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ELECTRON MICROSCOPY OF ISOLATED RAT TESTICULAR CELLS GROWN *IN VITRO*

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Summary. Suspensions of viable testicular cells, obtained from two groups of rats (one group treated for ten days with 50 I. U. of serum gonadotropins (HCG) daily; one group not previously treated), were cultured for ten days. Numerous cells adhered to the glass to form a confluent monolayer and remained in good condition after ten days. This monolayer contained two cell types identified by electron microscopy: fibroblastic cells, and Leydig cells. The relative proportion by which these two cells contributed to the monolayer was related to the condition of the donor when the culture was initiated. Fibroblastic cells were more abundant when the animal was not previously treated with HCG. However, Leydig cells almost exclusively formed the monolayer when cultures were begun with testicular cells of HCG-treated rats. Gonadotropins, on the other hand, did not seem capable of acting *in vitro*.

Introduction

The study of testicular physiology is seriously handicapped, since the testes are composed of a number of different cell types. It is difficult to investigate metabolic or other parameters of specific cell types under these conditions. However, if one were able to isolate each type of cell and maintain it in culture, it would provide an advantageous approach to this problem.

It has been observed (Steinberger, 1965) that in cultures of rat testicular cells, the germinal elements remain suspended in the culture medium while others adhere to the glass surface of the culture vessel and grow as a monolayer. The latter cells remain in good condition for at least ten days without a transfer. Employing cytochemical techniques, Sreinberger, Steinberger and Vilar (1966) have shown that under specific experimental conditions, certain cells in the monolayer exhibit positive reactions for Δ^5-3β-hydroxysteroid dehydrogenase. Since it has been demonstrated that in testes of rodents, this reaction is positive only in Leydig cells (Wattenberg, 1958; Baillie and Griffiths, 1964), it was tentatively assumed that such a monolayer was partly composed of Leydig cells. However, since some cells reacted negatively, it was questioned as to whether they were Leydig cells or cells of some unrelated type, since negative cytochemical reaction does not exclude the possibility that weak enzymatic activity may be taking place in these cells.

The present communication describes the results of electronmicroscopic studies of rat testicular cells grown in monolayers under various experimental conditions.

Material and Methods

Ten-day-old rats, raised in our laboratory, were divided into two groups. One group received 50 I. U. human chorionic gonadotropin (HCG) twice daily for ten days. The other group, used as controls, received no treatment. All animals were sacrificed at the age of 20 days; their testes were removed and those from each group were pooled and prepared for culture. Technique for culturing has been previously described (Steinberger, 1965). Briefly, finely minced

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testes, without tunica albuginea, were incubated at 31° C for 10 to 15 min with 0.25% trypsin solution in phosphate buffer, pH 7.2. The nondissociated tissue fragments were separated by gravity. The supernatant, containing predominantly single cells, was centrifuged at 900 rpm and the sedimented cells were resuspended in culture medium to a concentration of 6 to 8×10^5 cells per ml. Petri dishes or 4-oz. bottles were seeded with aliquots of this suspension and incubated at 31° C for 10 days in an atmosphere of 95% air and 5% CO_2. The culture medium was maintained at pH 7.0 and changed on the seventh day. Eagle's minimal essential medium was used, to which 10% calf serum, several amino acids, sodium pyruvate and antibiotics were added. Half the cultures in each group was grown in medium containing 50 I. U./ml. of HCG.

