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Is There a Local Feedback from the Seminiferous Tubules Affecting Activity of the Leydig Cells?[1]

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ABSTRACT

Silastic tubing containing cyproterone acetate or braided silk sutures impregnated with WIN-18446 or ORF-1616 were inserted under the tunica albuginea of the testes. Spermatogenesis was suppressed in tubules in the immediate vicinity of the implants, but the interstitial tissue associated with the atrophic tubules appeared more abundant. Electron micrographs showed hypertrophy of the smooth endoplasmic reticulum and other evidence of Leydig cell stimulation. Interstitial tissue in the same testis at a distance from the implants showed no evidence of stimulation. The results are discussed in relation to the possibility of a local feedback from the tubules modulating sensitivity of the Leydig cells to circulating gonadotropin.

INTRODUCTION

The demonstration that very high local concentrations of testosterone are required to maintain spermatogenesis has given added significance to the close topographical relationships of the Leydig cells to the seminiferous tubules. This observation has stimulated renewed interest in the organization of the interstitial tissue; the species differences in the abundance of Leydig cells and their arrangement with respect to the blood vessels, lymphatics and the tubules.

The classical concept of control of androgen synthesis by hypophyseal luteinizing hormone and the feedback regulation of LH by circulating testosterone has been amply confirmed in recent years. Our confidence that this well established regulatory mechanism can account for all alterations in the structure and function of the testis, may cause us to overlook local tissue interactions that may modify the sensitivity of target cells to tropic hormones or otherwise influence their physiological responses.

It is a common observation after administration of antifertility drugs which cause regression of the seminiferous tubules, that the interstitial tissue seems to be more abundant (Figs. 1, 2). This appearance has generally been attributed simply to an aggregation and consolidation of the Leydig cells as a consequence of tubular regression. The descriptive term "pseudohypertrophy" is sometimes used, implying that the change in Leydig cell volume is relative, not absolute and that the difference is more apparent than real. This traditional interpretation was based upon examination of histological sections with the light microscope which does not resolve the cytoplasmic organelles and therefore provides no information bearing upon the physiological state of the Leydig cells. Observations with the electron microscope, on the other hand, suggest that under conditions of tubular damage, the clusters of Leydig cells not only become more prominent, but appear to be stimulated as evidenced by hypertrophy of the smooth endoplasmic reticulum. The conventional interpretation to explain this change is that testicular damage leads to an elevation of gonadotropin which in turn stimulates the Leydig

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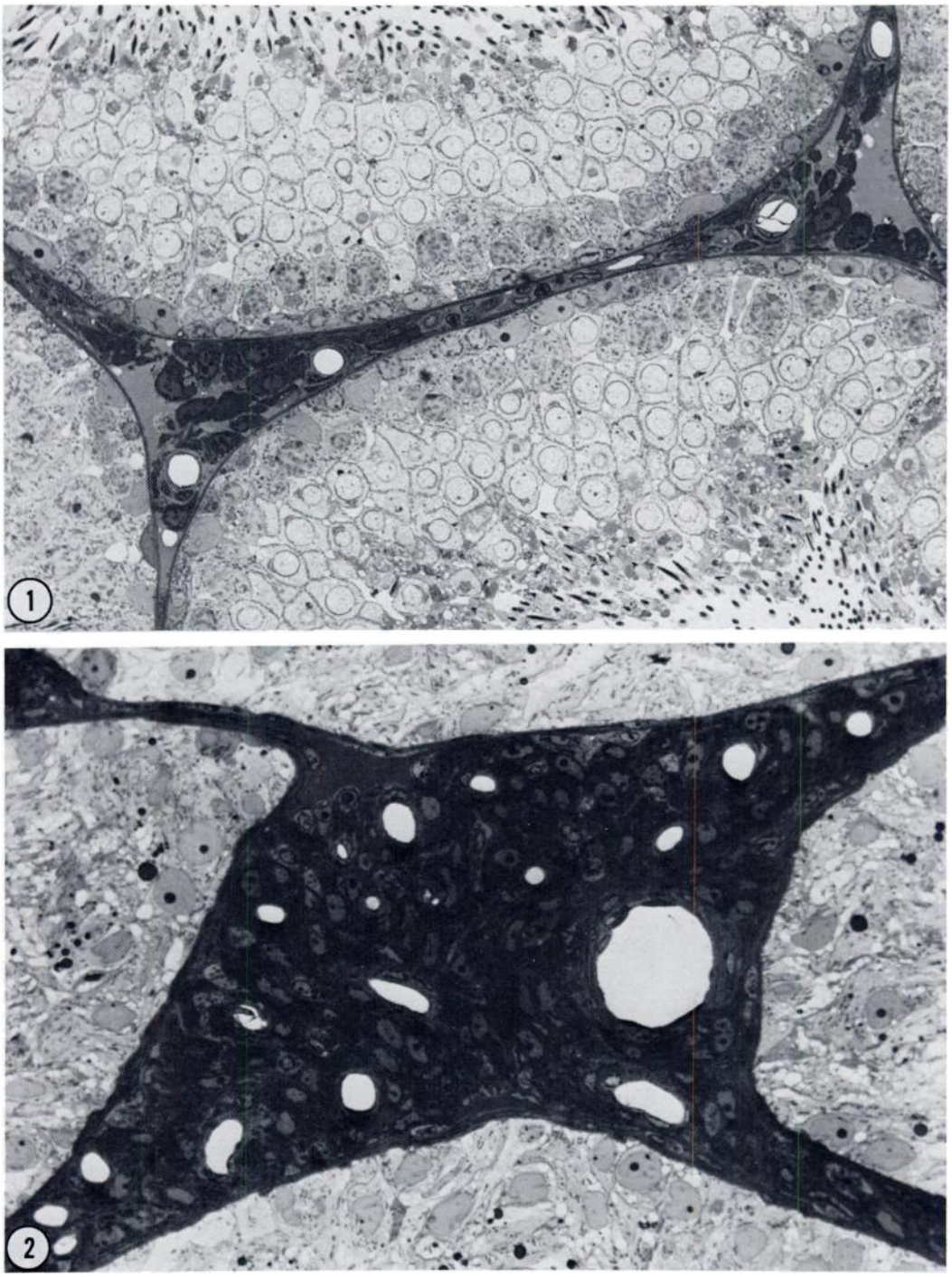


FIG. 1. Photomicrograph of testis implanted with cyproterone acetate for 30 days. This field is from tissue excised from the testis some distance away from the implanted capsule. The seminiferous tubules are normal in appearance and the interstitial tissue is present in normal abundance.

