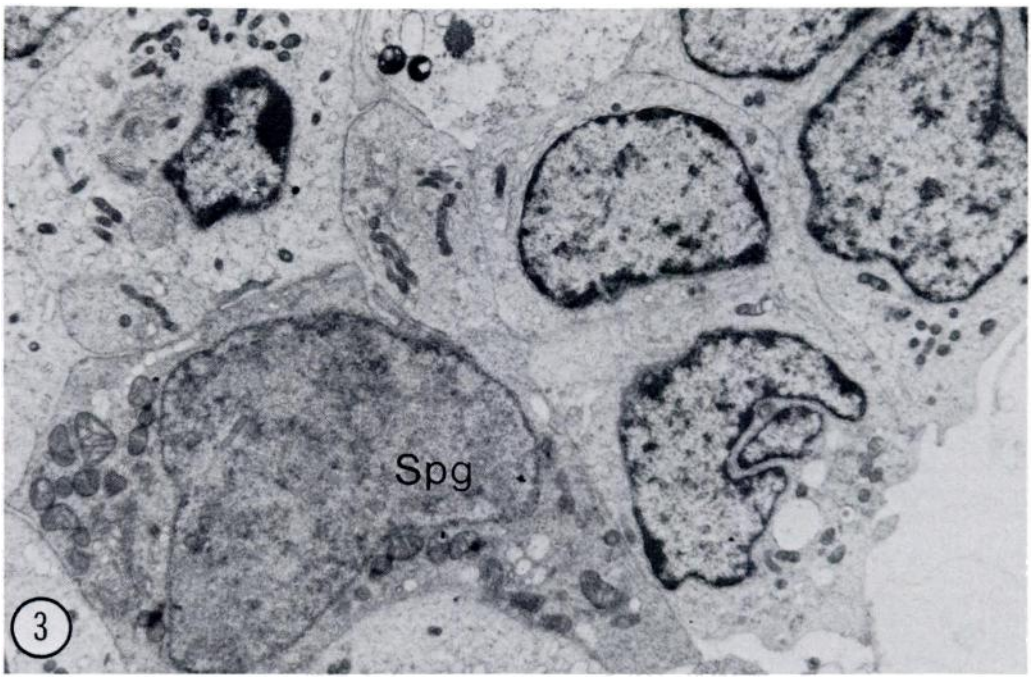
After the isolation procedures, the cell aggregates consist mainly of supporting cells (95.3% supporting cells, 2.4% germ cells, 0.2% peritubular cells and 2.1% unidentified cells). Occasionally small clumps of aggregated peritubular cells contaminate the preparations. Spermatogonia often show various degrees of ultrastructural impairment, such as increased density of the chromatin, cytoplasmic vacuoles and irregular cell borders. However, the isolated supporting cells show the same ultrastructure as that observed in sections of intact testes (Fig. 3). The straight, epithelial cell borders and the typical slender mitochondria of supporting cells allow a clear distinction between these cells and other testicular cell types.

Changes Resembling Maturation in Ultrastructure of the Supporting Cells During Culture in Serum-Enriched Medium Containing FSH

Cell nucleus. Twenty-one h after plating, the nuclei of the supporting cells are less irregular and the chromatin is paler than in the nonplated aggregates. Mitosis of supporting cells is frequently observed (Fig. 4). After 4 days, the chromatin is nearly homogeneous and nucleoli are enlarged (Fig. 5). By 6 days in culture, nuclear infoldings are clearly observed in many cells. Ten and 15 days after plating, a nearly mature nucleus is present in these cells, with the exception that no paranucleolar heterochromatin bodies are observed (Figs. 6 and 7). Nucleoli are centrally located and have a diameter of about 2.5 μm. Extensive nuclear infoldings have developed. After culture of cells for 8–15 days, the nucleolus attains its largest size and shows a mesh-like structure in which granular, fibrillar and amorphous components are observed (Figs. 6, 7).

Cytoplasm. As early as 21 h after plating, the presence of "dark" and "light" supporting cells becomes evident. The light cells (Figs. 5 and 6) show a relatively sparse distribution of the organelles in a lighter cytoplasmic matrix and they possess a greater abundance of vacuoles. These vacuoles do not appear to have any structural contents. With increasing age of the cultures, the light type of supporting cell predominates over the dark one. However, nuclei, mitochondria and cell borders do not show any significant difference between these 2 cell types (Fig. 6).

The amount of cytoplasm in the support cells appears to increase in volume during the first 10 days in culture (compare Figs. 3, 4 with Figs. 6, 8). The mitochondria become longer and more frequently branched, but their structure does not change. Cup-shaped mitochondria are not observed in these cultures. The endoplasmic reticulum of the dark supporting cells is mainly composed of flattened cisternae with clear contents, while in the light cell type it forms vacuoles and small vesicles. The number and size of the light vacuoles increase during culture. There is no indication of any specific growth of Golgi complex and no evidence of secretory granules. Primary and modified lysosomes are relatively scarce at early stages, but after 4–6 days in culture the number of autophagic or heterophagic vacuoles increases.



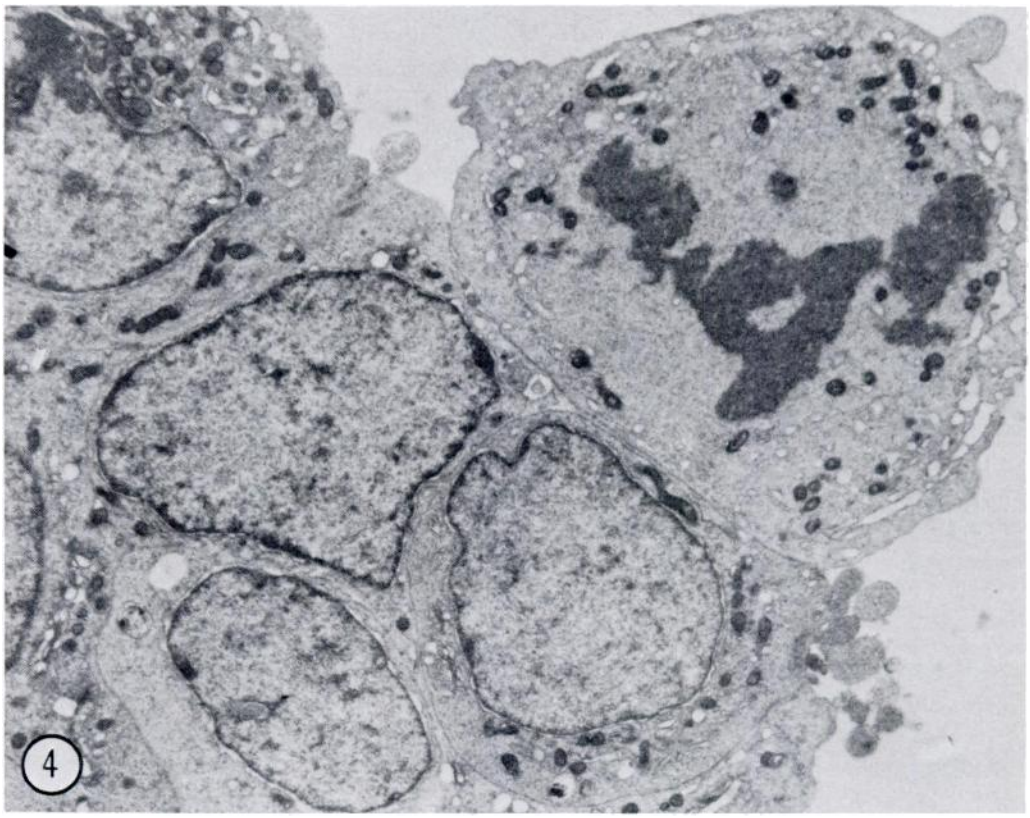


FIG. 3. Isolated cell aggregate from 10-day-old rat fixed before plating in culture. The supporting cells maintain their ultrastructural features. A spermatogonium (Spg) has a shrunken appearance. X 7,700.

