Proc. Nat. Acad. Sci. USA
Vol. 72, No. 5, pp. 1838–1842, May 1975

Structural Changes Induced by Follicle-Stimulating Hormone or Dibutyryl Cyclic AMP on Presumptive Sertoli Cells in Culture

(rat testis/controls of spermatogenesis/Sertoli cell ultrastructure)

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Communicated by Charles H. Best, February 18, 1975

ABSTRACT **Cells isolated from testes of 20-day-old rats, maintained in primary culture in a defined medium, are shown to respond to follicle-stimulating hormone or 3′:5′-cyclic AMP with characteristic morphological changes. No response is observed in cells treated with luteinizing hormone or 5′-AMP. The cells form a monolayer, and have been identified as presumptive Sertoli cells structurally by identification of unique tight junctions in electron micrographs of the preparations, along with other ultrastructural properties characteristic of Sertoli cells *in situ*. These cells do not undergo mitosis. The presumptive Sertoli cells are shown to be morphologically and functionally different from peritubular fibroblasts grown in parallel cultures. Fibroblasts have high rates of mitosis, do not respond to follicle-stimulating hormone, and frequently form multilayers. Other information on the biochemical responses of the cells is cited, which supports the conclusion that the cultured cell preparations consist primarily of Sertoli cells.**

In recent years it has become clear that Sertoli cells are intimately associated with the processes of spermatogenesis. Sertoli cells are thought to be involved in the formation and maintenance of the blood–testis barrier and in the secretion of tubular fluid (1–3). Follicle-stimulating hormone (FSH) increases 3′:5′-cyclic AMP (cAMP) production by preparations enriched in Sertoli cells, but not by germinal or Leydig cells (4–6). Sertoli cells have also been implicated in the *in vivo* (7, 8) and *in vitro* (9) production of testicular androgen-binding protein. The hypothesis has been advanced that hormones controlling spermatogenesis may act in part by influencing Sertoli cell functions (2, 10). In attempts to test this hypothesis, and to be able to investigate eventually the possible relationships between germinal cells and Sertoli cells, we have isolated Sertoli cells from the rat testis and have examined their behavior in primary culture in the presence and absence of FSH.

MATERIALS AND METHODS

Cell Preparation. Testes from ten 20-day-old Wistar rats were decapsulated, placed into Hanks' buffer, and cut into 0.5 mm segments by a Mickel tissue slicer. Testicular fragments (approximately 1.6 g wet weight) were digested at 32° in 50 ml of Hanks' buffer containing 0.25% trypsin (Grand Island Biological) and 10 μg/ml of DNase (type I, Sigma Chemical). After incubation for 30 min with shaking (60 oscillations/min), soybean trypsin inhibitor was added. Partially digested seminiferous tubules were harvested, passed through a wire grid (1 mm mesh), washed several times with Hanks' buffer, and then subjected to collagenase digestion (1 mg/ml, Sigma Chemical, Type I) in 20 ml of Hanks' buffer for 30 min. The tubule fragments were harvested by low-speed centrifugation, washed twice with Hanks' buffer containing 1% bovine serum albumin (Pentax Chemical) and were dispersed by gentle agitation (20 times) with a pasteur pipette in Ca^{2+}- and Mg^{2+}-free Hanks' buffer containing 0.1 mM ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate EGTA (Sigma). Cell aggregates were then washed with 1% serum albumin in Hanks' buffer, and collected by filtration through a double layer of 200 μm mesh nylon cloth. The aggregates, ranging in size from 60 to 100 μm in diameter, were resuspended in 1.0 ml of Eagle's minimum essential medium (Gibco), supplemented with antibiotics and non-essential amino acids, as in Medium A of Steinberger *et al.* (11), with the exceptions that the final glutamine concentration was 4 mM, and no sodium pyruvate was present. Unless noted otherwise, no serum was added to the preparations.

Culture Conditions. Aliquots of the above suspensions were plated into 60 mm Falcon polystyrene petri dishes at a density of 10^4 aggregates per dish, and 5 ml of culture medium was added. Vessels were incubated at 32° in a water-saturated atmosphere of 95% air and 5% CO_2. The medium, together with unattached cells, was removed 24 hr later, and 5 ml of fresh culture medium was added. In some experiments to be reported, the culture medium was supplemented with 10% calf serum. Cells were incubated for various periods of time in the presence or absence of hormones or other agents described in *Results*, and the medium was changed at 48 hr intervals.