FIG. 2. Photomicrograph of a field from the same testis, but in an area adjacent to the cyproterone acetate filled Silastic capsule. The seminiferous tubules show marked involution and virtual absence of germ cells. The interstitial tissue is hyperplastic, consisting of a compact agglomeration of Leydig cells traversed by numerous blood vessels.

cells.

In the present study, experiments were designed to produce localized tubular regression. Under these conditions, ultrastructural evidence of Leydig cell stimulation was found in the immediate vicinity of damaged tubules, but not elsewhere in the same testis. Since a localized effect cannot be explained on the basis of an elevation of circulating gonadotropin, we suggest the possibility that there is a local feedback by a diffusable agent from the tubules to the Leydig cells which limits activity of the Leydig cells or modulates their sensitivity to gonadotropin.

MATERIALS AND METHODS

Silastic capsules containing cyproterone acetate (Lot 50,402) (4) were implanted into one or both testes of 11 adult, 300 g, male rats of the CD strain from Charles River Laboratories. In addition, 10 control rats were implanted with empty capsules or capsules containing solvent only. The capsules were Dow-Corning medical grade Silastic tubing No. 602–23 (1.47 mm i.d., 1.97 mm o.d.), about 5 mm long and filled with 2 mg cyproterone acetate as the dry powder or dissolved in benzl benzoate-castor oil mixture (Neumann, personal communication). The open ends of the tubing were sealed with Silastic medical grade adhesive A. A piece of surgical silk thread 6-0 was attached to one end with the adhesive. Capsules and contents were sterilized in an autoclave at 100°C for 30 min.

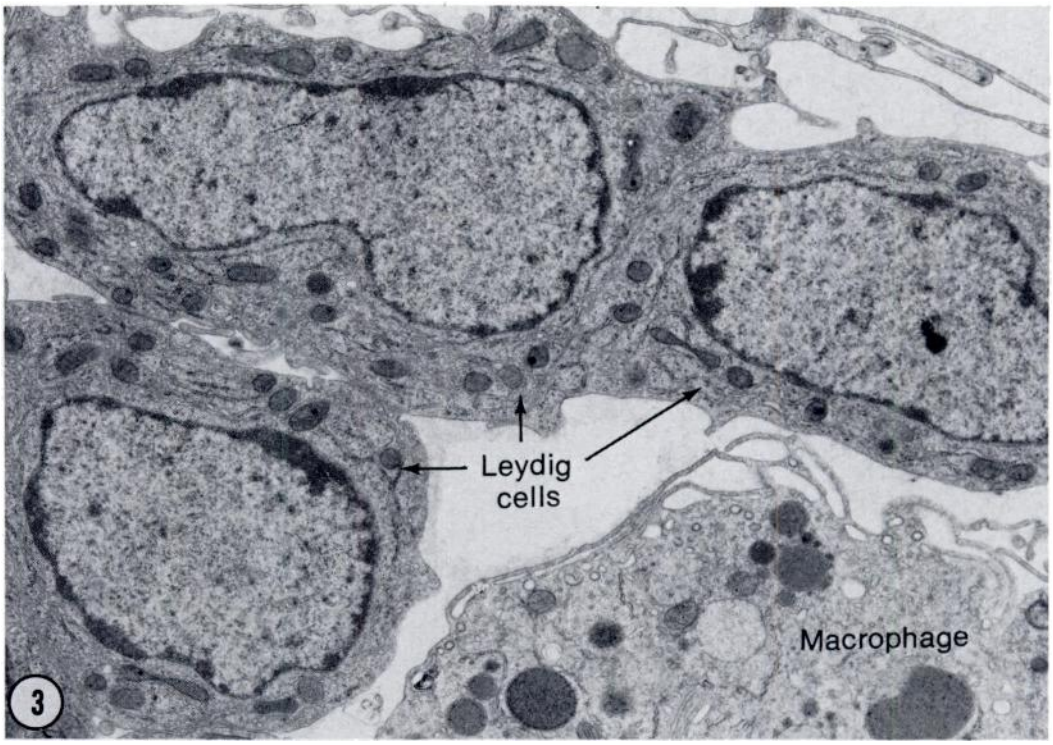
To eliminate the remote possibility that the Silastic (poly-dimethylsiloxone) might have sufficient structural similarity to steroid hormones to be responsible for changes attributed to the contents of the capsule, parallel experiments were carried out using braided surgical silk impregnated with the test compounds cyproterone acetate, WIN-18446 (a bis-dichloroacetyl diamine) and ORF-1616 (a dinitropyrole). Deknatel braided surgical silk No. 2 was cut into short segments and lipid materials extracted by several changes of analytical grade acetone. The silk was then impregnated with the antifertility compounds suspended in sesame oil. Working under a dissecting microscope to avoid damage to superficial blood vessels, 5 mm long segments of the drug-impregnated silk were implanted under the tunica albuginea through a small hole made with a hypodermic needle. Four rats were used for each compound and their testes were processed for morphological studies 30 days after implantation.

At the end of the experiments, the testes which contained implants were fixed by perfusion through the internal spermatic artery using 4% glutaraldehyde in 0.1 M s-collidine buffer, pH 7.3 plus 20 mM calcium chloride. Small blocks were cut from areas adjacent to the implants. In some specimens, portions of Silastic capsules or silk were included in the blocks. Tissues from areas distant from the implants were also studied for comparative purposes. The blocks were then postosmicated in 1% OsO_4 in the same buffer and embedded in epon-araldite mixture. Sections exhibiting pale gold interference colors were cut with a diamond knife on a Porter-Blum MT-1 microtome and stained with half saturated uranyl acetate in 50% acetone followed by lead citrate (Venable and Coggeshall, 1965).