FIG. 4. Cell aggregates from 10-day-old rats cultured for 21 h (medium B with FSH). A supporting cell is in mitosis. Cells in interphase show a decrease in chromatin condensation. X 7,700.

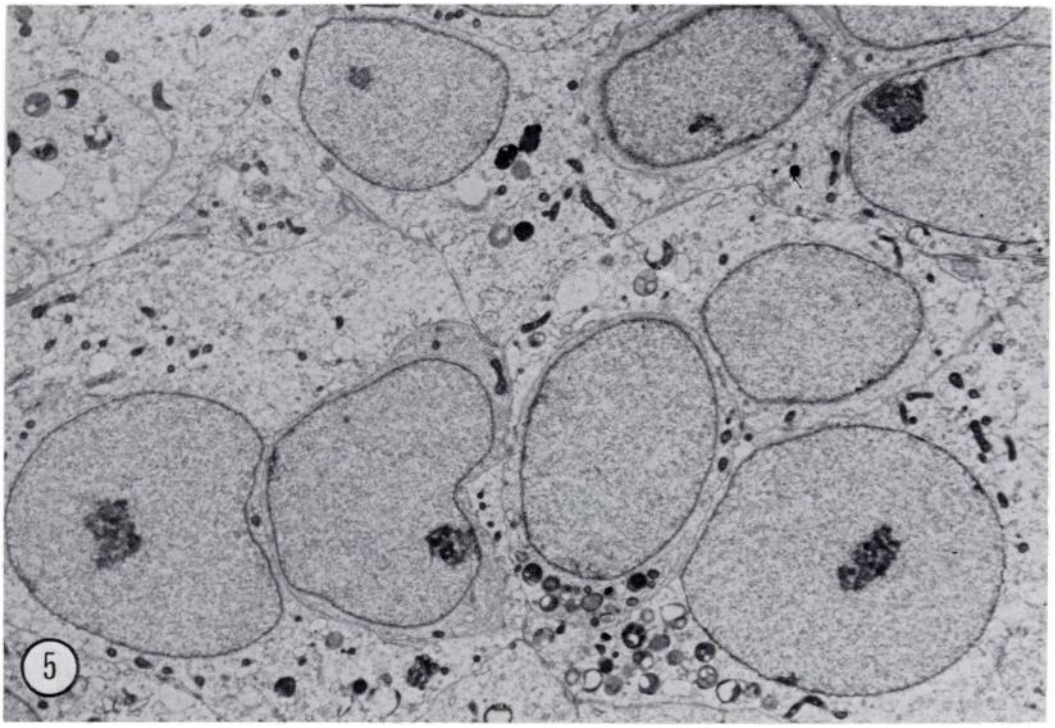


FIG. 5. Supporting cells from 10-day-old rats cultured for 4 days in medium B with FSH. The chromatin is almost homogeneous and nucleoli have enlarged in size. ×4,200.

Lipid droplets are very scarce in recently plated cultures and increase in frequency during culture. Microtubules are frequent in both types of supporting cells and they are preferentially located near the luminal surface (Figs. 8, 9). Microfilaments are scarce, especially in the light type and they are also preferentially located in the cytoplasmic extensions of the luminal surface (Fig. 9).

Polarized structures in the supporting cells. The use of vertical sections show that some structures of the supporting cells are preferentially differentiated in the luminal region, corresponding to the surface opposite to the site of cell attachment (Figs. 8, 9). These luminal differentiations are not altered in their location by changing the plastic support (Petri dish) for a millipore filter, or by plating the culture on top of a Millipore or Nucleopore filter supported by a metal grid and bathed by the medium from the undersurface. The luminal differentiations include: a) luminal cytoplasmic extensions (generally finger-like) (Figs. 8, 9); b) junctional complexes in the borders of neighboring cells (Figs. 10, 11); and c) preferential accumulation of cytoplasmic structures: microtubules, microfilaments and vesicles (Figs. 9, 10).

The cytoplasmic extensions (Fig. 9) have generally a finger-like appearance, 0.4–0.8 μm wide and up to 2 μm long. The cytoplasm inside these extensions contains thin filaments and occasional microtubules and vesicles. During progressive culture from 4–15 days, the number, length and irregularity of the cytoplasmic extensions increase. The junctional complexes are found exclusively near the luminal end of the intercellular spaces (Fig. 10). These complexes extend for distances up to 0.5 μm along the cell border. The intercellular space is filled with dense material and the adjacent cytoplasmic membranes approach each other, touching at isolated points (Fig. 11). Dense, fibrillar material is layered on the cytoplasmic side of each membrane. Peripheral to the dense fibrils, a single layer of flattened cisternae runs parallel to the membranes (Fig. 10). In addition to these complete junctional complexes, numerous partial or incomplete junctional differentiations are observed in

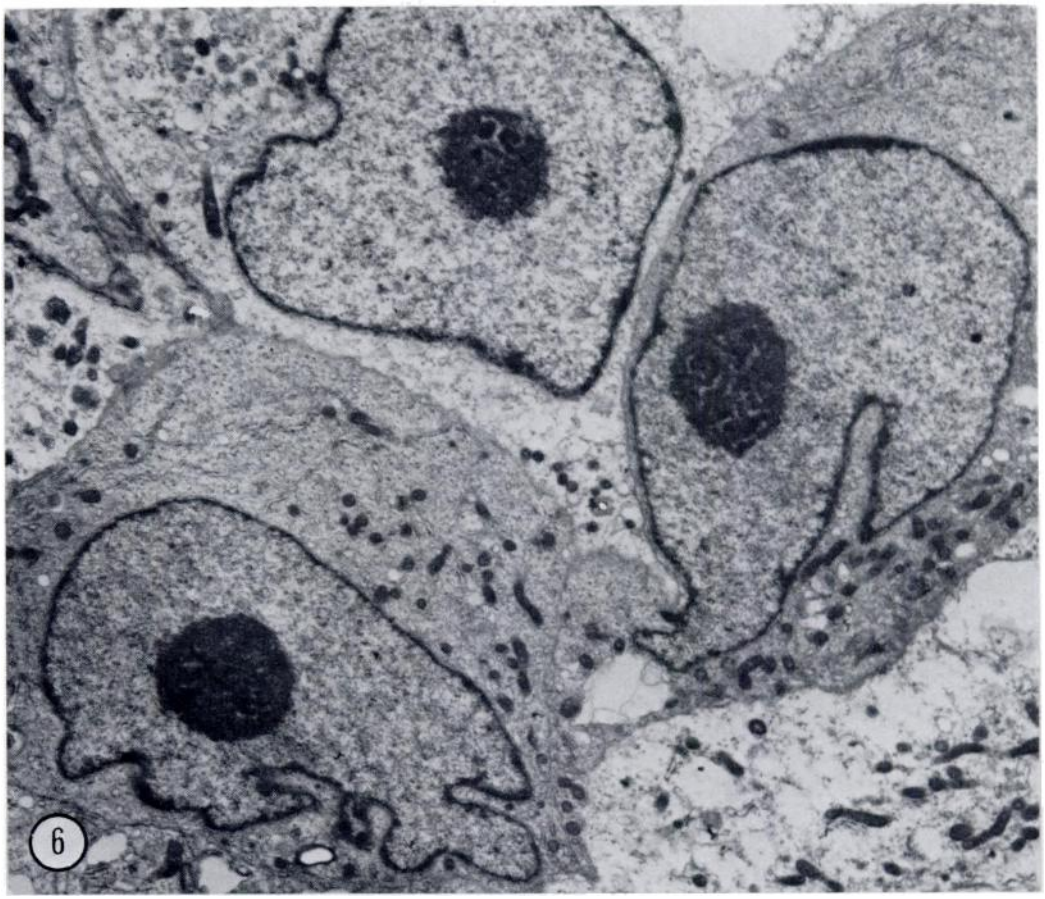


FIG. 6. Nearly full nuclear differentiation of supporting cells after 10 days in culture (medium B with FSH). The light and dark cell types are present. ×8,700.

