Zeitschrift für Zellforschung 78, 221—[redacted] (1967)

703812

# AN ELECTRON MICROSCOPIC STUDY OF CULTURED RAT TESTICULAR FRAGMENTS

Oscar Vilar, Anna Steinberger, and Emil Steinberger[redacted]
Division of Endocrinology and Reproduction, Albert Einstein Medical Center Research Laboratories, Philadelphia, Pa.

Received November 15, 1966

*Summary.* Fragments of testicular tissue of 26-day-old rats were grown as organ cultures during one to six weeks. Electron microscopic studies showed that these tissues can be maintained *in vitro* for prolonged periods of time, although the most differentiated elements (Leydig and spermatic cells other than spermatogonia) fail to continue their development and degenerate rather rapidly. The connective tissue structures preserve their usual architecture, but the basal membrane of the tubules appears extremely folded and detached from the epithelium. After four weeks in culture, spermatogonia without differentiating are still present, and among them the presence of a "more primitive" type is noted. Sertoli cells are well preserved and ultrastructurally they present the characteristics of the adult type. The possibility exists that differentiation of these two lines of cells may be achieved *in vitro* if the factors necessary for their growth and differentiation are recognized and incorporated in the culture system.

## Introduction

A number of attempts have been made in the past to maintain organized testicular tissue in culture (Champy, 1920; Dux, 1940; Mendelsohn, 1938; Lostroh, 1960. In these studies, the duration of culture was limited to 1–3 weeks. Recently, culture conditions have been described which permit *in vitro* maintenance of testicular tissue fragments for a number of months (Steinberger et al., 1964a, b). Most of the germinal elements gradually degenerate, but the tubular architecture remains well preserved and certain cells in the seminiferous tubules remain viable.

This study was undertaken to obtain information concerning the ultrastructure of testicular fragments grown in culture for varying periods of time. It was anticipated that a study of the subcellular characteristics will enable us to observe changes which could not be detected with light microscopy and determine which cell types remain viable in culture.

## Material and Methods

Testes from 26-day-old Long-Evans hooded rats, bred in our laboratories, were used in this study. Testicular fragments approximately 1 mm³ in size were grown as organ cultures, using previously described technique (Steinberger et al., 1964a, b). After one to six weeks of cultivation, multiple fragments were fixed either in buffered osmium tetroxide solution (Caulfield, 1957) for one hour at —4° C or in buffered 2.5% glutaraldehyde for two hours, followed by overnight storage in 0.1 M cacodylate buffer, pH 7.2—7.4 with 0.2 M sucrose, and a second fixation with 1% osmium tetroxide (Sabatini, Bensch et al., 1963). Postfixation was also carried out in 2% uranyl acetate, at room temperature. The tissue was dehydrated with ethanol containing 0.1% phosphotungstic acid and embedded in a mixture of Epon 812, Dodecenyl Succinic Anhydride, 1—2 Cyclohexane dicarboxylic anhydride and N-N-dimethylbenzylamine (Vilar, Steinberger et al., 1966). After soaking tissues in this mixture overnight at 37° C, polymerization was done for two days at 60° C. Silver and gold

REPOSITORY DUF - MacRESTAL
COLLECTION Markey Files
BOX No. 2 of 6
FOLDER Univ of Washington II
(45-1) 1781 Alvin C. Paulsen
1001666

sections, obtained with the Porter-Blum microtome, were stained with lead citrate (REYNOLDS, 1963) and observed with an RCA EMU-3 electron microscope.

*Acknowledgments.* The authors wish to thank Miss LYNN PROCHORENKO for her technical assistance.

This work was supported by U.S. Public Health Service Grants No. HD 01039 and No. HD 00399.

## Results

The testicular tissues used to initiate the cultures contained Leydig cells in the intertubular spaces and spermatogenesis proceeded to the cap phase of spermatid development (Fig. 1). After three days in culture, many interstitial, as well as intratubular cells showed degenerative changes. Earliest changes were noted in the vascular endothelium, the Leydig cells and the spermatids. Later, spermatocytes and some spermatogonia showed similar changes. The main ultrastructural alterations were: cytoplasmic swelling, destruction of mitochondria, dissolution of RNA granules, vacuolization of the cytoplasm and appearance of complex cytoplasmic inclusions; in the nucleus, chromatin showed dense aggregates and the nuclear membrane showed progressive dissolution (Fig. 2). Eventually, all cellular features were distorted, wide intercellular spaces were formed due to retraction of the necrotic cells, and the original cell types could no longer be recognized. The debris were rapidly cleared from the lumen of the seminiferous tubules.

By the fourth week, definite and specific histologic characteristics of the cultured fragments were established, which were maintained until the time of termination of the culture. In addition, the surface of the explant has shown frequently formation of a sheath-like structure composed of numerous cells and collagen fibers. The ultrastructural characteristics of this sheath resembled the intertubular connective-tissue and will be described below.