RESULTS

The general organization of the interstitium of normal rat testes has been described previously (Fawcett et al., 1973; Mori and Christensen, 1978). In brief, it consists of Leydig cells clustered around blood vessels centrally situated in the angular intertubular spaces and bathed peripherally by extensive peritubular lymphatic sinusoids. The finer details of the ultrastructure of rat Leydig cells are more difficult to study by routine methods than are those of other species because of their dense cytoplasmic matrix and the consequent poor definition of their membranous organelles. In micrographs of thin sections, the contrast can be somewhat enhanced photographically. The nucleus is usually round or oval with a prominent nucleolus and abundant heterochromatin located mainly adjacent to the nuclear envelope (Fig. 3). The cytoplasm contains mitochondria with a dense matrix and sparse foliate cristae, an inconspicuous Golgi apparatus, short cisternae of ribosome-studded endoplasmic reticulum and a few lysosomes and peroxisomes (Fig. 3). The agranular or smooth endoplasmic reticulum is relatively abundant and forms a loose meshed network of irregularly anastomosing tubules (Figs. 4,5). Its profiles are often difficult to discern owing to the density of the cytoplasmic matrix (Fig. 3). The smooth endoplasmic reticulum in the Leydig cells of rats seems to be less extensively developed than in the guinea pig and other common laboratory and domestic animals. Nevertheless it is reported to occupy nearly 24% of the cytoplasmic volume (Mori and Christensen, 1978).

Implants of empty or solvent-filled Silastic capsules residing in the interstitum of the testis for periods of 1–2 months result in no major changes in the architectural organization or cytology of the testis other than an accumulation of fibrous tissue organized around the capsules. Some localized regressive changes in the germinal epithelium were observed in some control animals due to unavoidable trauma to the seminiferous tubules or damage to their blood supply. The resulting tissue response mimics, to a limited extent, the alterations



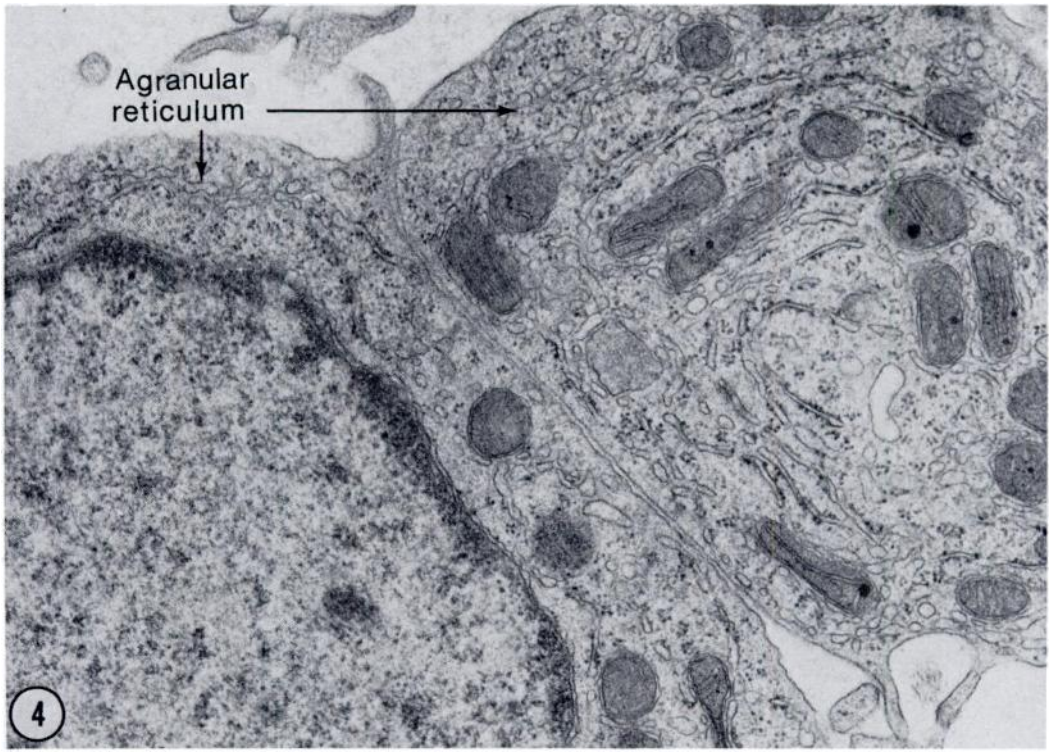


FIG. 3. Electron micrograph of interstitial tissue from a normal rat testis including parts of 3 Leydig cells and a macrophage.

FIG. 4. Normal rat Leydig cells at higher magnification showing sparse tubular elements of agranular endoplasmic reticulum and cisternae of granular reticulum.