Morphological Techniques. Culture flasks were examined periodically with a Nikon inverted microscope with phase optics, and representative fields were photographed. For scanning electron microscopy, samples were fixed in 2% glutaraldehyde in 0.1 Millonig phosphate buffer at pH 7.4 for 30 min at 32°, followed by post-fixation in similarly buffered 1% osmium tetroxide at room temperature for an additional 30 min. The preparations were then freeze-dried, using liquid nitrogen and a lyophilizer under vacuum. A thin film of gold was plated on the preparation in a vacuum evaporator, and preparations were examined with a Cambridge Stereoscan Mark 2A electron microscope operated at 20 kV.

For transmission electron microscopic studies, the samples were fixed in 3% glutaraldehyde and postfixed as described above, except that the postfixation period in buffered 2%

Abbreviations: FSH, follicle-stimulating hormone; cAMP, 3′:5′-cyclic adenosine monophosphate; Bt_2cAMP, dibutyryl cAMP.

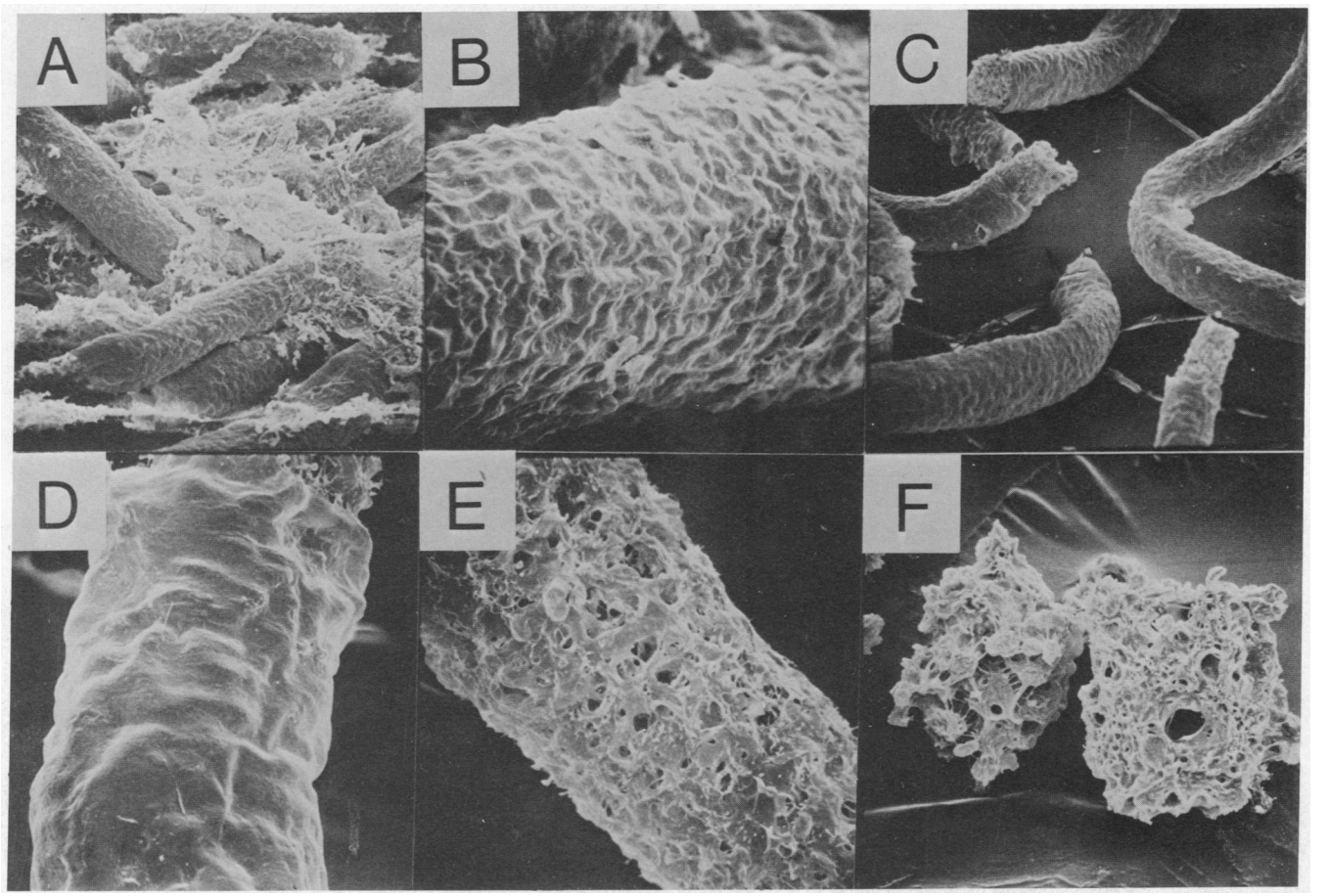

FIG. 1. Scanning electron photomicrographs of seminiferous tubule fragments prior to and at different stages during sequential enzymatic treatments described in *Methods*. (A) Crude testicular preparation after mincing testes from 20-day-old rats, illustrating segments of seminiferous tubules and masses of interstitial cell clumps (×192). (B) A higher magnification (×960) of a seminiferous tubule segment, showing the external surface of the lamina propria and attached material. (C) Testicular preparation after trypsin digestion and subsequent washing. Note removal of interstitial and supratubular material (×192). (D) A segment of seminiferous tubule after trypsin digestion (×960). (E) A segment of seminiferous tubule after collagenase digestion and subsequent washing, illustrating removal of peritubular elements (×960). (F) Fragments of cell aggregates ready for plating (×960).