which the intercellular space is narrowed and filaments are present in the adjacent cytoplasm. When cells are plated on top of millipore filters supported by grids (see Methods), the same luminal differentiations develop after 8–10 days, but the finger-like extensions appear flattened against the cell surface and the luminal region contains more microfilaments.

On the other hand, the basal region, corresponding to the surface facing the plastic support of the supporting cells, does not show cytoplasmic extensions of similar structure. Junctional complexes are absent in the basal region and there is no accumulation of microtubules and microfilaments. On the contrary, extensive interdigitations having an even and clear intercellular space are frequently found in the basal region.

Effects of Culture in Less Complete Medium on Maturation-Like Changes of the Supporting Cells

When cells from testes of 10-day-old rats are cultured in basal medium A, the ultrastructural changes occurring are relatively slight. The nuclear infoldings are minimal and the nucleoli remain small. Only a single type of supporting cell is evident, similar to the dark type described above, with the exception that there is a higher frequency of autophagic vacuoles, microfilaments and the presence of glycogen deposits. A significant number of mitochondria shows degenerative changes. The luminal cytoplasmic differentiation is only slightly developed, complete junctional complexes are absent and the cytoplasmic extensions are scarce in number and poorly developed. Micro-

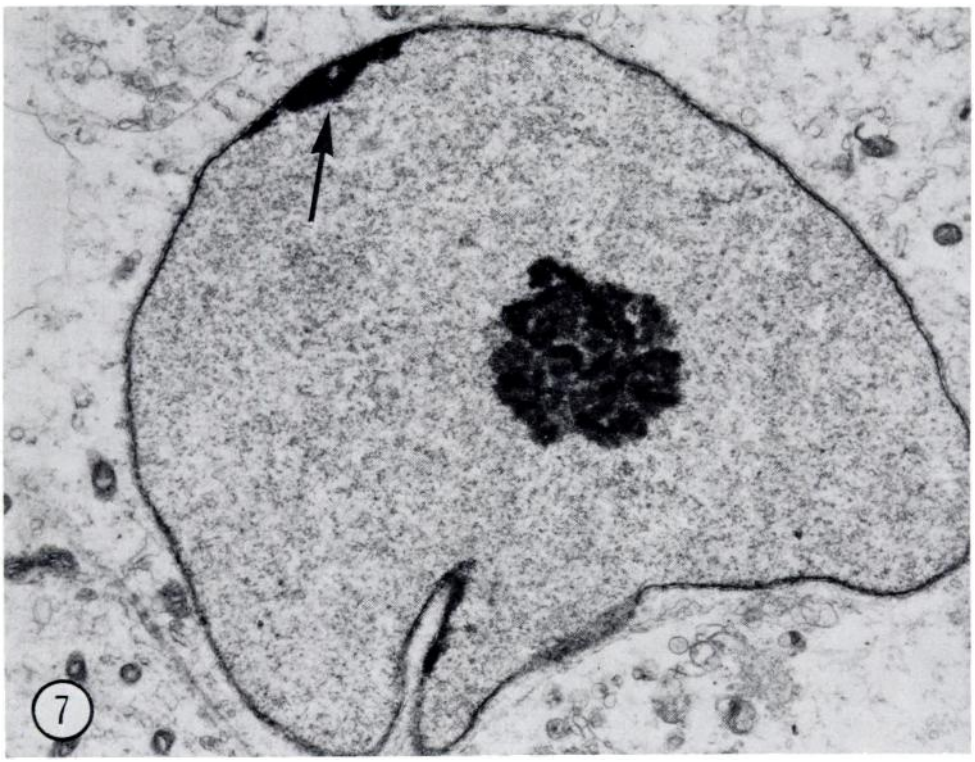


FIG. 7. A supporting cell from 10-day-old rats after 15 days in culture (medium B with FSH) shows the large nucleolus and a flattened mass of peripheral, dense chromatin (arrow). X 13,500.

filaments predominate in the luminal region, while the cell borders near the luminal surface have only occasional small patches of dense filaments.

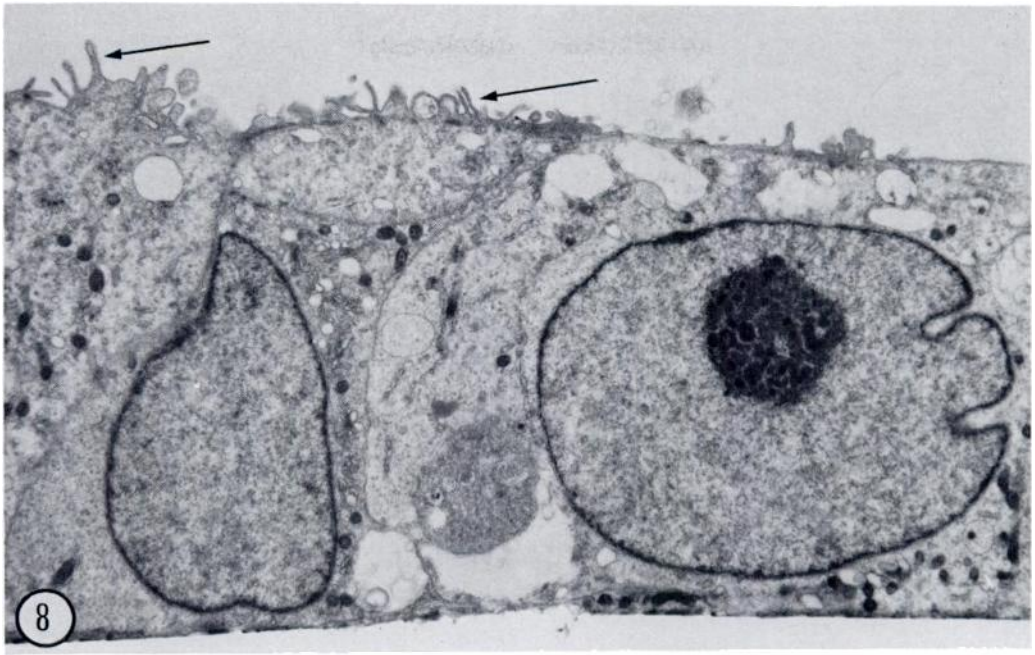
Addition of FSH to basal medium A allows some degree of nuclear maturation after 5 days in culture, enhances the formation of the smooth endoplasmic reticulum and prolongs the life-span of the supporting cells. Changes resembling maturation are considerably less extensive than those reported above in cells cultured in serum-enriched medium, but are more pronounced than those observed in cells cultured in basal medium alone.