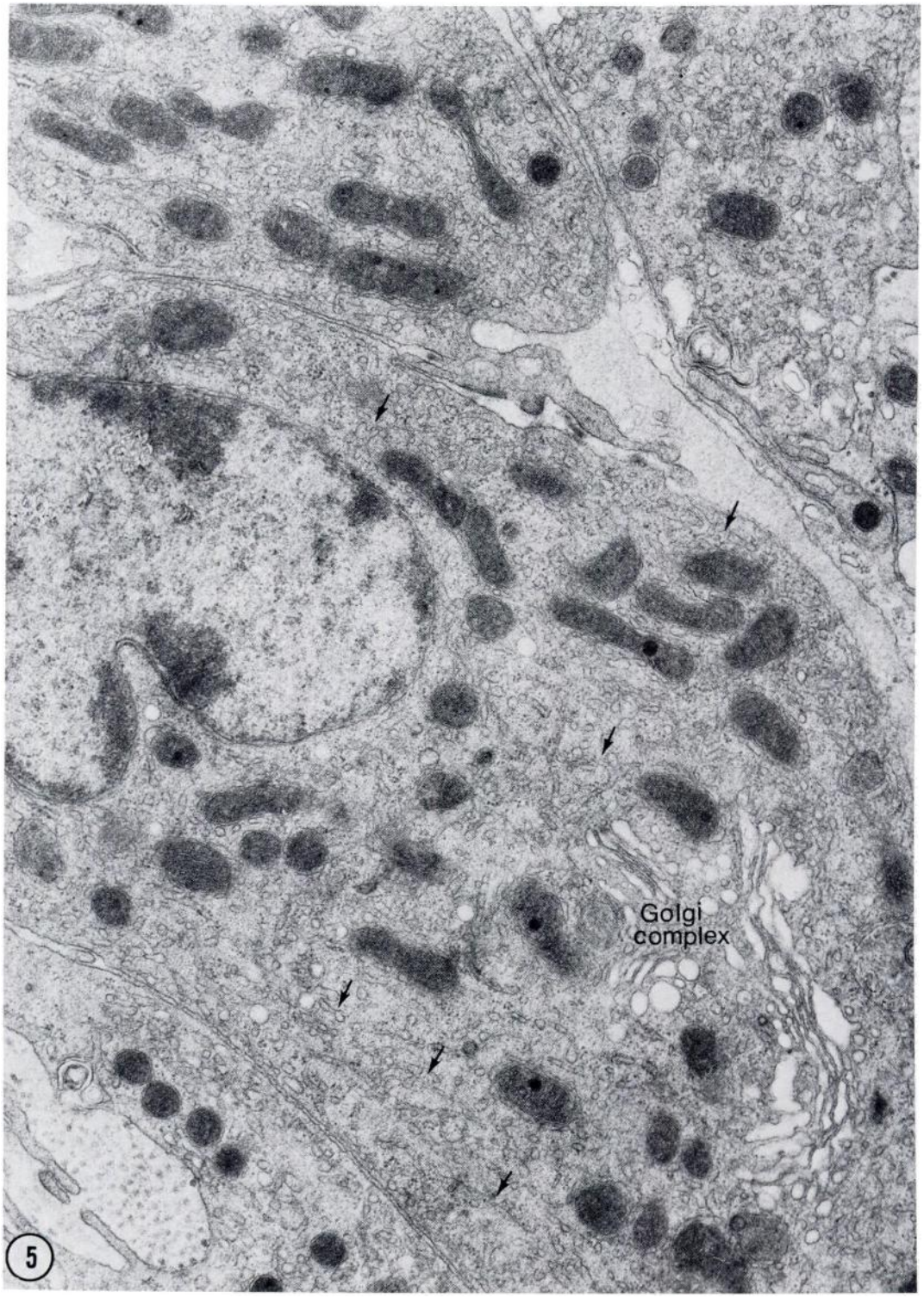


FIG. 5. Electron micrograph of a control area of rat interstitial tissue showing a Leydig cell with a normal complement of smooth endoplasmic reticulum (at arrows), a prominent Golgi complex and mitochondria of rather uniform size with a dense matrix.

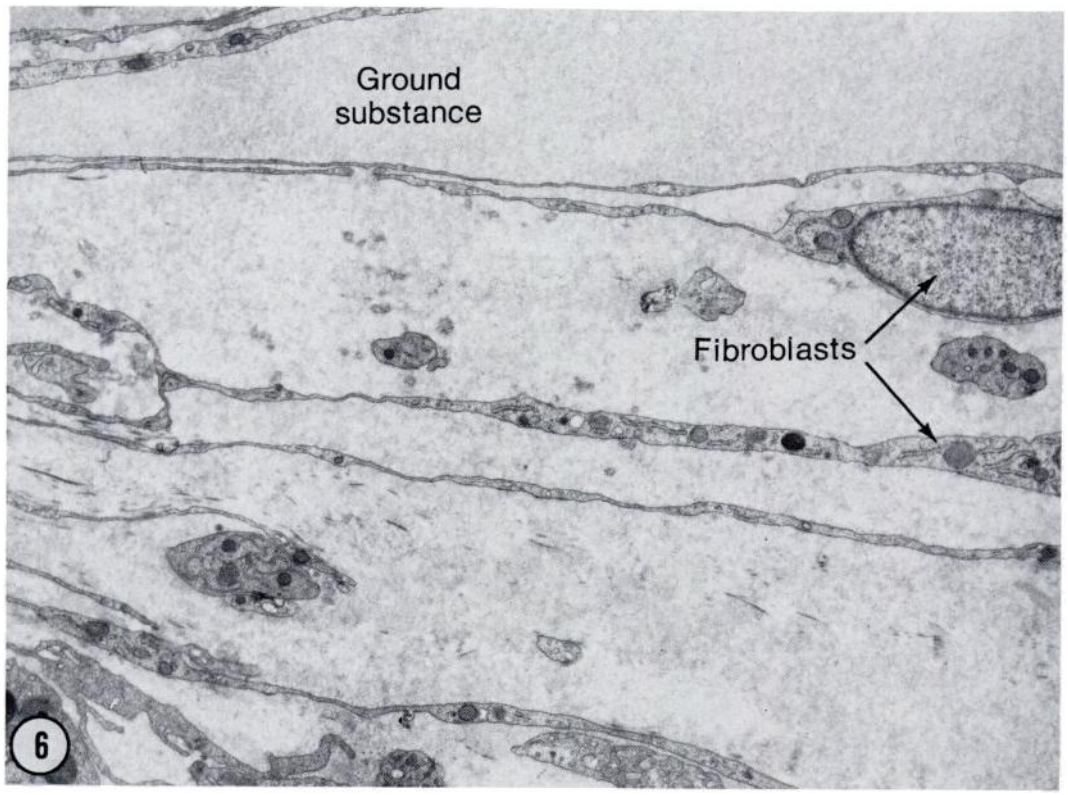
found in CA-implanted testes. Involuted seminiferous tubules can usually be recognized on direct visual examination as slender white strands originating from the area of the implant. On microscopic examination, they appear depleted of germinal cells and are often associated with hypertrophic interstitial cells.