Preparation for Electronmicroscopy: Cells were released from the glass by trypsin, spun down and resuspended in fresh medium, to which osmium tetroxide (5%) was added to give a final concentration of 1%. The resulting osmolar concentration and pH were close to those of the medium. After one hour's fixation in an ice-bath, the suspension was centrifuged to obtain a firm pellet. Each pellet contained cells pooled from several bottles or Petri dishes. The pellets were divided into 1 mm. fragments and post-fixed in 2% uranyl acetate. Dehydration was accomplished with ethanol containing 1% phosphotungtsic acid. Tissues were embedded in a mixture containing 10 ml. Epon 812; 25 ml. dodecenyl succinic anhydride; 1.3 ml. of 1-2-cyclohexane dicarboxylic anhydride and 0.45 ml. N-N-dimethylbenzylamine. After soaking cells in this mixture overnight, at 37° C, polymerization was carried out at 60° C for 48 hours. Glutaraldehyde, in a final concentration of 2,5%, was alternately used as a fixative. Fixation was carried out for two hours and the pellets were stored overnight at 0—4° C in 0.22 M sucrose in cacodylate buffer, pH 7.2; refixed in 1% osmium tetroxide (CAULFIELD, 1957), dehydrated through ethanol and embedded in Epon. Silver and gold sections, obtained with a Porter-Blum microtome, were stained with lead citrate (REYNOLDS, 1963) and observed with an RCA EMU-3 electron microscope. The electron microscopic observations represent the study of four experiments.

Results

In testes from both groups of rats, spermatogenesis proceeded to the stage of primary spermatocytes. The interstitial spaces of the testes from untreated rats contained small clusters of fibroblasts and occasional Leydig cells around the arterioles and capillaries (Fig. 1). In testes of HCG-treated animals, the interstitial tissue showed marked hyperplasia with most cells showing Leydig-cell characteristics (Fig. 2). When isolated cells were grown on the glass surface, they formed a confluent monolayer and presented the appearance shown in Fig. 3. Although it was obvious that they did not correspond to the spermatic cells, their identification with the remaining cellular types was not feasible. Furthermore, no specific histologic characteristics or method of growth was observed which enabled us to differentiate cultures arising from the two different types of donors.

Electronmicroscopic studies of cultures initiated from either HCG-treated or untreated animals revealed two types of cells. In one type, the lobulated nucleus was surrounded by a nuclear membrane having no differential characteristics. The karyoplasm was uniformly granulated, except for clumps of granules attached to the nuclear membrane, making its outlines sharply defined. A single compact nucleolus appeared in contrast to the rest of the karyoplasm. The cytoplasm revealed somewhat irregular, dilated cisterns. Their number was not uniform, some cells having very few cisterns, others having the cytoplasm filled with them. Smooth surface vesicles, frequently containing electron-dense material, were observed in the area of the plasma membrane, suggesting that these cells were incorporating materials actively. The endoplasmic reticulum was otherwise poorly developed and only a few vesicular outlines, showing RNA granules, were seen (Fig. 4). In contrast, such granules were formed in larger numbers and were scattered throughout

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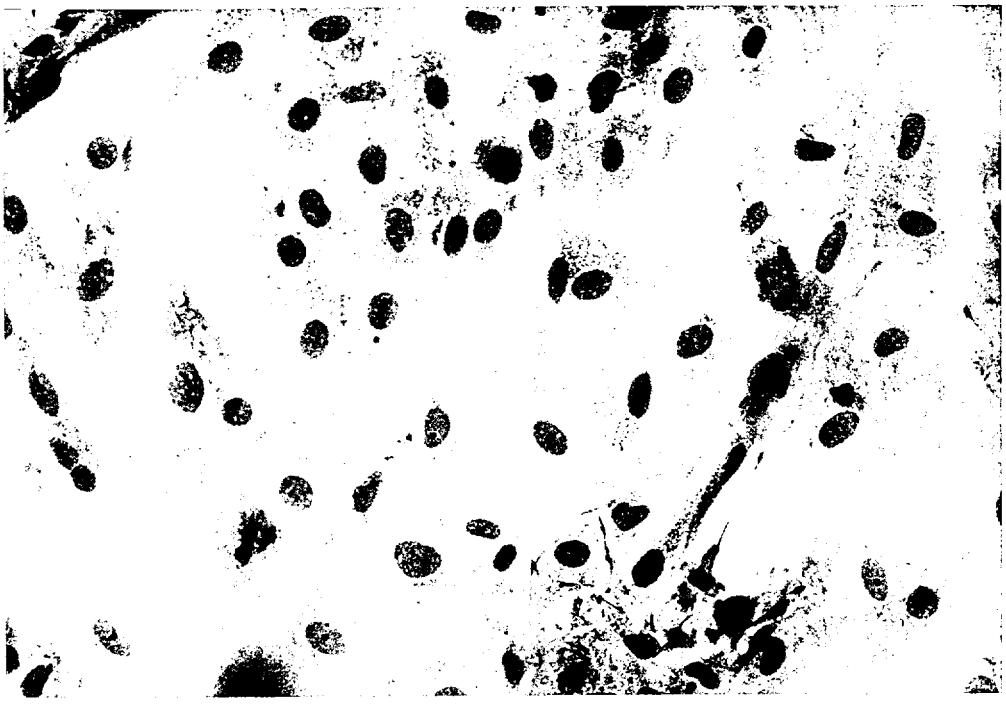