Deletion of FSH from the serum-enriched medium does not have striking effects on ultrastructural changes occurring in cultured supporting cells. Maturation takes place to nearly the same extent as that described above in cells cultured in serum-enriched medium containing added FSH, with the exception that nucleoli do not become as large and the junctional complexes are not as frequent or extensive. Mitotic activity is maintained, however, in supporting cells cultured in the presence of FSH (vide infra) (Table 1).

TABLE 1. Mitotic indices of cultured supporting cells from 10-day-old rats.

Culturing conditions	Time after plating		
	21 h	10 days	15 days
B Medium + FSH	0.48%	0.45%	0.12%
B Medium	0.74%	<0.01%*	<0.01%*

*No mitosis recorded in 10^4 nuclei from pooled cultures.



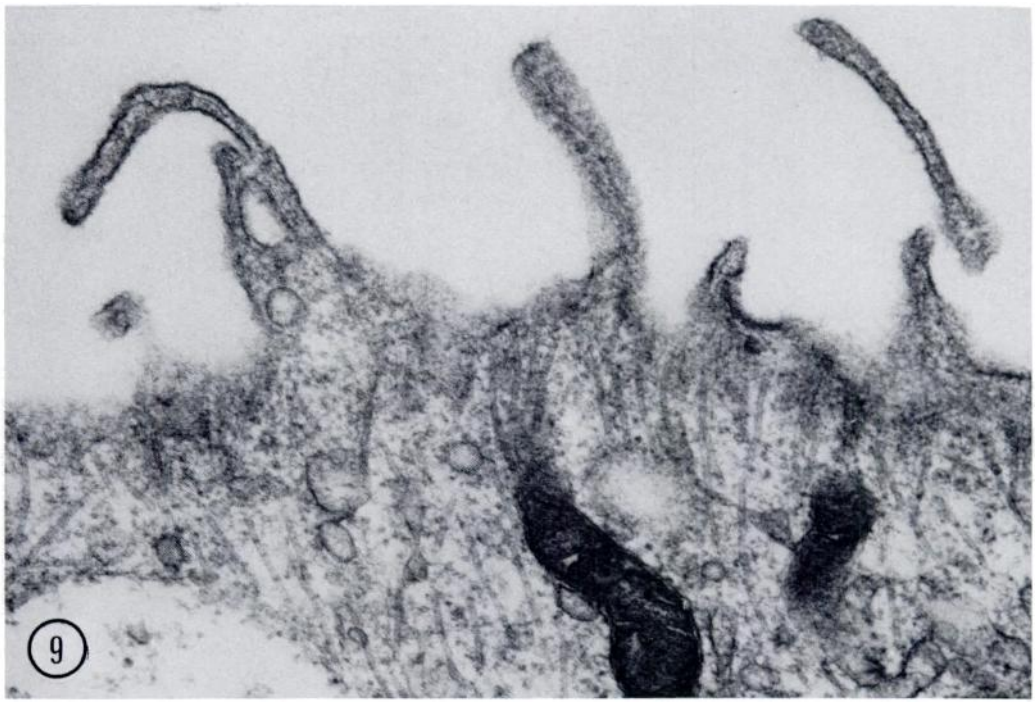


FIG. 8. Vertical section through supporting cells cultured for 10 days in medium B with FSH. Cytoplasmic luminal extensions (arrows). X 8,800.

FIG. 9. The luminal region of supporting cells after 10 days in culture (medium B with FSH) shows the finger-like extensions and the accumulation of microtubules and filaments. X 60,000.

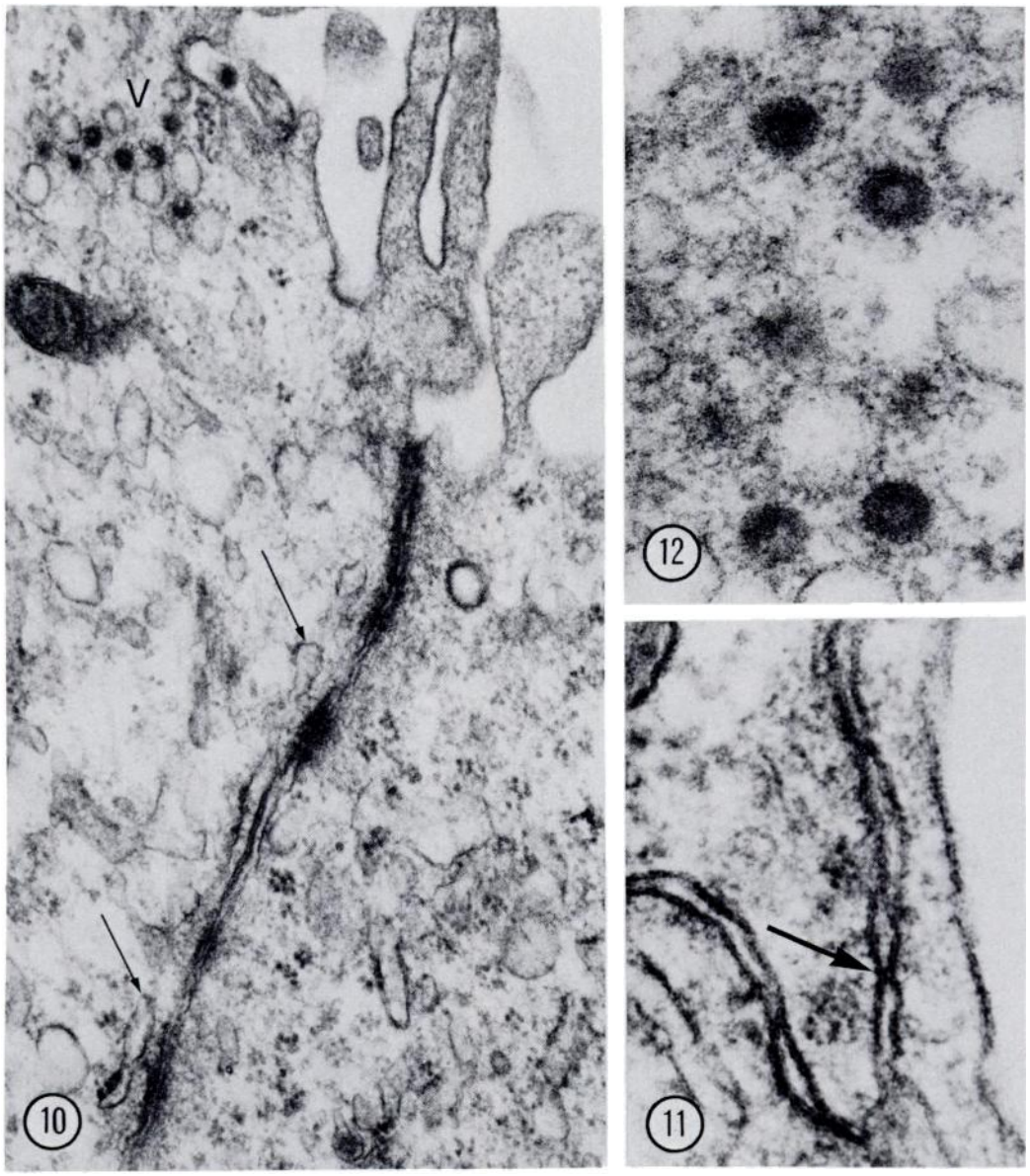


FIG. 10. Junctional complex near the luminal surface of supporting cells after 10 days in culture (medium B with FSH). Flattened cisternae (arrows) and virus-like particles (V). ×60,000.