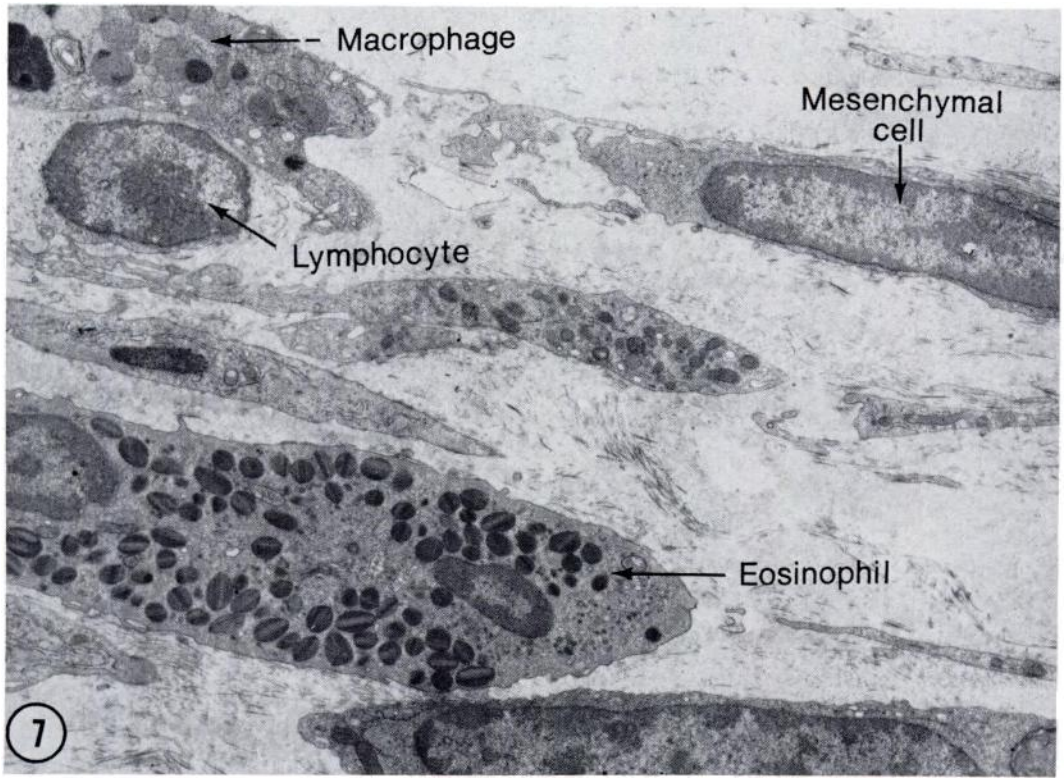
In testes bearing implants of cyproterone acetate (CA), the tubular involution is similar, but more extensive and is consistently found in all the specimens studied. The seminiferous tubules consist of Sertoli cells and are completely devoid of germ cells. The diffusion of effective concentrations of CA and its histopathological effects are restricted to a few mm around the implant and the remaider of the testis does not appear to be affected by this treatment. The most significant feature of these testes is a dramatic hyperplasia of the interstitial tissue adjacent to the involuted tubules. Increased numbers of Leydig cells intermingled with some fibroblasts and wandering cells form a compact tissue irrigated by a rich network of blood vessels (Fig. 2). In contrast to the normal architecture of rodent testes, lymphatic sinuses are not present in these areas.

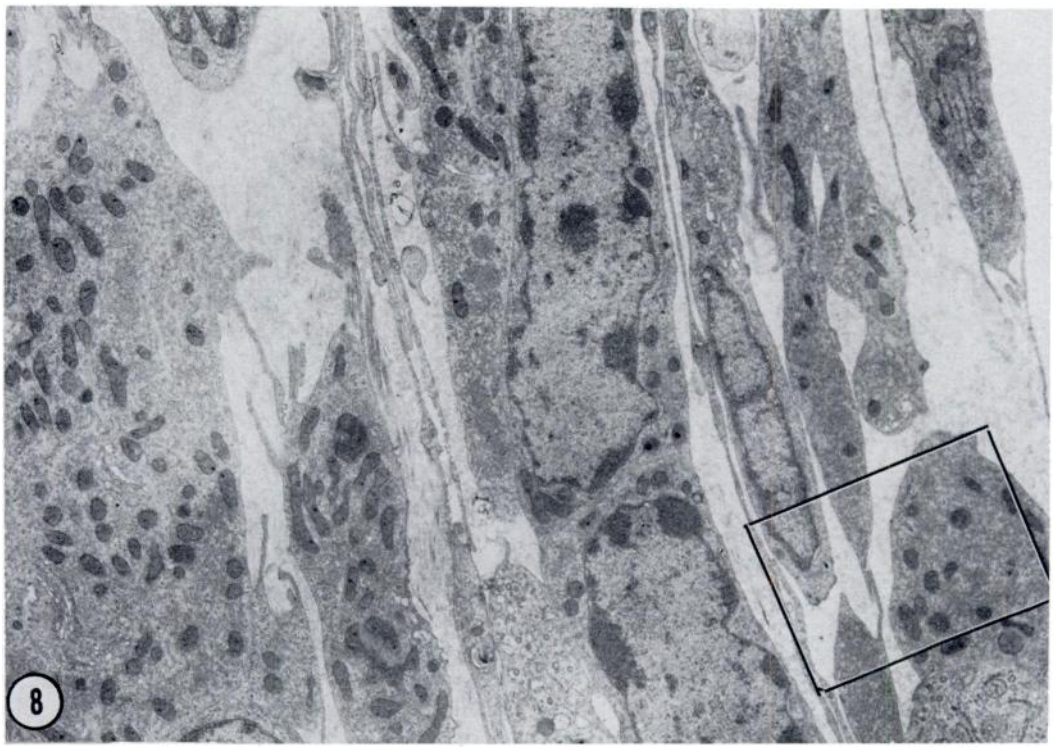
In testes from control animals implanted with empty capsules, a connective tissue reaction develops around the Silastic. Thirty days after insertion it appears as a multilayered cellular investment of elongated mesenchymal cells and fibroblasts arranged in an orderly parallel fashion with an abundance of extracellular matrix containing sparce, randomly oriented bundles of collagen fibrils (Fig. 6). This tissue is usually infiltrated by macrophages and a few eosinophils and lymphocytes (Fig. 7). The local connective tissue reaction to the Silastic implants, which is well established at 30 days, becomes somewhat thinner at 60 days as a result of closer aggregation and consolidation of its components. Moving outward from the Silastic, Leydig cells first appear in a transition zone peripheral to the fibrous capsule where cells are clustered irregularly around newly formed blood vessels. These Leydig cells are irregularly shaped and often surrounded by a thick layer of extracellular material rich in collagen. Cytologically they do not differ significantly from those of normal testes described above (Fig. 4), except in those testes where mechanical injury to seminiferous tubules seems to have caused some degree of Leydig cell stimulation.