Fig. 3. 10-day culture of testicular cells. Growth of cells on the glass surface. PAS an space hemalum, × 300

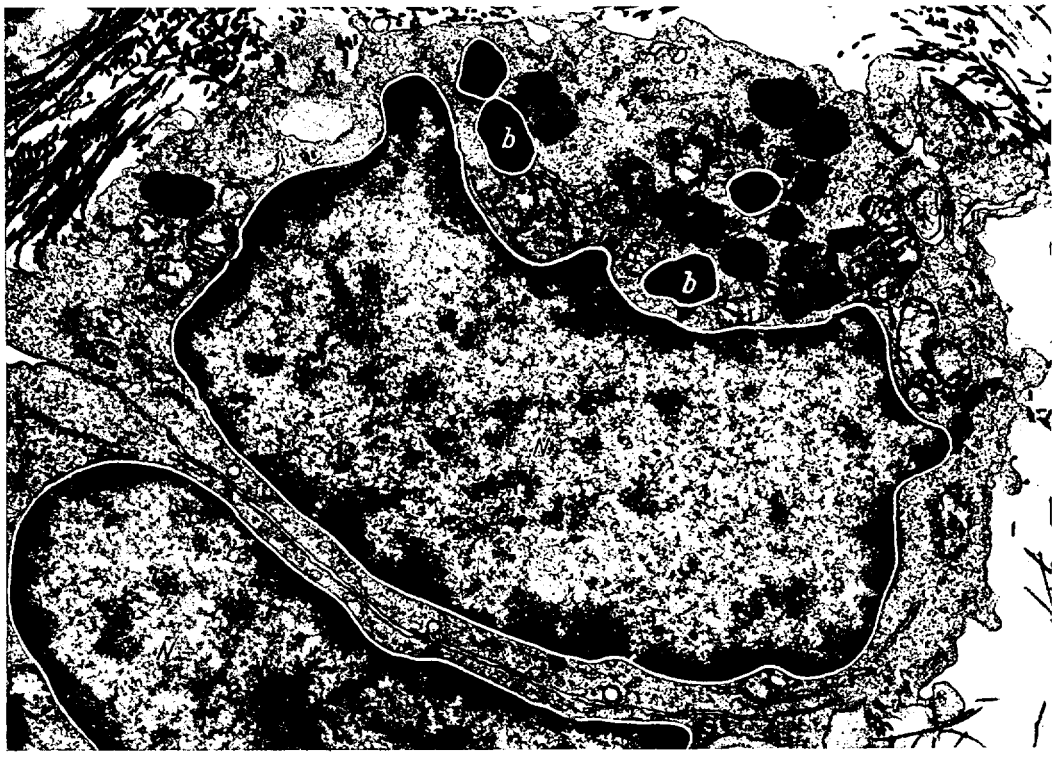

Fig. 4. Caulfieds' fixation and Epon embedding as in all the following photographs. Electron micrograph of two cells from a monolayer derived from testes of untreated rat. Nuclei of fibroblastic cells (*N*); dense bodies (*b*) and collagen fibers (*f*). Note that cytoplasm has few vesicular profiles. Magnification × 20,000