FIG. 11. Tight junction (arrow) near the luminal surface of supporting cells after 8 days in culture (medium B with FSH). ×176,000.

FIG. 12. Virus-like particles in the cytoplasm of cultured supporting cells. ×180,000.

Presence of Virus-Like Particles in Cultured Supporting Cells

In the course of our investigations, we repeatedly detected the presence of virus-like particles in the cytoplasm of supporting cells, frequently located near the luminal surface (Fig. 10). These particles, having an average diameter of 52 nm, appear round, with suggestion of an hexagonal outline (Fig. 12). They show an ill-defined 7 nm thick layer covering a round core. The particles are generally found in clusters interspersed with small vesicles and a finely fibrillar material. The particles have not

been observed inside the nucleus, or in other cell types. Among the supporting cells, these particles have been found equally in cells cultured in the presence or absence of serum.

Mitotic Activity of the Supporting Cells

Both germ cells and supporting cells show mitotic activity in sections prepared from intact testes of 10-day-old rats. However, the mitotic activity of the epithelial colonies in preparations cultured for several days is exclusively ascribable to the supporting cells. The cell aggregates contain only 2.4% germ cells at the time of plating and most of them do not survive after 5 days in culture. With respect to the influence of various types of culture media, continued mitotic activity is observed only in supporting cells maintained in the presence of added FSH in medium A or B. In preparations cultured in the absence of added FSH, no supporting cells are found in mitosis at any time after the third day in culture (Table 1).

After 21 h in culture, a moderate quantity of supporting cells is in mitosis (Table 1) and the highest proportion is found in cultures kept in basal medium. However, the latter cultures show few, if any, mitotic supporting cell at Day 4 and afterwards (Table 1). On the other hand, a small but significant number of supporting cells is in mitosis after 10 and after 15 days in culture in the presence of FSH (Table 1). The addition of serum was not necessary for this mitotic activity.

Mitotic supporting cells are identified on the basis of the following features: a) epithelial borders with an even 15–20 nm wide intercellular space; b) abundant, slender mitochondria, generally less than 0.3 μm in width and showing irregularly arranged cristae; c) absence of dilated cisternae containing moderately dense material; d) absence of large microfilament bundles; and, occasionally, e) presence of polarized (luminal) structures.

All phases of mitosis are present in supporting cells at various times of culture (Figs. 13–15). Mitotic cells do not show spindle abnormalities. However, a rather frequent observation is the presence of virus-like particles *(vide supra)* associated with the pericentriolar area near one of both spindle poles (Fig. 15). During prophase the large nucleolus becomes divided into small (0.1–0.2 μm) masses of dense, fibrillar material and the chromatin condenses into wide threads, without any indication of especial regional condensation (Fig. 14). During metaphase and anaphase (Fig. 13), the chromosomes are associated with microtubular bundles. In the poles, pairs of centrioles are located as usual, except that the astral region may contain virus-like particles. During telophase (Fig. 15) the chromatin usually becomes very densely packed. The cell border of the mitotic cells frequently has many interdigitations with neighboring supporting cells, but no junctional complexes are detected. Mitotic supporting cells correspond most frequently to the light type. The mitotic cells show cytoplasmic changes similar to those of the interphase supporting cells during prolonged time in culture.

Observations on the Culture of Sertoli Cells from 20-Day-Old Rats

Sertoli cells prepared from testes of 20-day-old rats and cultured for 5 days in medium C with added FSH, show essentially the same morphology as those of supporting cells from 10-day-old rats cultured for 15 days in medium B with added FSH (Fig. 16). In these 2 cases, Sertoli cells are of the same chronological age.

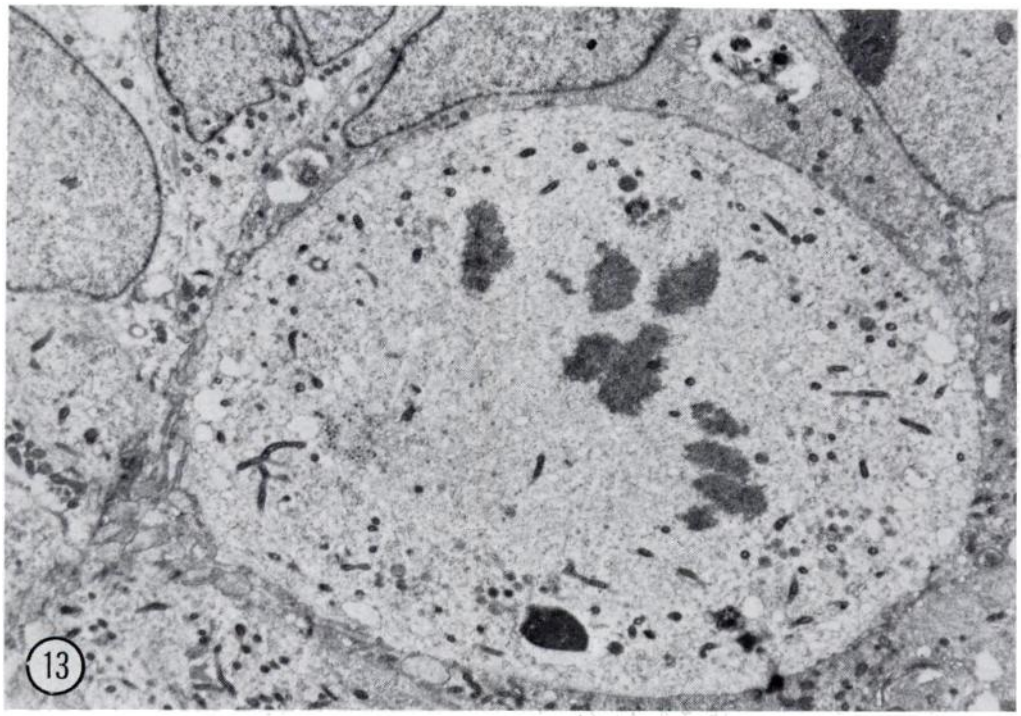
No mitotic activity is detected in Sertoli cells prepared from 20-day-old rats and cultured in basal medium A without FSH, or in medium C with added FSH. In another series of experiments, cells from testes of 20-day-old rats were cultured for 48 h in medium D, after which time, the medium was changed to basal medium A containing FSH and cultured for an additional 72 h. In these preparations some mitotic Sertoli cells are present (Fig. 17). The mitotic index of Sertoli cells in these preparations (0.15%) is similar to that obtained in the supporting cells from 10-day-old rats and cultured for 15 days in the presence of FSH (0.12%; Table 1). Preparations from 20-day-old rats contain a significantly higher population of germ cells. Mitotic spermatogonia (Fig. 18) are frequently observed in these preparations.