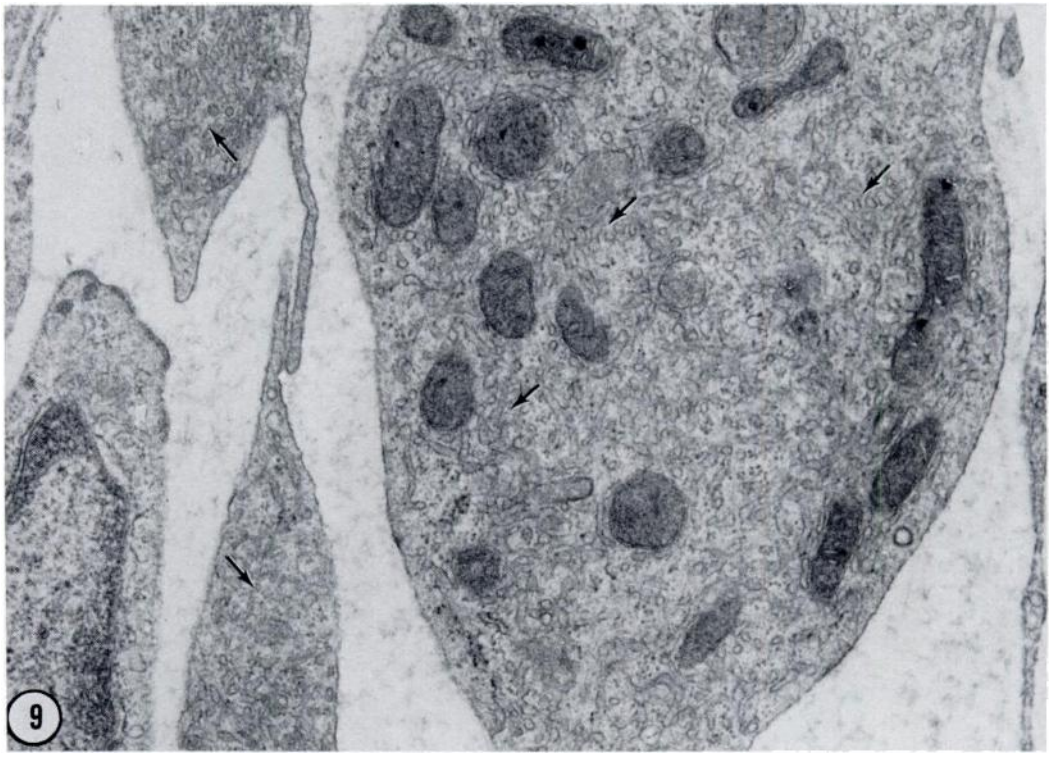
In testes implanted with cyproterone acetate, on the other hand, there is a marked proliferation of Leydig cells forming a very compact cellular tissue between the involuted seminiferous tubules. The Leydig cells in these areas exhibit a wide range of cell size and shapes. Some are larger than normal and seem to have increased the volume of their cytoplasm. Others are relatively small and fusiform and bear a superficial resemblance to mesenchymal cells, but have the unmistakable cytoplasmic characteristics of steroid secreting cells (Figs. 8, 9). The occurrence of these fusiform elements strongly suggests that in addition to aggregation and hypertrophy of preexisting Leydig cells, there is a recruitment of new cells by differentiation from mesenchymal precursors. The mature cells exhibit ultrastructural evidences of hyperactivity including a nucleus of irregular outline, an enlarged Golgi region and polymorphous mitochondria with tubular as well as lamellar cristae. The presence of crystalline inclusions in the mitochondrial matrix is not a rare occurrence. The most striking change, however, is seen in the endoplasmic reticulum. The rough form is somewhat more extensive than usual and is in continuity at many points with the smooth reticulum (Fig. 10). The latter is markedly hypertrophied, occupying nearly all the cytoplasm available between the other organelles and inclusions. Not infrequently, the smooth reticulum is organized in parallel arrays of fenestrated cisternae in the peripheral regions of the cells (Fig. 11). Such cisternae often form large concentric systems of membranes (Fig. 12), a configuration rarely, if ever, observed in normal rat testes or indeed in the experimental testes in sites at a distance from the Silastic implants.

FIGS. 6, 7. In control animals receiving testicular implants of empty Silastic capsules, the tissue response consists of fibroblasts oriented parallel to the capsule, abundant ground substance with small bundles of collagen fibrils and some infiltration of the area by macrophages, eosinophils and occasional lymphocytes. There is no significant change in the Leydig cells.