*Interstitial Spaces.* The endothelial cells and the Leydig cells disappeared rapidly and failed to regenerate. Serial observations of different age cultures showed no evidence of transformation of these cells into other cell types. After four weeks in culture (Fig. 3), only one type of cell remained in the interstitial spaces — the adult fibroblast. This cell had slender fusiform shape with cytoplasm forming a narrow rim around the nucleus. The nucleus was relatively large, showing smooth invaginations and rather uniform karyoplasm, except for the periphery, where dense granules attached to the nuclear membrane sharply defined the boundaries of the nucleus. Golgi apparatus was not particularly outstanding and the endoplasmic reticulum was represented by few vesicles with ribosomes attached to them. These ribosomes formed clusters throughout the cytoplasm. Numerous inclusion bodies with a single bordering membrane and of various diameters were observed. Most commonly the inclusion bodies were vacuoles containing few vesicular or membranous profiles over an empty background, dense cytosomal bodies containing filamentous material, or whorl-forming membranes arranged in parallel fashion ("myelin figures"). The cytoplasm also contained irregular osmophilic droplets which were interpreted to be lipids.

The number of fiber bundles with the transverse periodicity of 640 Å, corresponding to collagen, appeared to be substantially augmented in the extracellular spaces among seminiferous tubules (Fig. 4).

1001667

Fig. 1. Testicular Tissue prior to cultivation. Periodic Acid-Schiff-hemalum. ×550

*Seminiferous Tubules. The Tubular Wall.* The anatomical structure of the wall of the seminiferous tubules presented the same elements as seen in the adult testes (Fig. 5). From the germinal epithelium toward the intercellular spaces, several distinct layers were recognized: 1. a 0.1—0.2 μ thick, homogenous continuous coat of moderate density, the basement membrane; 2. a clear zone containing groups of collagen fibers; 3. a cellular layer composed of extremely elongated cells showing numerous interdigitating processes. The ultrastructure of the later cells was characteristic of adult fibroblasts, although in a more aceentuated form. Adjacent to the inner zone, the surface of these cells was coated with a material, probably muco-polysaccharide in nature, similar to the basement membrane in its electron density and appearance. The architecture of the tubular wall (Fig. 5) differed from the normal in the following respects: 1. the basement membrane, although of normal thickness, formed numerous folds and appeared to be separated from the cells of the seminiferous epithelium by wide irregular spaces containing scattered granular material of unknown nature; this folded appearance was more accentuated in tubules with reduced diameter and was probably responsible for the apparent thickening of the membrane under the light