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Fig. 1. Testis of an untreated 20-day-old rat. Periodic Acid Schiff (PAS) and hemalum, × 300

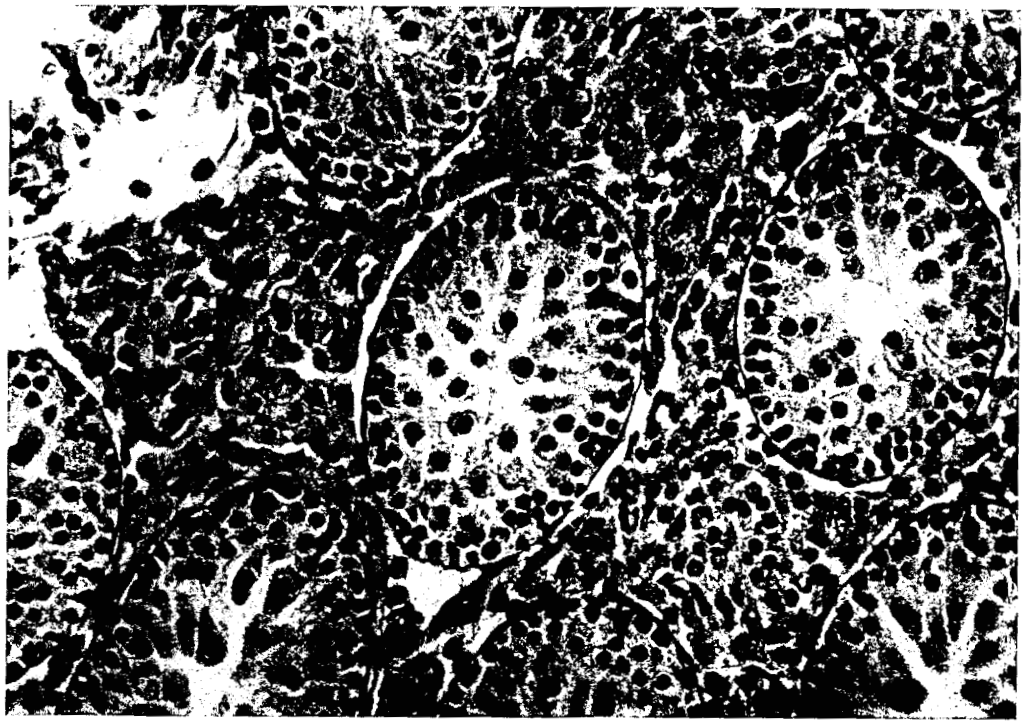

Fig. 2. Testis of a 20-day-old rat treated with HCG. Note hyperplasia of interstitial cells. PAS and hemalum, ×300

the cytoplasm forming rosettelike clusters. Fine filaments were seen in the cytoplasm (Fig. 5). They were more conspicuous when other cytoplasmic components

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Fig. 5. Fibroblastic cells form a monolayer derived from testes of untreated rat. Fine filements are seen in the cytoplasm, as well as small inclusion bodies. Magnification, ×61,500

appeared to be washed away, probably a consequence of manipulation prior to fixation. Golgi complex was not prominent and a small number of mitochondria, with dense matrix and the usual internal structure, were present. Lipids were rarely seen but numerous single membrane-bordered inclusion bodies with varied diameters and nonhomogenous appearance were observed. They showed either the characteristics of vacuoles, containing few vesicular or membranous profiles over an empty background, or the features of cytosomal "dense bodies", with a dense homogenous matrix in which denser membranous or filamentous profiles contrasted sharply. Cytosomal bodies, resembling myelin figures, were seen infrequently. Dense packeted bundles of collagen fibers were seen very close to the outer surface of these cells. The subcellular characteristics of this type of cell, present in our cultures, are similar to those of the intertubular connective tissue fibroblasts. Nevertheless, the fibrilogenic activity was remarkably higher, probably due to the *in vitro* conditions (Fig. 6).