Implants of braided silk impregnated with cyproterone acetate, WIN-18446 or ORF-1616 proved to be a useful alternative to Silastic capsules. Their insertion is less traumatic and they are well tolerated. They probably lack the uniform rate of release of the drugs that is expected of Silastic implants and the tissue reaction to the silk is somewhat different. A granulation tissue composed of undifferentiated mesenchymal cells, fibroblasts and multinucleate, foreign body, giant cells develops around each strand of the thread. The effects of all 3 antifertility agents on the neighboring seminiferous tubules were similar; an involution of the epithelium and loss of germ cells. The reaction of the interstitial tissue to cyproterone acetate impregnated thread was comparable in all respects to that induced by the drug when contained in Silastic tubing. In the experiments with WIN-18446, there was also a remarkable proliferation and stimulation of the Leydig cells. ORF-1616 caused evident stimulation of the Leydig cells as assessed in electron micrographs, but there was no significant proliferation.

DISCUSSION

Proliferation and hypertrophy of testicular interstitial cells associated with degeneration of the germinal epithelium is not a new finding. Such changes have been recorded in a variety of experimental conditions. At the turn of the century Maximov (1899) described Leydig cell hyperplasia after aseptic focal traumatic lesions of the seminiferous tubules. In the early observations on testis grafts (Steinbach and Sand, 1921) and on the cryptorchid testis (Moore, 1924), it was reported that the intertubular tissue frequently consisted of "hypertrophic" interstitial cells. More recently, involution of seminiferous tubules of rat testis associated with an apparent increase in the volume of the interstitial tissue has been reported after administration of the antifertility drugs WIN-18446 (Reddy and Svoboda, 1967; Drobeck and Coulston, 1962) and ethionine (Goldberg et al., 1959); Benson and Clare, 1966). Testicular atrophy and interstitial cell hyperplasia are well known sequelae of parental administration of cadmium and a high proportion of such animals ultimately develop Leydig cell tumors (Gunn et al., 1963; Roe et al., 1964). Interpretation of the observations of apparent interstitial cell hypertrophy or hyperplasia has varied. Some have considered it merely a relative change in volume due to the atrophy of the seminiferous tubules (Drobeck and Coulston, 1962), while others have been convinced that there is an absolute increase in Leydig cell volume. Indeed, Reddy and Svoboda (1967) manually dissociated the tubules from the interstitial tissue in rats treated 8 weeks with bis (dichloroacetyl-diamine) and compared their wet and dry weights with those of control animals and concluded that there was more than 100% increase in interstitial tissue in the treated rats. Incorporation of tritiated thymidine suggested that at least part of this increase was due to cell proliferation. The possibility that the interstitial tissue hyperplasia was due to increase in the number of cells other than Leydig cells (Iturriza and Irusta, 1969) is difficult to exclude.

Studies of the cryptorchid testis have often led to reports of increase in the volume of the interstitial tissue and in the number of Leydig cells (Hanes and Hooker, 1937; Clegg, 1961). It is known that thermal damage to the seminiferous tubules in the cryptorchid testis causes an elevation of circulating gonadotropins (Altwein and Gittes, 1972; Walsh and Swerdlow, 1973; Steinburger and Chowdhury, 1974). Those who interpret the hypertrophy of the interstitial tissue after testicular damage as an absolute rather than a relative change in Leydig cell number and volume, explain this change as a logical consequence of increased hypophyseal release of LH due to impairment of testosterone secretion in the intraabdominal testis.

In a recent ultrastructural study of the effects of experimental cryptorchidism, Kerr (1977) found hypertrophy and mitotic proliferation of Leydig cells, enlargement of the

FIG. 8. In addition to large Leydig cells near cyproterone implants, there are numerous smaller elongate cells bearing a superficial resemblance to fibroblasts, but their nuclear and cytoplasmic characteristics are those of Leydig cells. The area in the rectangle is shown at higher magnification in Fig. 10.

FIG. 9. At higher magnification the area indicated in Fig. 7 shows an abundance of smooth endoplasmic reticulum in these spindle-shaped cells. It is suggested therefore that they represent new Leydig cells that have differentiated from mesenchymal precursors.