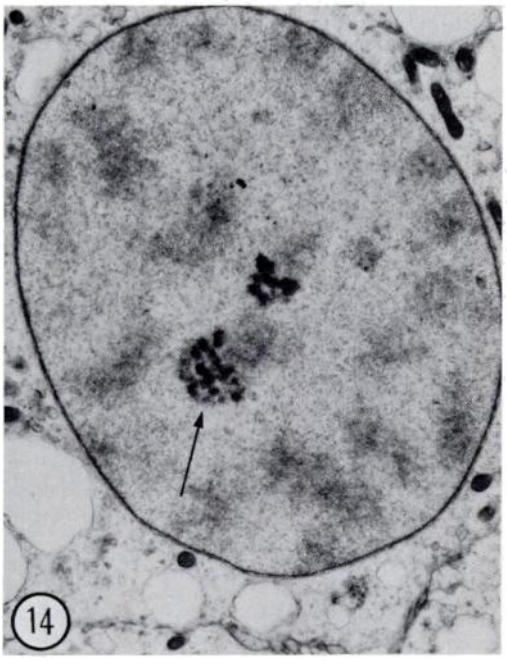
DISCUSSION

Ultrastructural Identification of Sertoli Cells in Cell Cultures

The characteristic ultrastructural features of supporting cells in culture which permit their identification include the following criteria:

1) Epithelial borders with an even intercellular space (15–20 nm wide) except at the





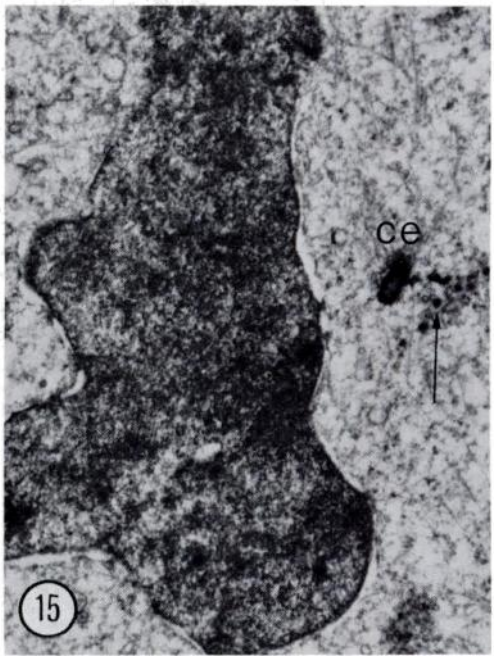
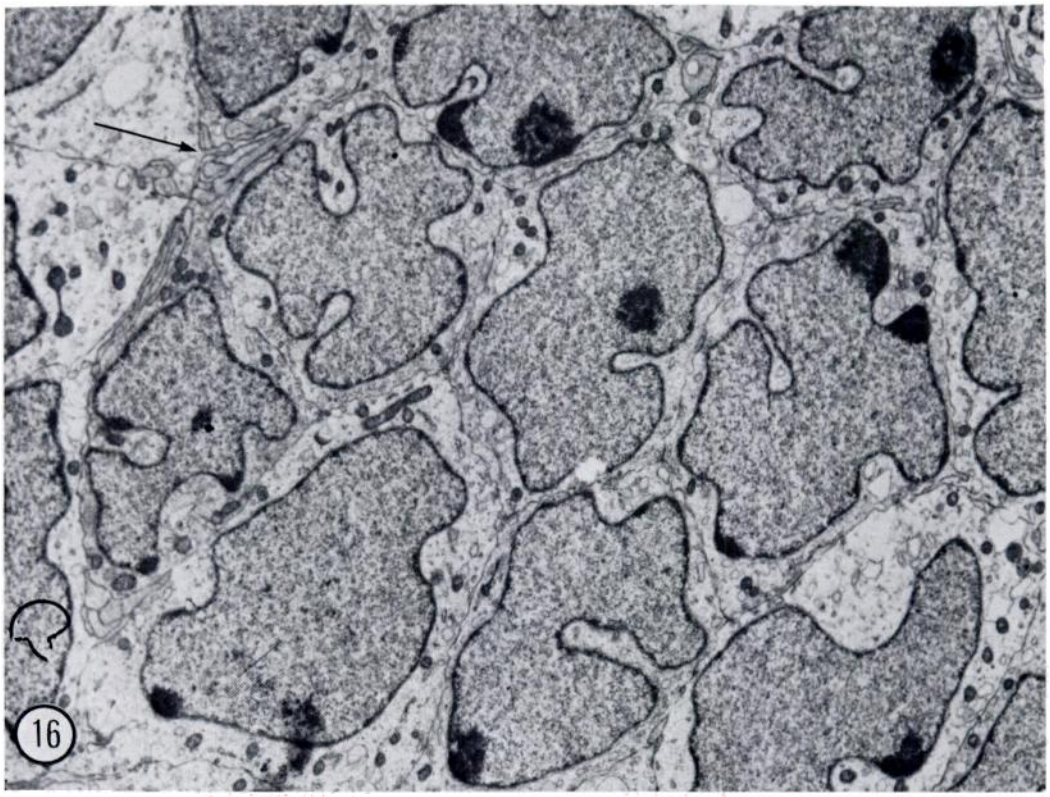
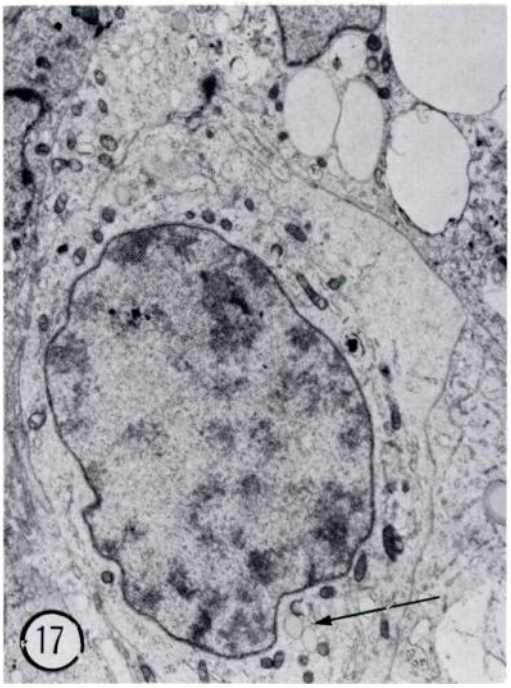


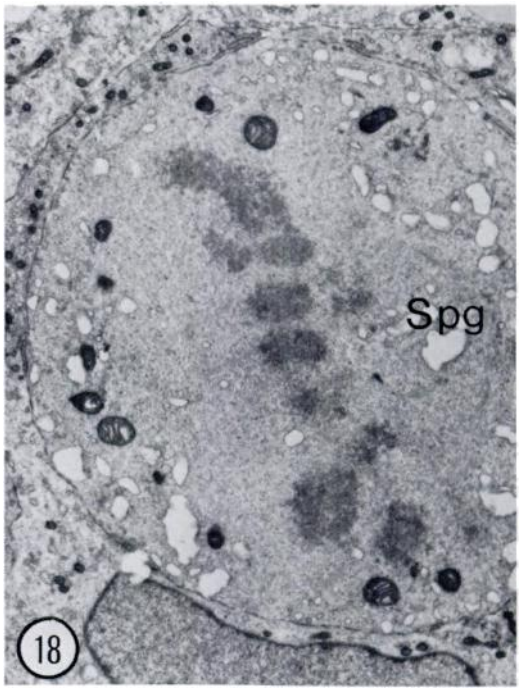
FIG. 13. Mitotic supporting cell from 10-day-old rat preparations after 10 days in culture (medium B with FSH). ×6,600.

FIG. 14. Supporting cell in prophase from a 10-day-old rat preparation cultured for 10 days in medium B with FSH. The arrow points to nucleolar material. ×9,300.

FIG. 15. Supporting cell in telophase in a preparation from 10-day-old rats cultured for 15 days in medium B with FSH. Virus-like particles (arrow) are near the centriole (ce). ×18,000.







location of junctional complexes and at sites of luminal differentiations.
2) Abundant, slender (less than 0.3 μm wide) mitochondria with dark matrix and irregularly arranged cristae.
3) A distinctive endoplasmic reticulum lacking dilated cisternae, with an increasing abundance of smooth relative to rough endoplasmic reticulum during culture.
4) A distinctive abundance and distribution of microfilaments.
5) An irregular outline of the nucleus which progressively develops more infoldings during culture and a nucleolus which becomes progressively large.