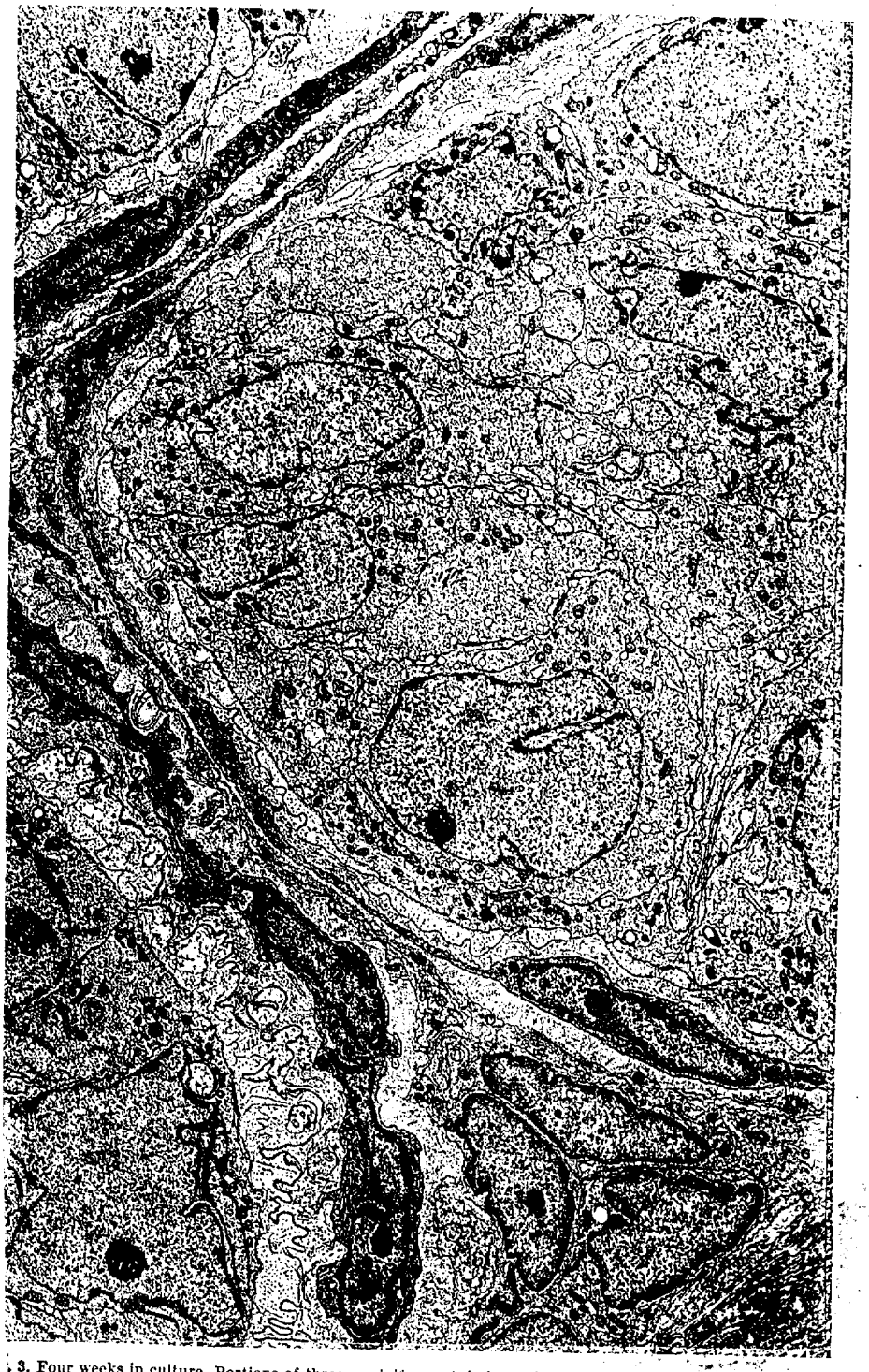
1001668

Fig. 2. Testicular tissue kept 3 days in culture. Note two degenerating spermatic cells (*g*) and between them the cytoplasm of a Sertoli cell (*S*) which shows no outstanding changes. ×24,000

microscope. 2. The collagen fibers appeared to be increased in number, forming more densely packed bundles.

*The Seminiferous Epithelium.* The epithelium was composed mainly of long, thin triangular palisading cells, lying adjacent to the basement membrane (Figs. 3, 5). The nucleus contained deep indentations which imparted to it an almost lobulated appearance. Large clumps of chromatin-like material were attached to the nuclear membrane. The nucleolus was round and prominent. It appeared granular with scattered patches of lesser densities. Outlines of the apical portions of these cells were more complex in tubules showing no lumen (Fig. 6). The cells, however, retained their anatomical unity, showing the usual intercellular spaces of 200 Å. Desmosome-like structures were occasionally seen where two cells adhered to one another (Fig. 6). The cytoplasmic matrix presented a network of delicate fibrils which showed no defined orientation, the Golgi apparatus formed by smooth-surfaced vacuoles and fine vesicles was barely discernible and the endoplasmic reticulum showed numerous tortuous and flattened saccules, as well as small vesicles which were primarily agranular. Ribosomes appeared usually in clusters of two to five (larger aggregates were rare). The chondrioma consisted of small, viliform and dense-matrix mitochondria, which were either branched or in the form of biconcave disks. Lipid inclusions were observed in most cells. They formed dense irregular-outlined droplets or round vacuoles, moderately dense at the periphery, with empty centers. Various types of inclusion bodies were frequently seen. They were limited by a single, or occasionally double, membrane and contained varying amounts of membranous profiles, filaments, dense material

1001669



3. Four weeks in culture. Portions of three seminiferous tubules and the interstitial tissue are shown. Sertoli cells (*S*) only are seen in the intratubular epithelium; (*f*) fibroblast. ×7,800