The second type observed in the monolayer was characterized by larger cells with a homogenous round or ovoid nucleus containing one, two or even three larger and irregularly-shaped nucleoli composed of several large central masses, projecting narrow and twisted strands. In the cytoplasm, lipid inclusions appeared as round dense droplets, some cells showing large ones, others containing few or none (Fig. 7). Cytosomal bodies were scarce and of various sizes, contents and density of the background matrix. The Golgi complex was moderately well developed, com-

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Fig. 6. Fibroblastic cells in a monolayer derived from testes of untreated rats. The production of collagen fibers is very high. Note numerous inclusion bodies (dotted lines) and cisterns (arrows) in the cytoplasm of the cells; (*N*) nucleus Magnification, × 8,500

posed of a few small vesicles and flat saccules arranged in parallel fashion in the juxtanuclear zone (Fig. 8). Most of the mitochondria were round or ovoid in shape, seldom elongated and branched; their size showed marked variations. This was probably due to the sensitivity of the mitochondria to the fixation and embedding procedures. Swollen and bizarre large mitochondria, exceeding 1 μ in diameter,

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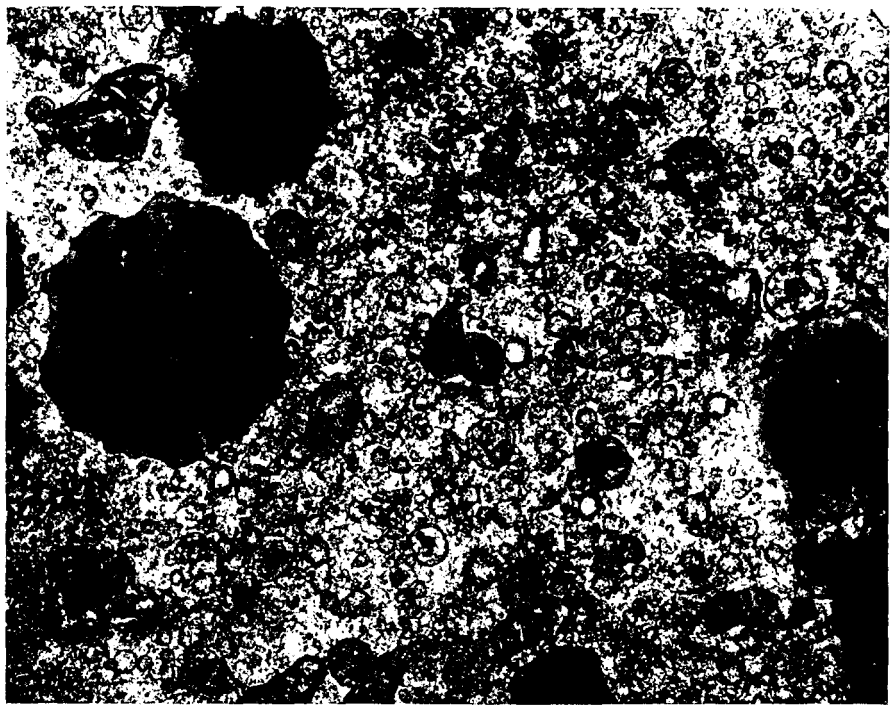

Fig. 7. Cell from a monolayer derived from testes of rats treated with HCG. Lipid droplets, as well as a well-developed system of smooth-surfaced vesicles are seen. Magnification, × 26,000

were quite frequent. The matrix was normally low in density and the inner membrane often presented fine tubules that tended to run radially. A limited number of dense particles, presumably ribosomes, occurred in the cytoplasmic matrix in clusters of four; they were rarely associated with membranes. The endoplasmic reticulum demonstrated a few elongated membranous profiles dispersed in the cytoplasm, but the most constant and outstanding feature was the presence of numerous small, smooth-surfaced vesicles, most of which appeared to be empty under the electron microscope; their numbers varied from cell to cell. Even in the same cell, there was frequently a tendency for these vesicles to be more closely packed in areas where other organelles did not occur (Fig. 7). The cellular boundaries were smooth but the plasma membrane occasionally revealed slender projections reminiscent of microvilli (Crabo, 1963). None or few of the fine collagen fibers were seen and the ultrastructural characteristics of these cells conformed to those of mature, active Leydig cells.