These combined structural components are not present in any other cells in testicular cultures, but they do have ultrastructural features characteristic of rodent Sertoli cells *in vivo* (Fawcett, 1975; Gilula et al., 1976). In cultures of cells prepared from testes of 10-day-old rats, we did not observe any cells having the ultrastructural characteristics of Leydig cells (Christensen, 1975) or endothelial cells from blood vessels (Haudenschild, 1975).

Germ cells and peritubular myoid cells, which constitute a small percentage of the cells in culture, could readily be distinguished from Sertoli cells by the differences between the ultrastructural features of Sertoli cells described above and those of other testicular cells in culture (Vilar et al., 1966, 1967; Bressler and Ross, 1973).

Changes Resembling Maturation in Ultrastructure of Supporting Cells During Culture

Changes *in vivo* in Sertoli cell structure during rodent testicular development have been described by several authors (Flickinger, 1967; Novi and Saba, 1968; Vitale et al., 1973; Gilula et al., 1976). Changes of a similar nature which resemble maturation have now been shown to take place *in vitro* during culture in serum-enriched medium of Sertoli cells from immature testes (Figs. 1–10). The changes in structure include the development of a large nucleolus within an irregularly-shaped nucleus having a "mature" type of chromatin distribution; the presence of increased amounts of smooth endoplasmic reticulum; development of increased numbers of vacuoles and vesicles in the cytoplasm; and the formation of junctional complexes in the near-luminal regions of intercellular borders. These maturation-like events are most complete in cells cultured in serum-enriched medium containing added FSH. In contrast, only slight changes occur when cells are cultured in basal medium in the absence of FSH. The presence of the light type of supporting cells is dependent on the addition of FSH and the extent of smooth endoplasmic reticulum is also greatly influenced by this treatment. It is possible that the light cell type may represent a stage of stimulation of the smooth endoplasmic reticulum elicited by FSH.

The Maintenance of Mitotic Potentiality and Its Dependence on FSH

Although there is strong evidence that supporting cells *in vivo* cease to divide about 15 days after birth in the rat (Clermont and Perey, 1957; Hilscher and Makoski, 1968; Steinberger and Steinberger, 1971) and that a similar inhibition occurs in organ culture (Steinberger and Steinberger, 1971), recent observations have shown that the addition of FSH to cultures of immature Sertoli cells stimulates DNA synthesis in a fraction of these cells (Griswold et al., 1976, 1977). Furthermore, Courot (1970) has claimed that the number of supporting cells in the prepuberal lamb is dependent on the presence of gonadotrophic hormones. Nagy (1972) reported that [^{3}H]-thymidine continues to be incorporated into nuclear DNA of Sertoli cells of rats up to 40 days of age. In the present observations, a small but significant number of supporting cells

FIG. 16. Sertoli cells from 20-day-old rats cultured for 5 days in medium C with FSH. The nuclear morphology is mature. In the cytoplasm there are complex interdigitations (arrow). X7,800.

FIG. 17. Sertoli cell in prophase in a preparation from 20-day-old rats cultured for 5 days (2 days in medium D and 3 days in medium A with FSH). The cytoplasm shows typical mitochondria and lipid droplets (arrow). X6,000.

FIG. 18. Mitotic spermatogonium (Spg) in a preparation from 20-day-old rats cultured for 5 days in medium C with FSH. The size and morphology of the mitochondria in the spermatogonium are different from those of the mitochondria of neighboring supporting cells. X6,000.

from 10-day-old rats continues to divide up to 15 days after plating, provided FSH is present in the medium. The frequency of mitoses decreases slowly with time in culture (see Table 1). This mitotic activity is dependent on the presence of FSH in the medium. Cells cultured in the absence of FSH in the medium, with or without added serum, do not show mitosis after the third day in culture. This dependency on added FSH is at variance with the conclusion of Steinberger et al. (1970) that ". . .regulation of Sertoli cell division may be an autonomous process not dependent on the hormonal milieu".

The low mitotic frequencies observed in FSH-treated cultures suggest that the number of supporting cells able to undergo mitosis is a small fraction of the cell population. This suggestion agrees with data on DNA labelling in cultures from 20-day-old rats (Griswold et al., 1976). These data show that only a minor fraction (approximately 5%) of the FSH-treated cells is able to become labelled during exposure for 24 h to [^{3}H]-thymidine and that this DNA synthesis is abolished in cells obtained from testes of rats over 40 days of age (Griswold et al., 1977).

These data suggest that the stimulatory effect of FSH on mitosis of Sertoli cells is superimposed on a progressive, nonreversible repression of mitosis that affects the population in a nonsynchronous way. Speculatively, the total number of mitotic divisions that a Sertoli cell can undergo may have an upper limit which cannot be exceeded. Other observations of mitosis in cultured supporting cells from 20-day-old rats have been described elsewhere (Griswold et al., 1977).

Polarized Structures in Cultured Supporting Cells

Cells cultured for 8–10 days in serum-enriched medium containing added FSH develop junctional complexes similar in structure, but smaller in size, to those present in mature Sertoli cells (Figs. 10, 11). It is tentatively assumed that at earlier stages of formation, these complexes consist of a narrowing of the intercellular space that becomes filled with dense material. A similar step-wise process of junction formation may exist *in vivo*, as suggested by Novi and Saba (1968) and by Gilula et al. (1976). The location of the junctions and the other luminal differentiations in cultured Sertoli cells could not be changed experimentally by changing the support, or the location of the medium (see Results). The junctions are essential and dynamic components of mature Sertoli cells and their location *in vivo* is restricted to the basal portion of the seminiferous epithelium (Flickinger, 1967; Dym and Fawcett, 1970; Russell, 1977). It is conceivable that the luminal region of the cultured supporting cells corresponds to the basal region of Sertoli cells *in vivo*. On the other hand, the development in epithelial lined ducts of juxtaluminal tight junctions is generally the case *in vivo*. Perhaps, Sertoli cells, which are exceptions to this rule *in vivo*, may follow the generality when cultured in an artificial environment.

Observations presented above have shown that the base of the supporting cells *in vivo*, at 10 days of age, is rich in microfilaments and vesicles, but that cultured supporting cells have a higher concentration of such structures in the luminal regions. Furthermore, no evidence of basement membrane formation is observed in the basal region of supporting cells in culture. The formation of cytoplasmic extensions in the luminal region (Figs. 8, 9) is a common feature of cultured epithelial cells. However, the presence of these extensions does not necessarily indicate the direction of fluid flow in some cultured, secreting cells, as shown by the formation of "domes" in cultures of normal and neoplastic mammary gland cells (Pickett et al., 1975). If the luminal region of the cultured supporting cells is structurally organized in a manner similar to that of the basal region of the cells *in vivo*, the failure of these cultures to act as a supporting cell layer for the development of advanced germ cells (unpublished observations) is more readily interpreted. The possible role of Sertoli cells as "nurse cells" with regard to germ cell differentiation (Vilar et al., 1962) must be investigated with alternate approaches. Further data on the significance of polarized structures in cultured Sertoli cells could possibly be obtained through the use of the trans-filter "induction" techniques (Meier and Hay, 1975).

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REFERENCES

Bressler, R. S. and Ross, M. H. (1973). On the character of the monolayer outgrowth and the fate of the peritubular myoid cells in cultured mouse testis. Exptl. Cell Res. 78, 295-302.