1001670

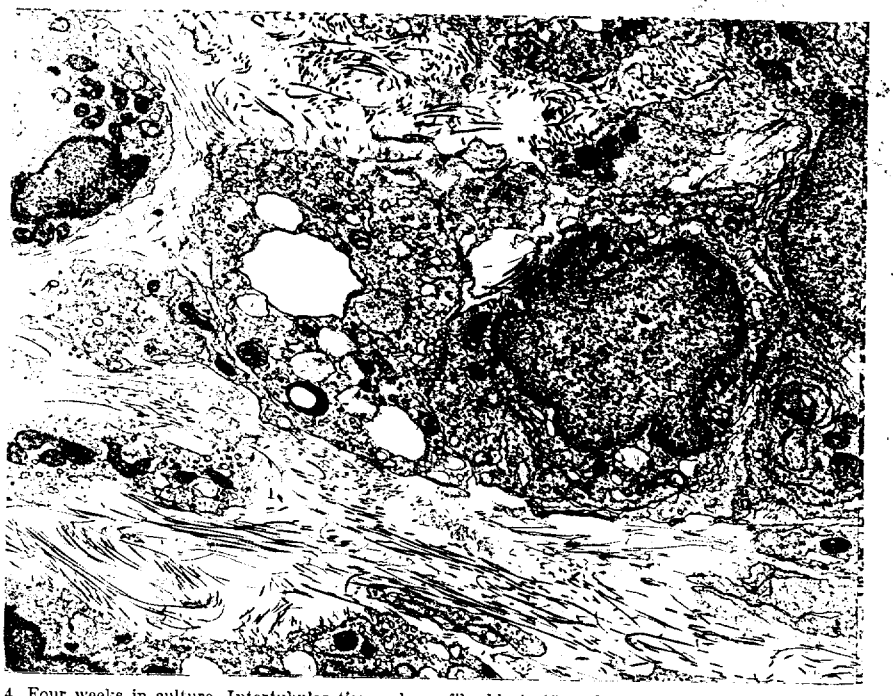
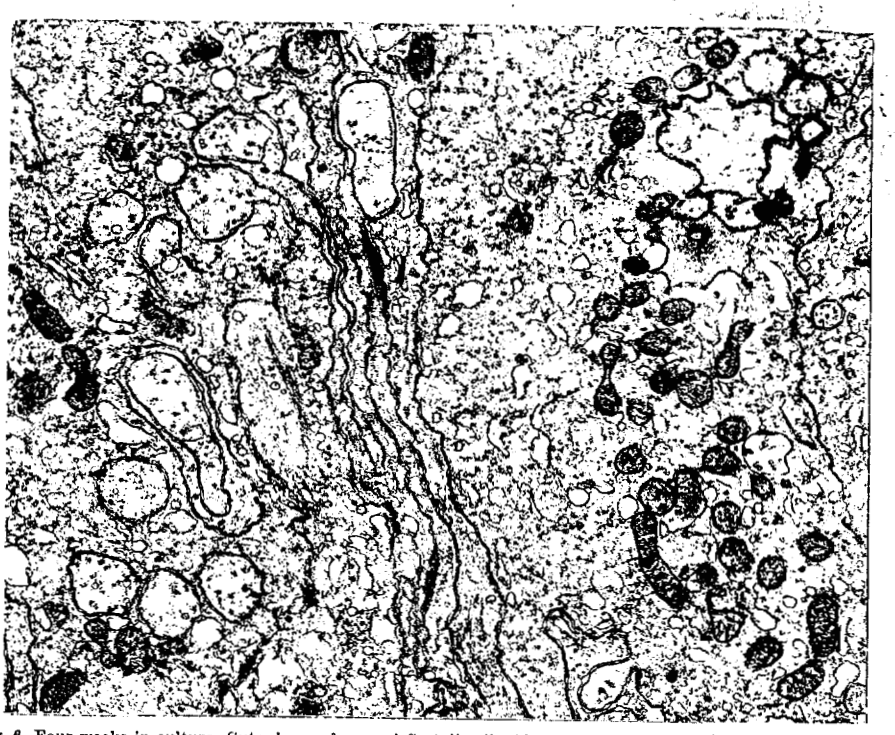


Fig. 4. Four weeks in culture. Intertubular tissue shows fibroblasts (*f*) and numerous collagen fibers (*ret*). No Leydig cells are seen. ×12,000

Fig. 5. Four weeks in culture. Seminiferous tubule wall. Note the abnormally folded basement membrane (*bm*), numerous collagen fibers and fibroblast (*f*) showing an extracellular coating (*c*). In the tubular epithelium, portions of Sertoli cells (*S*). ×17,600

1001671



g. 6. Four weeks in culture. Cytoplasm of several Sertoli cells. Narrow branches of some cells articulate in the center. Note the "desmosomelike" (*d*) and "striated" structures (*mt*). × 22,000