osmium tetroxide was 60 min. The preparations were then dehydrated with ethanol, transferred into propylene oxide, and embedded in Epon 812/Araldite. Some cells were embedded *in situ* on the petri dish, and sectioned either horizontally or vertically with respect to the monolayer (12). Other cell preparations were scraped from the petri dish with a rubber policeman after fixation, centrifuged, and subsequently treated for embedding. Thin sections (600 Å) were double stained with uranyl acetate and lead hydroxide, then examined and photographed with an RCA electron microscope. Thick sections (2.5 μm) were stained with 1% toluidine blue for light microscopy studies. The mitotic index in the presence of colcemid was measured by the method of Hsu (13).

RESULTS AND DISCUSSION

Initial Partial Purification of Testicular Cell Preparation. The scanning electron photomicrographs in Fig. 1 illustrate the crude testicular preparation after mincing (panels A and B), trypsin digestion, (panels C and D), and after further treatment with collagenase (panel E). Finally, after agitation with a pasteur pipette, the tubular fragments were dispersed into aggregates containing primarily Sertoli cells and germinal cells (panel F).

Properties of Aggregates and Cells in Culture. Control cell preparations cultured for 72 hr in the defined culture medium had the appearance under phase microscopy shown in Fig. 2A. Murphy observed that FSH administration *in vivo* results in a secretory hypertrophy of Sertoli cells of hypophysectomized rats, without apparent influence on other testicular cells (14). We, therefore, decided to examine the effects of FSH on our presumptive Sertoli cell preparation. Cells, grown under conditions identical to those shown in Fig. 2A for the first 48 hr and then cultured in the presence of FSH (5 μg/ml of NIH S-10 ovine FSH) for the next 24 hr, are shown in Fig. 2B. Cell nuclei appear darker, in association with changes in cell shape, and the surrounding cytoplasm appears scant. In cells exposed to FSH, the formation of multiple cytoplasmic extensions is evident. Consequently, the cell shape appears slender and more fibroblast-like (Fig. 2B). Similar effects were obtained with a more highly purified FSH (0.2 μg/ml of ovine FSH, courtesy of Dr. Harold Papkoff, having a potency 50 times greater than that of NIH FSH S-1). Even more striking effects of the same qualitative sort were observed in cells cultured in the presence of 0.1 mM dibutyryl cAMP (Bt_2cAMP) or 1.0 mM cAMP (Fig. 2C). Moreover, these morphological responses could be re-