Christensen, A. K. (1975). Leydig Cells. In: Handbook of Physiology. Section 7, Vol. V. Male Reproductive System. (D. W. Hamilton and R. O. Greep, eds.). American Physiological Society. Washington. pp. 57-94.

Clermont, Y. and Perey, B. (1957). Quantitative study of the cell population of the seminiferous tubules in immature rats. Amer. J. Anat. 100, 241-269.

Courot, M. (1970). Effects of gonadotrophins on the seminiferous tubules of the immature testis. In: The Human Testis. (E. Rosenberg and C. A. Paulsen, eds.). Plenum Press, New York. pp. 355-367.

Dorrington, J. H. and Armstrong, D. (1975). Follicle-stimulating hormone stimulates estradiol-17β synthesis in cultured Sertoli cells. Proc. Natl. Acad. Sci. U.S. 72, 2677-2681.

Dorrington, J. H. and Fritz, I. B. (1974). Effects of gonadotrophins on cyclic AMP production by isolated seminiferous tubule and interstitial cell preparations. Endocrinology 94, 395-403.

Dorrington, J. H., Roller, N. F. and Fritz, I. B. (1974). The effects of FSH on cell preparations from the rat testis. In: Hormone Binding and Target Cell Activation in the Testis. (M. L. Dufau and A. R. Means, eds.). Plenum Press, New York. pp. 237-241.

Dorrington, J. H., Roller, N. F. and Fritz, I. B. (1975). Effects of follicle-stimulating hormone on cultures of Sertoli cell preparations. Molec. Cell Endocr. 3, 57-70.

Dym, M. and Fawcett, D. W. (1970). Observations on the blood-testis barrier of the rat and on the physiological compartmentation of the seminiferous epithelium. Biol. Reprod. 3, 308-326.

Fawcett, D. W. (1975). Ultrastructure and function of the Sertoli cell. In: Handbook of Physiology. Section 7, Vol. V. Male Reproductive System. (D. W. Hamilton and R. O. Greep, eds.). American Physiological Society, Washington. pp. 21-55.

Flickinger, C. J. (1967). The postnatal development of the Sertoli cells of the mouse. Z. Zellforsch. 78, 92-113.

Fritz, I. B. (1977). Sites of action of androgens and FSH on cells of the seminiferous tubule. In: Biochemical Actions of Hormones. (G. Litwack, ed.). Vol. V. (In press.).

Fritz, I. B., Rommerts, F., Louis, B. G. and Dorrington, J. H. (1976). Regulation by FSH and dibutyryl cyclic AMP of the formation of androgen-binding protein in Sertoli cell-enriched cultures. J. Reprod. Fertil. 46, 17-24.

Gilula, N. B., Fawcett, D. W. and Aoki, A. (1976). The Sertoli cell occluding junctions and gap junctions in mature and developing mammalian testis. Devel. Biol. 50, 142-168.

Griswold, M. D., Mably, E. R. and Fritz, I. B. (1976). FSH stimulation of DNA synthesis in Sertoli cells in culture. Mol. Cell Endocr. 4, 139-149.

Griswold, M. D., Solari, A., Tung, P. S. and Fritz, I. B. (1977). Stimulation by FSH of mitosis and of DNA synthesis in cultured Sertoli cells prepared from testes of immature rats. Mol. Cell Endocr. 7, 151-165.

Haudenschild, C. C., Cotran, R. S., Gimbrone, M. A. and Folkman, J. (1975). Fine structure of vascular endothelium in culture. J. Ultrastructure Res. 50, 22-32.

Hilscher, W. and Makoski, H. B. (1968). Histologisch und Autoratiographische Untersuchungen zur "Praspermatogenese" und "Spermatogenese" der Ratte. Z. Zellforsch. 86, 327-350.

Means, A. R. (1975). Biochemical effects of follicle-stimulating hormone on the testis. In: Handbook of Physiology. Section 7, Vol. V. Male Reproductive System. (D. W. Hamilton and R. O. Greep, eds.). American Physiological Society, Washington. pp. 203-218.

Meier, S. and Hay, E. D. (1975). Stimulation of corneal differentiation by interaction between cell surface and intercellular matrix. I. Morphometric analysis of transfilter "induction". J. Cell. Biol. 66, 275-291.

Nagy, F. (1972). Cell division kinetics and DNA synthesis in the immature Sertoli cells of the rat testis. J. Reprod. Fertil. 28, 389-395.

Novi, A. M. and Saba, P. (1968). An electron microscopic study of the development of rat testis in the first 10 postnatal days. Z. Zellforsch. 86, 313-326.

Pickett, P. B., Pitelka, D. R., Hamamoto, S. T. and Misfeldt, D. S. (1975). Occluding junctions and cell behaviour in primary cultures of normal and neoplastic mammary gland cells. J. Cell Biol. 66, 316-332.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17, 208-212.

Russell, L. (1977). Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. Amer. J. Anat. 148, 313-328.

Sjostrand, F. J. (1967). Electron Microscopy of Cells and Tissues. I. Instrumentation and techniques. Academic Press, New York. pp. 287-294.

Steinberger, A. and Steinberger, E. (1971). Replication pattern of Sertoli cells in maturing rat testis *in vivo* and in organ culture. Biol. Reprod. 4, 84-87.

Steinberger, E., Steinberger, A. and Ficher, M. (1970). Study of spermatogenesis and steroid metabolism in cultures of mammalian testes. Rec. Prog. Hormone Res. 26, 547-588.

Steinberger, A., Heindel, J. J., Lindsey, J. N., Elkington, J. S., Sandborn, B. M. and Steinberger, E. (1975). Isolation and culture of FSH responsive Sertoli cells. Endocr. Res. Commun. 2, 261-272.

Terzakis, J. A. (1968). Uranyl acetate, a stain and a fixative. J. Ultrastructure Res. 22, 168-184.

Tung, P. S., Dorrington, J. H. and Fritz, I. B. (1975). Structural changes induced by follicle-stimulating

hormone or dibutyryl cyclic AMP on presumptive Sertoli cells in culture. Proc. Natl. Acad. Sci. 72, 1838-1842.

Vilar, O. M., del Cerro, Perez and Mancini, R. E. (1962). The Sertoli cell as a "bridge cell" between the basal membrane and the germinal cells. Exptl. Cell Res. 27, 158-161.

Vilar, O., Steinberger, A. and Steinberger, E. (1966). Electron microscopy of isolated rat testicular cells grown *in vitro*. Z. Zellforsch. 74, 529-538.

Vilar, O., Steinberger, A. and Steinberger, E. (1967). An electron microscopic study of cultured rat testicular fragments. Z. Zellforsch. 78, 221-233.

Vitale, R., Fawcett, D. W. and Dym, M. (1973). The normal development of the blood-testis barrier and the effects of clomiphene and estrogen treatment. Anat. Rec. 176, 333-344.

Welsh, M. J. and Wiebe, J. P. (1975). Rat Sertoli cells: a rapid method for obtaining viable cells. Endocrinology 96, 618-624.

RECOMMENDED REVIEWS

Fawcett, D. W. (1975). Ultrastructure and function of the Sertoli cell. In: Handbook of Physiology. Section 7, Vol. V. Male Reproductive System. (D. W. Hamilton and R. O. Greep, eds.). American Physiological Society, Washington. pp. 21-55.

Fawcett, D. W. (1976). The Male Reproductive System. In: Reproduction and Human Welfare. (R. O. Greep, M. A. Koblinsky and F. S. Jaffe, eds.). M.I.T. Press, Cambridge. pp. 165-277.