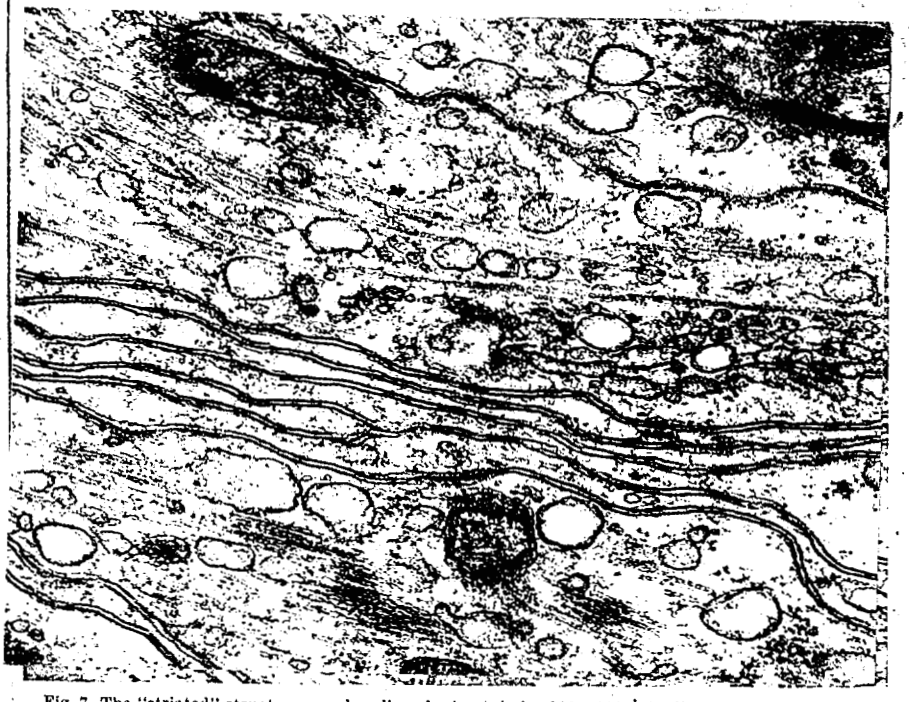


Fig. 7. The "striated" structures are bundles of microtubules 200—300 Å in diameter (*mt*). × 59,200

1001672

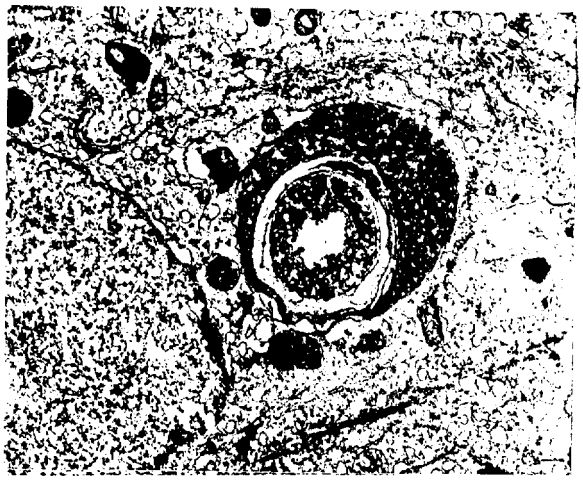
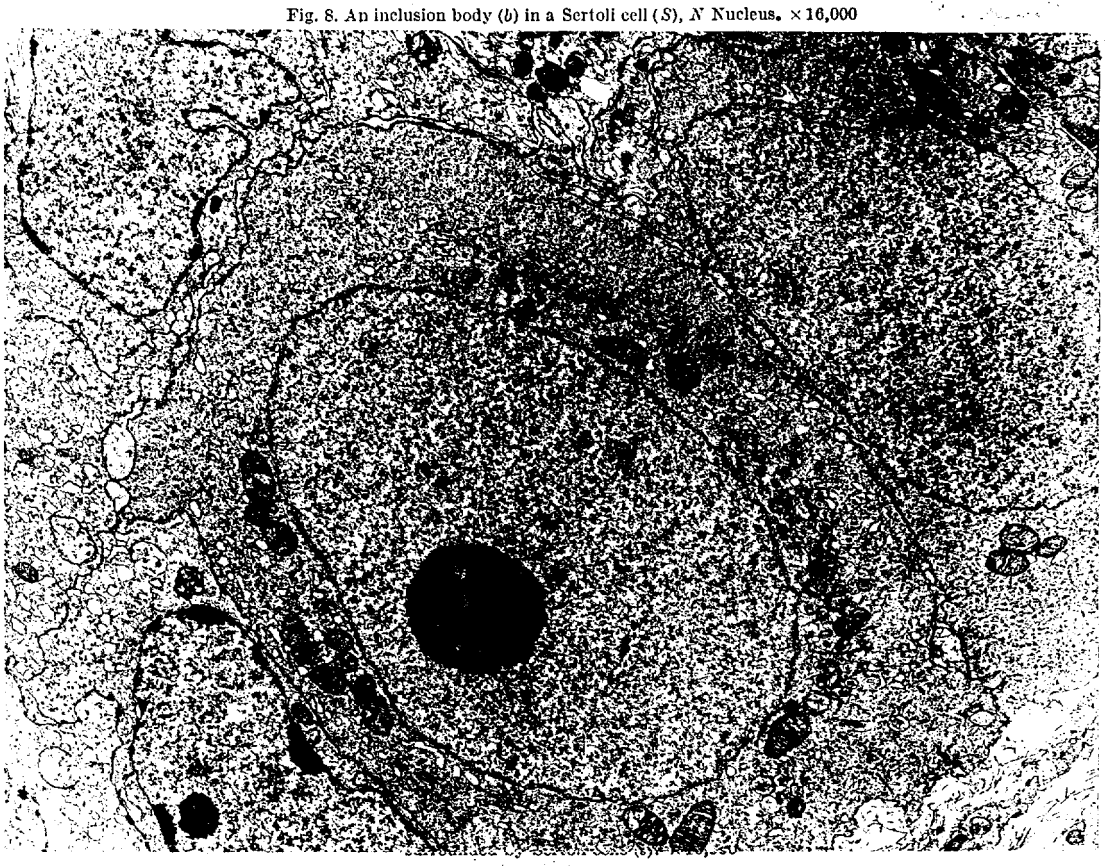