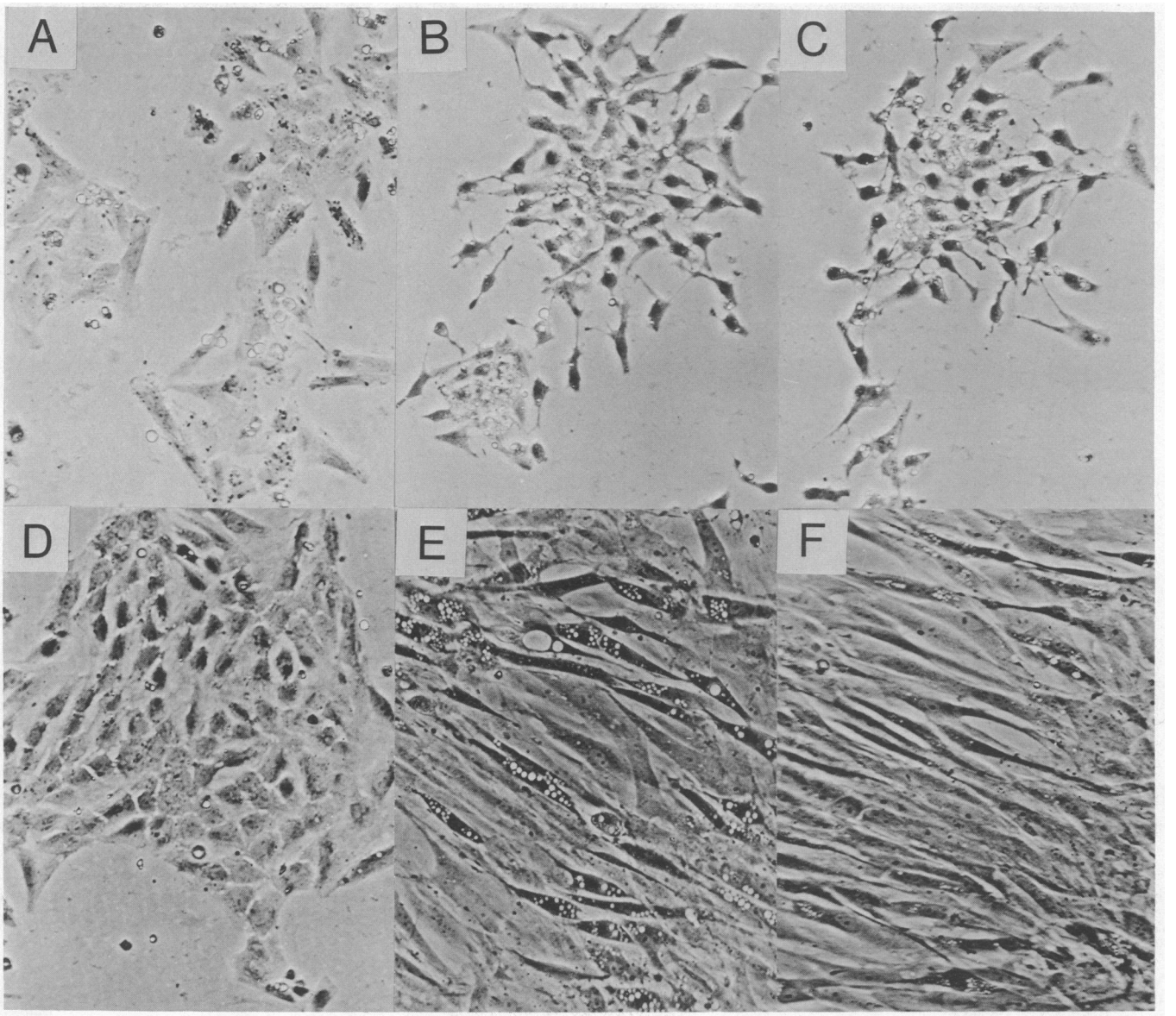

FIG. 2. Phase contrast photomicrographs (×380) of cultured Sertoli cells or fibroblasts. (A–D) Sertoli cell aggregates (1×10^4 per 60 mm Falcon tissue culture dish) were plated and cultured for 72 hr in standard culture medium, as follows: (A) Untreated cells (control); (B) Cells treated with 5 μg/ml of NIH S-10 ovine FSH for the last 24 hr; (C) Cells treated with 0.1 mM Bt_2cAMP for the last 24 hr; and (D) Cells grown in control medium enriched with 10% calf serum for an additional 2 days (5 days in culture). (E) Testicular fibroblasts were separated from testicular fragment cultures (17) by the technique of Owens (18) and subcultured for 5 days in control medium containing 10% calf serum. (F) Testicular fibroblasts prepared as in E but cultured with modified minimum essential medium not containing serum.

verted to normal form in standard culture medium supplemented with 10% calf serum (Fig. 2D). The structural differences between Sertoli cell cultures and fibroblasts grown in culture under these conditions are evident in Fig. 2E and F. The morphological changes induced by FSH in Sertoli cells were not duplicated in cells cultured in the presence of luteinizing hormone (NIH S-18 ovine LH) at concentrations up to 10 μg/ml, or in the presence of 1 mM 5′-AMP or 0.1 mM sodium butyrate. During the first 6 days of culture, the mitotic index was lower than 0.03%. This calculation is based upon observations on Sertoli cells cultured in the presence of colcemid (0.5 μg/ml) in Eagle's minimal essential medium alone or in Eagle's minimal essential medium plus calf serum.