Cells other than fibroblast or Leydig type normally present in the testes (spermatic, Sertoli and/or vascular endothelial), were not observed in these monolayers. They most probably did not attach to the glass, remaining in the suspension and degenerating. Mitoses were very infrequently observed after ten days in culture. The relative proportion of fibroblasts and Leydig cells in the monolayer differed in relation to the condition of the donor tissue.

1. Cultures of Testicular Cells from Untreated Rats. The cells were predominantly fibroblasts with numerous closely packed bundles of extracellular collagen fibers always associated with them. Only occasional cells in these monolayers demonstrated characteristic features of Leydig cells. When HCG (5 I.U./ml.) was added

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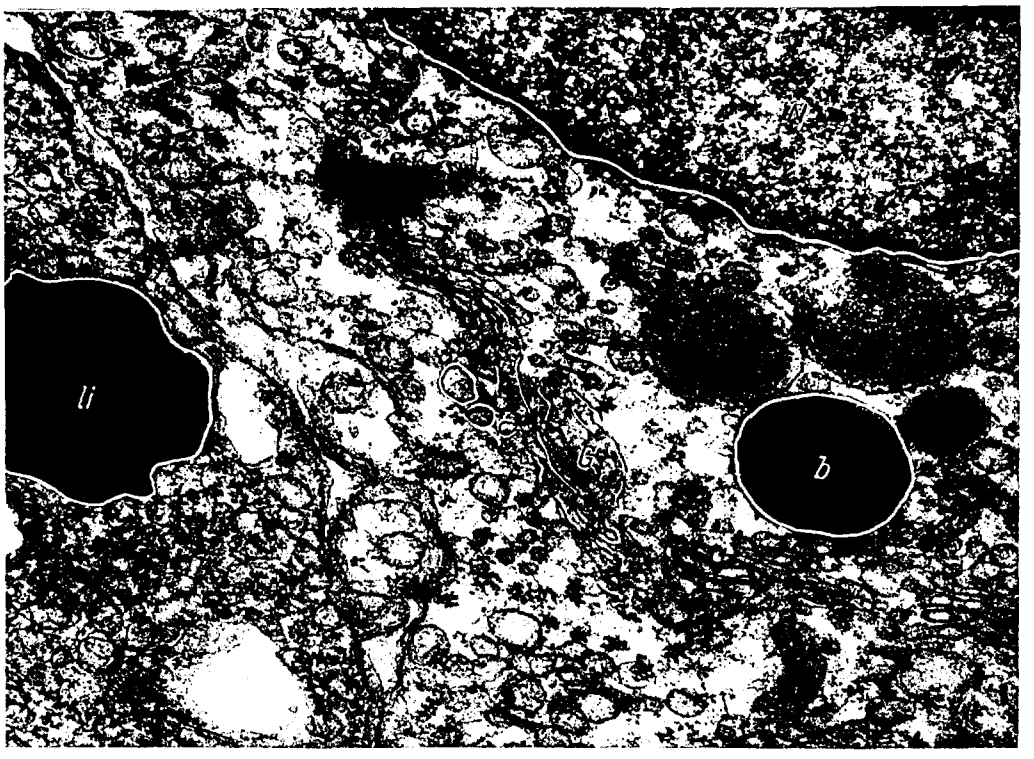

Fig. 8. Two Leydig cells from a monolyaer derived from testes of rats treated with HCG. Lipids (*li*) and dense inclusion bodies (*b*), as well as the Golgi system (*G*) are seen. Magnification, × 34,000

to the medium, the cells tended to round up and the continuity of the monolayer was frequently interrupted. However, the relative proportion of the cell types and their subcellular features remained unchanged.