Fig. 8. An inclusion body (*b*) in a Sertoli cell (*S*), *N* Nucleus. ×16,000



or clearly recognizable cytoplasmic organelles or nuclear debris (Fig. 7). The inclusion bodies were extremely large and numerous during the first two weeks of culture, at the time when many germinal cells were in the process of degeneration. Their number decreased rapidly after the third week in culture. Striated struc-

1001673

ig. 10. Four weeks in culture. Spermatogonia of a more mature type situated among several Sertoli cells. × 14,000

ures, radially arranged in relation to the basement membrane, were observed poradically in the apical part of the palisading cell (Fig. 6). At higher magnication, they appeared to be composed of paralleled microtubules, 200 to 300 Å n diameter, similar to the fibers of the "manchette" in the spermatids or the ilaments of the mitotic spindle of the meiotic spermatocytes (Fig. 8). They were present in all fragments and regardless of the fixation method used.

The electron microscopic appearance of the pallisading cells in the tubules of the cultured testicular fragments was characteristic of the adult Sertoli cells. Scattered among the Sertoli cells an ultrastructurally different cell type was also observed. These cells were larger, oval or polyhedric, some of them in contact with the basement membrane. They contained rather large, rounded nuclei with well-defined nuclear membranes and nucleoli consisting of knots of granular wads. The Golgi apparatus was a prominent organelle consisting of flat parallel sacs or cisterns, small rounded vesicles with dense content, and larger empty vacuoles.

1001674

Mitochondria differed strikingly from those of Sertoli cells. They were large, round or ovoid, well delineated against the background, containing well-defined cristae arranged in parallel or radial fashion. These cells were of the spermagonotial type and fell into two groups: 1. Large oval cells in direct contact with the basal membrane, with nuclei containing regularly distributed chromatin and one or two very prominent nucleoli; their cytoplasm contained numerous polyribosomes which made the cells appear darker and more outstanding than the surrounding Sertoli cells (Fig. 9); and 2. Smaller polyhedrical cells with reduced nuclear-cytoplasmic ratio, similar to spermatogonial cells seen in the puberal and adult ages. The second cell type was, in most cases, separated from the basal membrane by Sertoli cell cytoplasm. The distribution of the nuclear chromatin was less uniform and the nucleolus was distinctly smaller. The cytoplasm contained a conspicuous Golgi apparatus and numerous small vesicles, the ribosomes were less abundant than in the previously described cell type and were not associated with the small vesicles (Fig. 10).

## Discussion

This study shows that while fragments of testes from 26-day-old rats can be maintained *in vitro* for prolonged periods of time, not all structures survive in the same fashion. The most differentiated elements (Leydig cells and spermatic cells other than spermatogonia), fail to continue their development, and degenerate during the first several weeks of cultivation. The tubular diameter is reduced, and the lumen is no longer discernible. CHAMPY (1920) on the basis of study of cultures of rabbit testes concluded that gonads cultured *in vitro* "return to embryological, or more specifically, to prepuberal stage". Our study did not confirm this conclusion. The ultrastructure of intertubular fibroblasts, the tubular wall and the Sertoli cells did not revert to that found in the testes of immature or prepuberal animals (MANCINI, VILAR et al., 1964; PIERCE and BEALS, 1964). On the contrary, these structures could not be distinguished from those of the adult (FAWCETT and BURGOS, 1960; VILAR, PEREZ DEL CERRO et al., 1962; BRÖKELMANN, 1963; CRABO, 1963; BAWA, 1963; GARDNER and HOLYOKE, 1964; CHRISTENSEN, 1965). The changes observed in the testicular architecture were the result of disappearance of certain cells and in no way suggestive of de-differentiation.

The folding of the basement membrane of seminiferous tubules and its detachment from the adjacent germinal epithelium cannot be easily explained, since it has been shown repeatedly, even in cases of poorly preserved material, that the basal portions of the germinal epithelium remain firmly attached to the basement membrane of the seminiferous tubules (PEASE, 1960). Removal of calcium could soften the "cement" substances and facilitate the separation of the epitheliae, but the medium used contained calcium in adequate proportions. The separation possibly was induced mechanically by the lack of the proper anatomical relationships between the reduced tubular content and the tubular wall.

The remaining elements of the tubular wall exhibited features usually observed in the adult rat testes (LACY and ROTBLAT, 1960; LEESON and LEESON, 1963). CLERMONT (1958) described in the tubular wall cells resembling smooth muscle characteristics, which he considered to be responsible for the contractile movements of the tubules. We were unable to detect this type of cells. Similarly,

1001675

BURGOS (1959) and MANCINI et al. (1964) were unable to find these cells in normal human testes. Since it has also been suggested that Sertoli cells may be responsible for the motility of the seminiferous tubules (ROOSEN-RUNGE, 1951), this question has to remain open at the present.