Scanning electron photomicrographs of presumptive Sertoli cells grown under control conditions in standard culture medium alone for 72 hr are shown in Fig. 3A. In cells of the same age cultured for the last 24 hr in the presence of FSH (5 μg/ml), a dense network of connecting cellular extensions is evident (Fig. 3B). The cells grown in the presence of FSH appear elevated from the surface, with extensive outgrowth of cytoplasmic processes. Cells grown in the presence of Bt_2-cAMP had a similar appearance, with numerous cytoplasmic extensions making contact with adjacent cells and with the surface of the dish (data not shown). Control cells that had not been exposed to either FSH or Bt_2cAMP in culture were flat, and few cytoplasmic extensions were evident (Fig. 3A). The addition of 10% calf serum to the culture medium com-

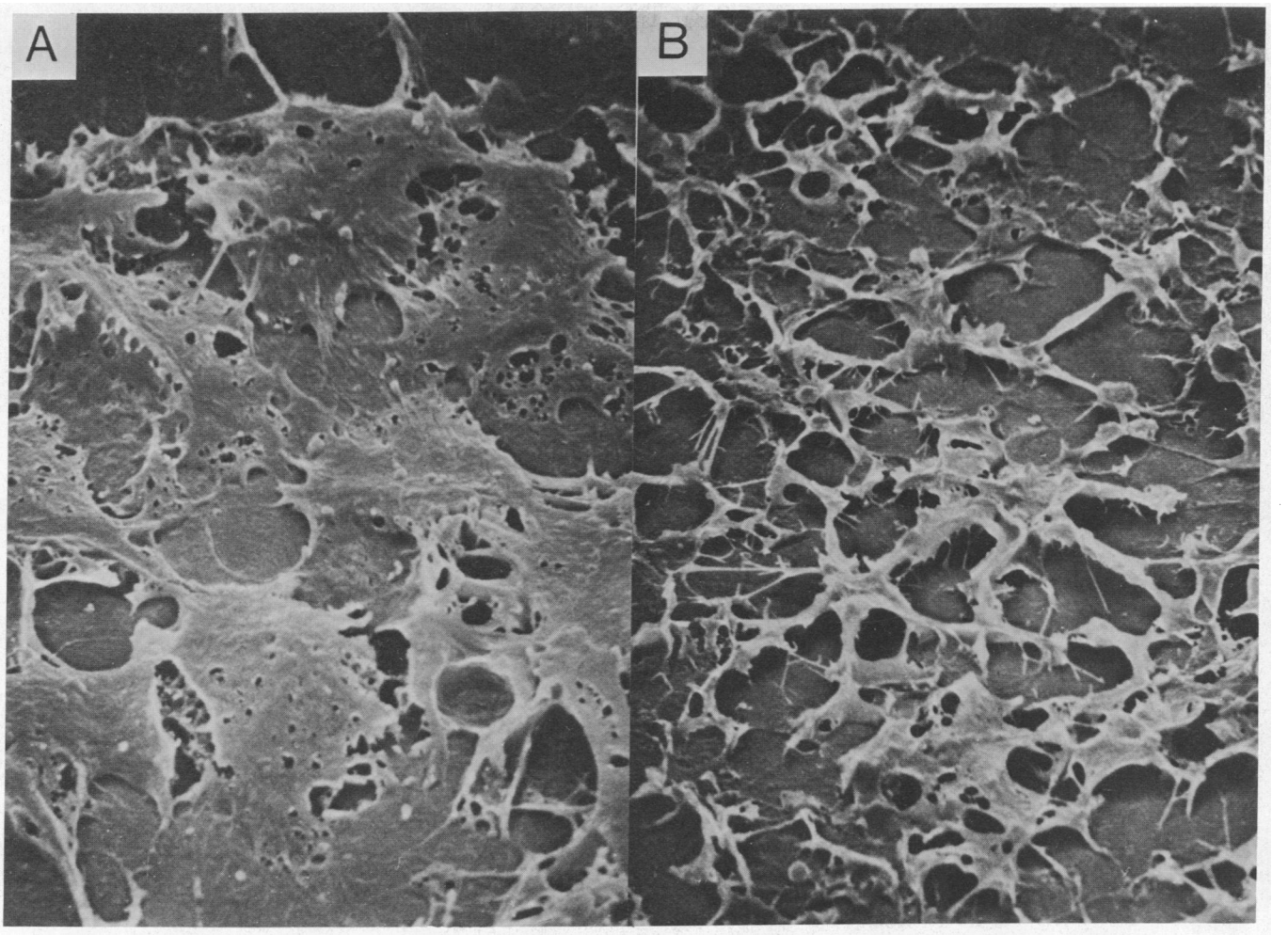

FIG. 3. Scanning electron micrographs of Sertoli cells cultured for 4 days in standard culture medium. (A) Untreated (control) cells; (B) Cells treated with 5 μg/ml of NIH S-10 ovine FSH for the last 24 hr. Magnification: $\times$3800.

pletely blocked the effects of FSH or Bt_2cAMP shown in Figs. 2 and 3.

A representative transmission electron micrograph of sections of presumptive Sertoli cells grown in culture medium containing 10% calf serum is shown in Fig. 4A. The indented nuclei, prominent nucleoli, and general morphological features evident have been described by others for Sertoli cells *in situ* in tubules (2, 3, 15). The nature of the tight junction is clearly evident in a higher magnification shown in Fig. 4B. The junction shown, with its associated cisterna, is characteristic of the unique tight junctions between Sertoli cells described by Fawcett (2) and Dym (3). Other ultrastructural features, such as the characteristic nucleolar structure reported in Sertoli cells fixed *in situ* (2, 3, 15), have been observed in our cultured cell preparations (unpublished observations, in preparation). The cell preparations are relatively uniform, with over 90% of the cells having the same general appearance when examined with the electron microscope. The remaining 5–10% of the cells are primarily spermatogonia or degenerating spermatocytes.

We have also investigated the properties of testicular fibroblasts grown in parallel cultures. Results of these studies revealed striking structural differences between presumptive Sertoli cells and testicular fibroblasts, which were evident with phase microscopy (compare Fig. 2A and D with 2E and 2F), and with electron microscopy. With scanning electron microscopy, fibroblasts of rat testis in culture were observed to form extremely thin cytoplasmic layers on the flask surface, and the presence of various stages in the cell cycle were clearly evident. The criss-crossing of cells, frequently seen in a multilayer of fibroblasts, was never observed in Sertoli cell cultures (unpublished observations, in preparation). Transmission electron microscopic examination of fibroblasts in culture showed ultrastructural properties similar to those reported for testicular fibroblasts *in situ* by Bressler and Ross (19). In addition, testicular fibroblasts in culture showed no structural changes after FSH addition under all conditions employed, and they had a high mitotic index when grown in the presence of serum (0.8–2.2%).

Biochemical responses of Sertoli-cell-enriched cultures have been described elsewhere. Production and release of androgen binding protein into the culture medium is stimulated by added FSH (9) or Bt_2cAMP (I. B. Fritz, F. Rommerts, G. Louis, and J. H. Dorrington, submitted for publication). Production of cAMP is increased by FSH but not by luteinizing hormone addition (6). An increased incorporation of labeled leucine into proteins of Sertoli cells cultured in the presence of FSH or Bt_2cAMP has also been observed (20).