2. Cultures of Testicular Cells from Rats Treated with HCG. The majority of cells showed the characteristic ultrastructure of Leydig cells, while relatively few cells with fibroblastic appearance were present. No different results were obtained even when HCG was added to the medium.

Discussion

Previous work, using light microscopy, established the fact that cultures initiated with suspension of rat testicular cells, yield a monolayer type of growth, and that some cells do not become part of the monolayer, remaining free-floating in the culture medium (STEINBERGER, 1965). Cytochemical determinations of Δ^5-3 β-hydroxysteroid dehydrogenase activity indicated that the monolayers might be at least partly composed of Leydig cells, their proportion varying according to the state of the donor tissue. In monolayers from testes of untreated 20-day-old rats, only occasional cells gave a positive reaction, whereas in monolayers from testes of rats treated with HCG, a large proportion of cells showed Δ^5-3 β-hydroxysteroid dehydrogenase activity. If one accepts the presence of this enzyme activity as a marker for Leydig cells, a proportion of cells in the monolayers still remain unidentified on the basis of light microscopy and cytochemistry (STEINBERGER, STEINBERGER and VILAR, 1966). In the present study, these cells

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have been identified as fibroblasts. If fibroblasts and Leydig cells remain viable, the question arises as to whether or not they are capable of accomplishing their specific functions when grown in culture under the above described conditions. This study has shown that fibroblasts are able to do so, since they were usually observed surrounded by many packeted collagen fibers. It is more difficult to determine whether the Leydig cells are also functional. It has been suggested (FAWCETT and BURGOS, 1960; CHRISTENSEN and FAWCETT, 1961; CHRISTENSEN 1965) that the agranular reticulum is probably the major site of hormone production in the Leydig cells, with the abundance of the agranular reticulum reflecting the degree of activity. If we accept this, the Leydig cells grown in culture appeared capable of producing steroids, since one of the characteristic features was the presence of a similar, well-developed agranular reticulum. Although it is very difficult to establish morphologically, that synthesis of steroids takes place in any given part of the cell, it is highly suggestive that Δ^5-3 β-hydroxysteroid dehydrogenase is present in the microsomal fraction of rat testes (SHIKITA, KAKIZAKI and TAMAOKI, 1964) and it was stated (CHRISTENSEN, 1965) that during homogenization, the abundant endoplasmic reticulum of the interstitial cells is the main contributor to the microsomal fraction. In this sense, the ultrastructural study parallels our histochemical results obtained in similar cultures (STEINBERGER, STEINBERGER and VILAR, 1966). Although we saw mitosis under the light microscope in the beginning of the cultures, we have not as yet determined whether the Leydig cells present in the monolayers are formed in culture by either differentiation from fibroblasts, or by division of other Leydig cells. It is believed that Leydig cells of most species are differentiated from fibroblasts of the intertubular connective tissue *in vivo*. Such development has been observed in the human testes, either in the fetus (BOUIN and ANCEL, 1903; GILLMAN, 1948), or at puberty (CHARNY, CONSTON and MERANZE, 1942; SNIFFEN, 1952; MANCINI, VILAR et al., 1963) and in testes of other species (RASMUSSEN, 1932) including bulls (HOOKER, 1948) and rats (ROOSEN-RUNGE and ANDERSON, 1932). We did not observe any intermediate stages of development of Leydig cells from fibroblasts. However, we cannot eliminate the possibility that such development could have occurred during the early stages of culture.

These studies have shown that it is possible to isolate and maintain in culture for several days, interstitial fibroblasts or Leydig cells, this providing an adequate system for the study of factors influencing the growth, differentiation and function of these cells. Previous treatment of the donor with HCG, to induce hyperplasia of the interstitial tissue, seemed essential for obtaining monolayers composed predominantly of Leydig cells. On the other hand, this hormone could not induce any *in vitro* change in the cellular components of the tissue cultures. No specific site of action for the gonadotropins on the testes has been demonstrated; their course of action remains unclear. It is possible that their activity may be mediated by some mechanism requiring conditions not present in our cultures.

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