The Sertoli cell retained its ultrastructural integrity and its specific characteristics throughout the culture period up to six weeks. At no time was mitotic activity observed, as reported by ESAKI (1928). Thus, the apparent increase in the number of Sertoli cells per cross-section could be attributed to the shrinking of the tubules. *In vivo*, following damage of germinal cells, the cellular debris are removed rapidly from the lumen of the seminiferous tubules. This clearing has been attributed either to the passage of the detritus into the lumen and from there into the epididymis, or to phagocytosis by the Sertoli cells (CLERMONT and MORGENTALER, 1955; ROOSEN-RUNGE, 1962; LACY, 1962; BRÖKELMANN, 1963). In the cultured tubules, the debris of the degenerating spermatic cells was also rapidly removed. Since presence of a flow mechanism is most unlikely in these isolated fragments, the removal of the debris must have been accomplished by phagocytic action of the Sertoli cells (VILAR, 1965).

The role of Sertoli cells in the physiology of germinal epithelium, and the factors that regulate their function is not known. Furthermore, their histochemical and histophysiological characteristics have never been well established, principally due to the difficulty in determining the boundaries of their cytoplasm in the germinal epithelium. Results of our study demonstrate that the organ culture system can be utilized for long-term maintenance of Sertoli cells and may provide a useful tool for their study under controlled environmental conditions.

On the other hand, the culture conditions employed in this study did not permit long-term maintenance of either the interstitial cells or the germinal epithelium cells other than the spermatogonial cells. Morphologically two types could be distinguished among the spermatogonial cells, one being a relatively large cell, similar to the "transitional" cell seen in the germinal epithelium of prepuberal rats (FRANCHI and MANDL, 1964), which most probably corresponds to the so-called "primitive" type A spermatogonia (STEINBERGER, STEINBERGER and PERLOFF, 1964a, b, c). The second cell type appeared to be more differentiated; however, it was not possible to identify it with any of the cell types described on the basis of light microscopy (LEBLOND and CLERMONT, 1952). Electronmicroscopic studies on spermatogenesis of immature and adult rats presently in progress in our laboratories may help in identification of this cell type.

Since spermatogonia, the stem cells of the spermatic line and the fibroblasts of the intertubular spaces which are considered the precursors of the Leydig cells (FAWCETT and BURGOS, 1955; MANCINI, VILAR et al., 1963) retain their morphologic characteristics in culture for at least six weeks, the possibility exists that differentiation of these two cell lines may be achieved *in vitro*, once the factors necessary for their growth and differentiation are recognized and incorporated in the culture system.

## References

BAWA, S. R.: Fine structure of the Sertoli cell of the human testes. J. Ultrastruct. Res. 9, 459—474 (1963).

BOUIN, P., et P. ANCEL: Sur la signification de la glande interstitielle du testicule embryonnaire. C. R. Soc. Biol. (Paris) 55, 1682—1684 (1903).



1001676

BRÖKELMANN, J.: Fine structure of germ cells and Sertoli cells during the cycle of the seminiferous epithelium in the rat. Z. Zellforschung 59, 820—850 (1963).

BURGOS, M.: Contribución al estudio de la membrana basal de los tubos seminíferos humanos. Rev. Soc. argent. Biol. 35, 309—314 (1959).

CAULFIELD, J. B.: Effect of varying the vehicle of $OsO_4$ in tissue fixation. J. biophys. biochem. Cytol. 3, 827—829 (1957).

CHAMPY, CH.: Quelques resultats de la méthode de culture des tissus VI. Le testicule. Arch. Zool. exp. et gén. 60, 461—500 (1920).

CHRISTENSEN, K. A.: Microtubules in Sertoli cells of the guinea pig testis. Anat. Rec. 151, 335 (1965) (Abstract).

CLERMONT, Y.: Contractile elements in the limiting membrane of the seminiferous tubules of the rat. Exp. Cell Res. 15, 438—440 (1958).

—, and H. MORGENTALER: Quantitative study of spermatogenesis in the hypophysectomized rat. Endocrinology 57, 369—382 (1955).

CRABO, B.: Fine structure of the interstitial cells of the rabbit testes. Z. Zellforsch. 61, 587—604 (1963).

DUX, C.: Recherches sur les cultures du testicule hors de l'organisme. Action de l'hormone gonadotrope. C. Arch. Anat. Micr. 35, 391—413 (1940).

ESAKI, S.: Über Kulturen des Hodengewebes der Säugetiere und über die Natur des Interstitiellen Hodengewebes und der Zwischenzellen. Z. mikr.-anat. Forsch. 15, 368—404 (1928).