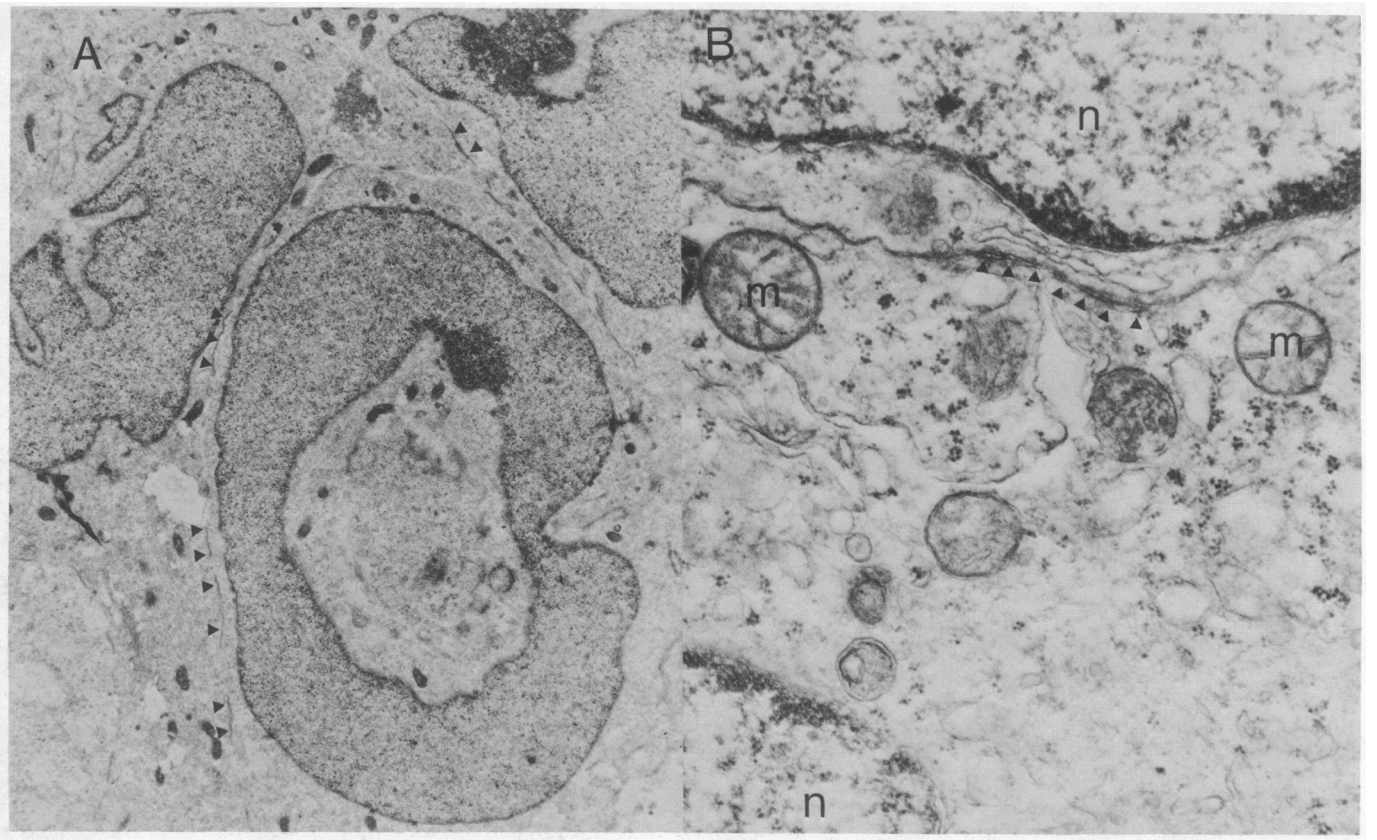

FIG. 4. Transmission electron micrographs of Sertoli cells cultured for 4 days in standard culture medium supplemented with 10% calf serum. Two representative sections of cells in monolayer *in situ* are shown. (A) (×6362), deeply indented nuclei are apparent, and probable cell junctions are indicated by arrowheads. (B) (×22,704), the characteristic junctional complexes between Sertoli cells are depicted by arrowheads, the nucleus is denoted by n and mitochondria by m.

These data suggest that the FSH effects that are mediated *in vivo* on testicular protein and RNA synthesis (16) and on androgen binding protein production (7–9) are probably a consequence of FSH actions on Sertoli cells. The duplication by added Bt_2cAMP of all actions elicited by FSH *in vitro* on Sertoli-cell-enriched cultures suggests that the FSH stimulation of adenylate cyclase activity in Sertoli cells (5, 6, 20) may be intimately associated with the biological actions of FSH.

We wish to express our thanks to Mr. Michael Welsh of the University of Western Ontario for useful discussions, and for showing us a copy of his manuscript in advance of publication (21). This paper describes a method for preparing Sertoli-cell-enriched preparations which is different from that which was simultaneously developed in this laboratory. We also wish to thank Dr. Martin Dym of Harvard for a careful reading of the manuscript and for his many suggestions. We are indebted to Ms. Krystyna Burdzy for excellent technical assistance, to Mr. Ernest Whitter for transmission electron micrographs, and to Mr. Eric Lin for scanning electron micrographs. This work was supported by grants from the Canadian Medical Research Council and the Banting Research Foundation.

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