FAWCETT, D. W., and M. H. BURGOS: Observations on the cytomorphosis of the germinal and interstitial cells of the human testis. Ciba Found. Coll. on Aging of Transient Tissues, vol. 2, p. 86—89. London: Little, Brown & Co. 1955.

— — Studies on the fine structure of the mammalian testis. II. The human interstitial tissue. Amer. J. Anat. 107, 245—254 (1960).

FRANCHI, L. L., and A. M. MANDL: The ultrastructure of germ cells in foetal and neonatal male rats. J. Embryol. exp. Morph. 12, 289—308 (1964).

GAILLARD, P. J., and W. W. VAROSSIEAU: The structure of explants from different stages of development of the testis on cultivation in media obtained from individuals of different ages. Acta neerl. Morph. 1, 313—327 (1938).

GARDNER, P. J., and E. HOLYOKE: Fine structure of the seminiferous tubule of the Swiss mouse. I. The limiting membrane, Sertoli cells, spermatogonia and spermatocyte. Anat. Rec. 150, 391—404 (1964).

LACY, D.: Certain aspects of testis structure and function. Brit. med. Bull. 18, 205—208 (1962).

—, and J. ROTBLAT: Study of normal and irradiated boundary tissue of the seminiferous tubules of the rat. Exp. Cell Res. 21, 49—70 (1960).

LEBLOND, C. P., and Y. CLERMONT: Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N.Y. Acad. Sci. 55, 548—573 (1952).

LEESON, C. R., and T. S. LEESON: The postnatal development and differentiation of the boundary tissue of the seminiferous tubules of the rat. Anat. Rec. 147, 243—260 (1963).

LOSTROH, A.: In vitro response of mouse testis to human chorionic gonadotropins. Proc. Soc. exp. Biol. (N.Y.) 103, 25—27 (1960).

MANCINI, R. E., O. VILAR, M. P. DEL CERRO, and J. C. LAVITRI: Changes in the stromal connective tissue of the human testis. A histological, histochemical and electro-microscopical study. Acta physiol. lat.-amer. 14, 382—391 (1964).

— — J. C. LAVIERI, J. A. ANDRADA, and J. J. HEINRICH: Development of Leydig cells in the normal human testis. A cytological, cytochemical and quantitative study. Amer. J. Anat. 112, 203—214 (1963).

MARTINOWITCH, P. N.: Development in vitro of the mammalian gonad. Nature (Lond.) 139, 413 (1937).

MENDELSOHN, W.: The cultivation of adult rabbit testicles in rolles tubes. Anat. Rec. 69, 355—359 (1938).

PEASE, D.: The basal membrane: substractum of histological order and complexity. 4th Internat. Conf. on Electron Microscopy, vol. II, p. 139—155. Berlin-Göttingen-Heidelberg: Springer 1960.

PIERCE jr., G. B., and T. F. BEALS: The ultrastructure of primordial germinal cells of the foetal testes and of embryonal carcinoma cells of mice. Cancer Res. 24, 1553—1567 (1964).

1001677

REYNOLDS, E. S.: The use of lead citrate at high pH as an electron opaque-stain in electron-microscopy. J. Cell Biol. 17, 208—212 (1963).
ROOSEN-RUNGE, E. C.: Motions of the seminiferous tubules of the rat and the dog. Anat. Rec. 109, 413 (1951) (Abstract).
— The process of spermatogenesis in mammals. Biol. Rev. 37, 343—377 (1962).
SABATINI, D. D., K. BENSCH, and R. J. BARRNETT: Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. 17, 19—58 (1963).
SNIFFEN, R. D.: Histology of the normal and abnormal testis at puberty. Ann. N.Y. Acad. Sci. 55, 609—618 (1952).
STEINBERGER, A., E. STEINBERGER, and W. H. PERLOFF: Mammalian testes in organ culture. Exp. Cell Res. 36, 19—27 (1964a).
STEINBERGER, L., A. STEINBERGER, and W. H. PERLOFF: Studies on growth in organ culture of testicular tissue from rats of various ages. Anat. Rec. 148, 581—589 (1964b).
— — — Initiation of spermatogenesis in vitro. Endocrinology 74, 788—792 (1964c).
VILAR, O.: An electron microscopical study of the phagocytosis of germinal cells by Sertoli cells in tissue cultures. Anat. Rec. 151, 428—429 (1965) (Abstract).
— M. P. DEL CERRO, and R. E. MANCINI: The Sertoli cell as a "Bridge cell" between the basal membrane and the germinal cells. Exp. Cell Res. 27, 158—161 (1962).
— A. STEINBERGER, and E. STEINBERGER: Electron microscopy of isolated rat testicular cells grown *in vitro*. Z. Zellforsch. 74, 529—538 (1966).

Dr. OSCAR VILAR
Albert Einstein Medical Center
Research Laboratories
York and Tabor Roads
Philadelphia, Pa. 19141, U.S.A.



1001678