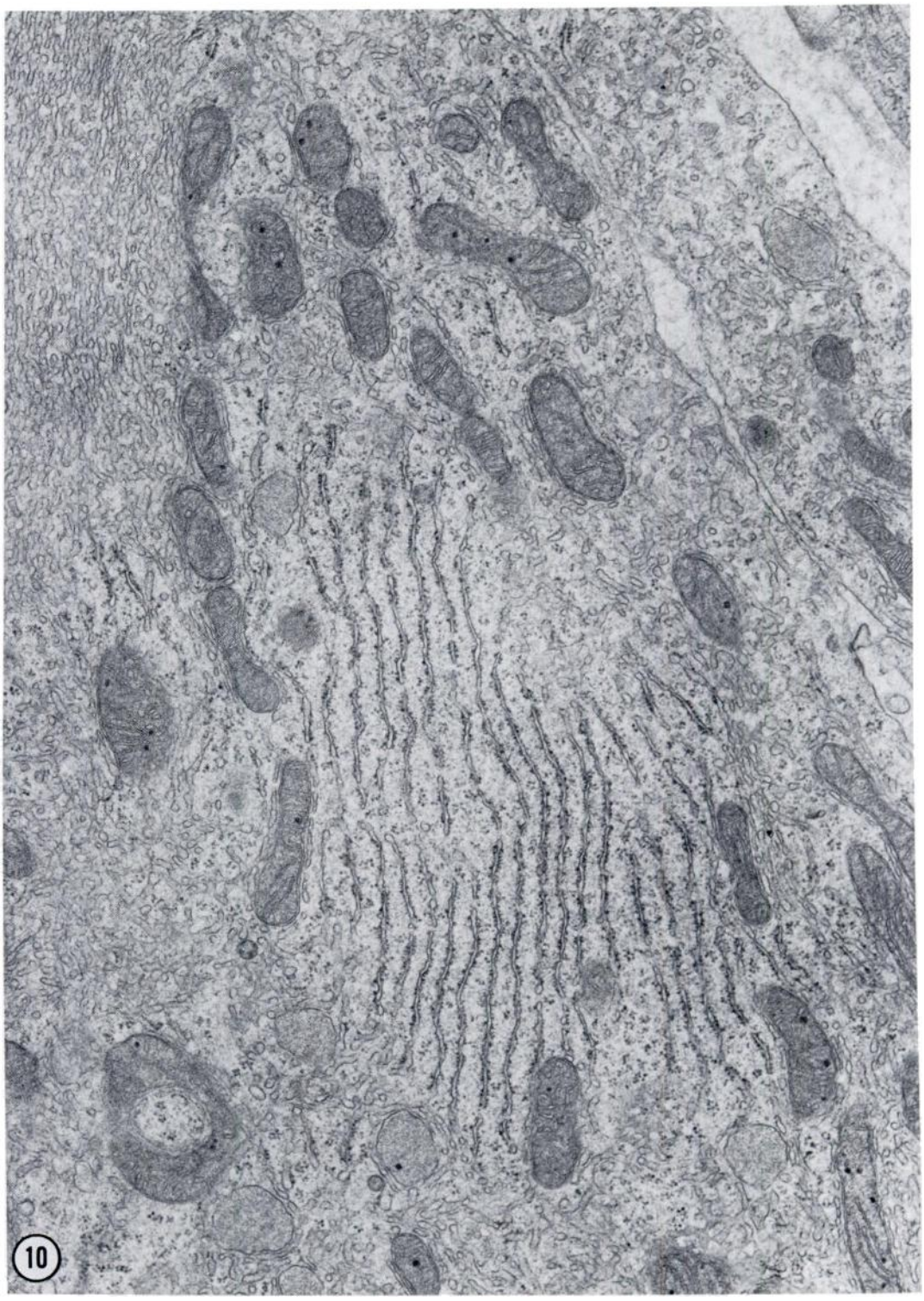


FIG. 10. Hypertrophic Leydig cells from an area near a Silastic implant containing cyproterone acetate. A parallel array of cisternae of granular reticulum is surrounded by cytoplasm crowded with tubular and cisternal elements of smooth endoplasmic reticulum. The membrane systems of the cytoplasm are much more extensive than in unstimulated Leydig cells.

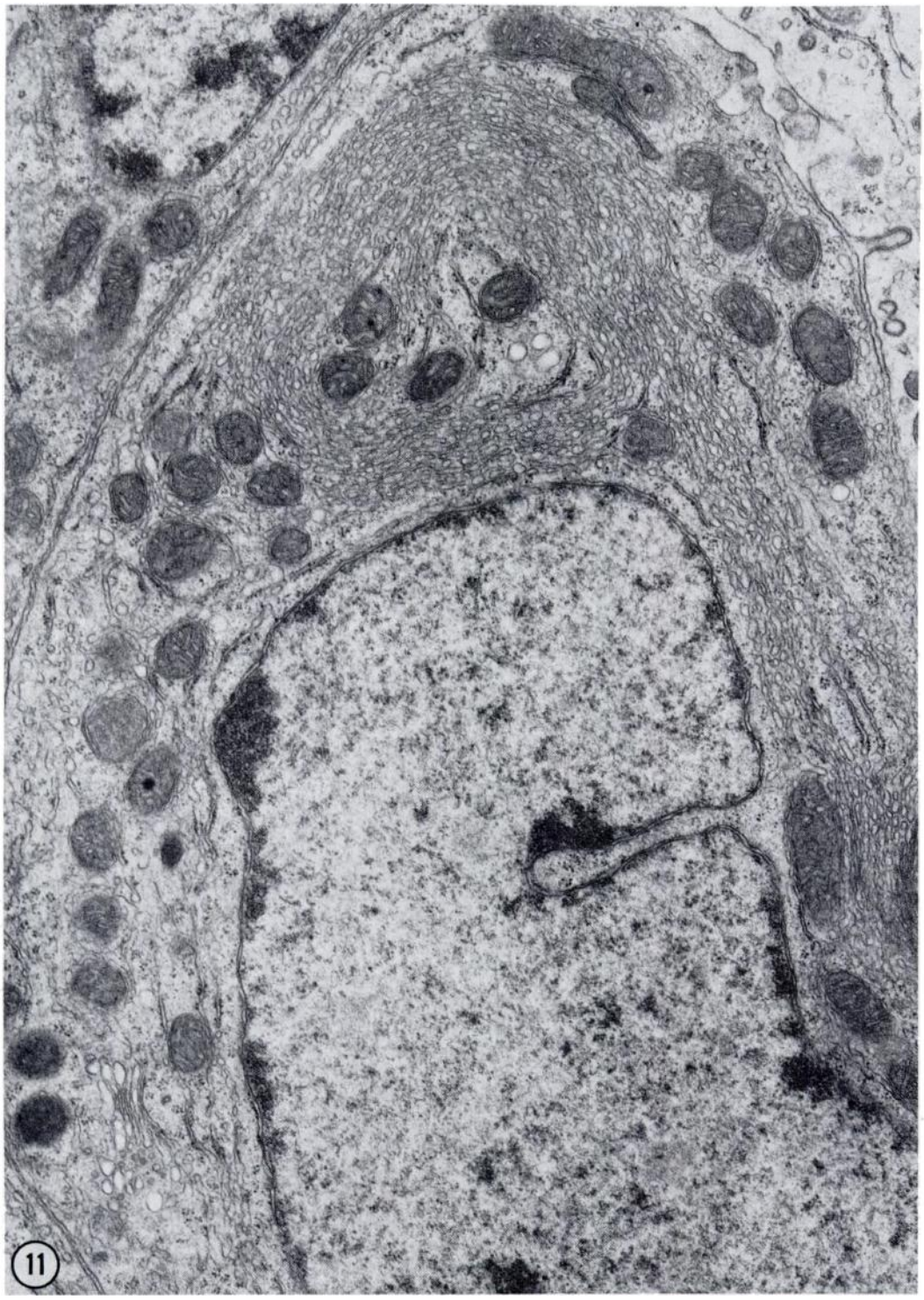


FIG. 11. Electron micrograph of a Leydig cell from an area near an implant of silk impregnated with WIN 18446. Concentric systems of cisternae of agranular reticulum such as that illustrated here are rarely seen in normal testis.

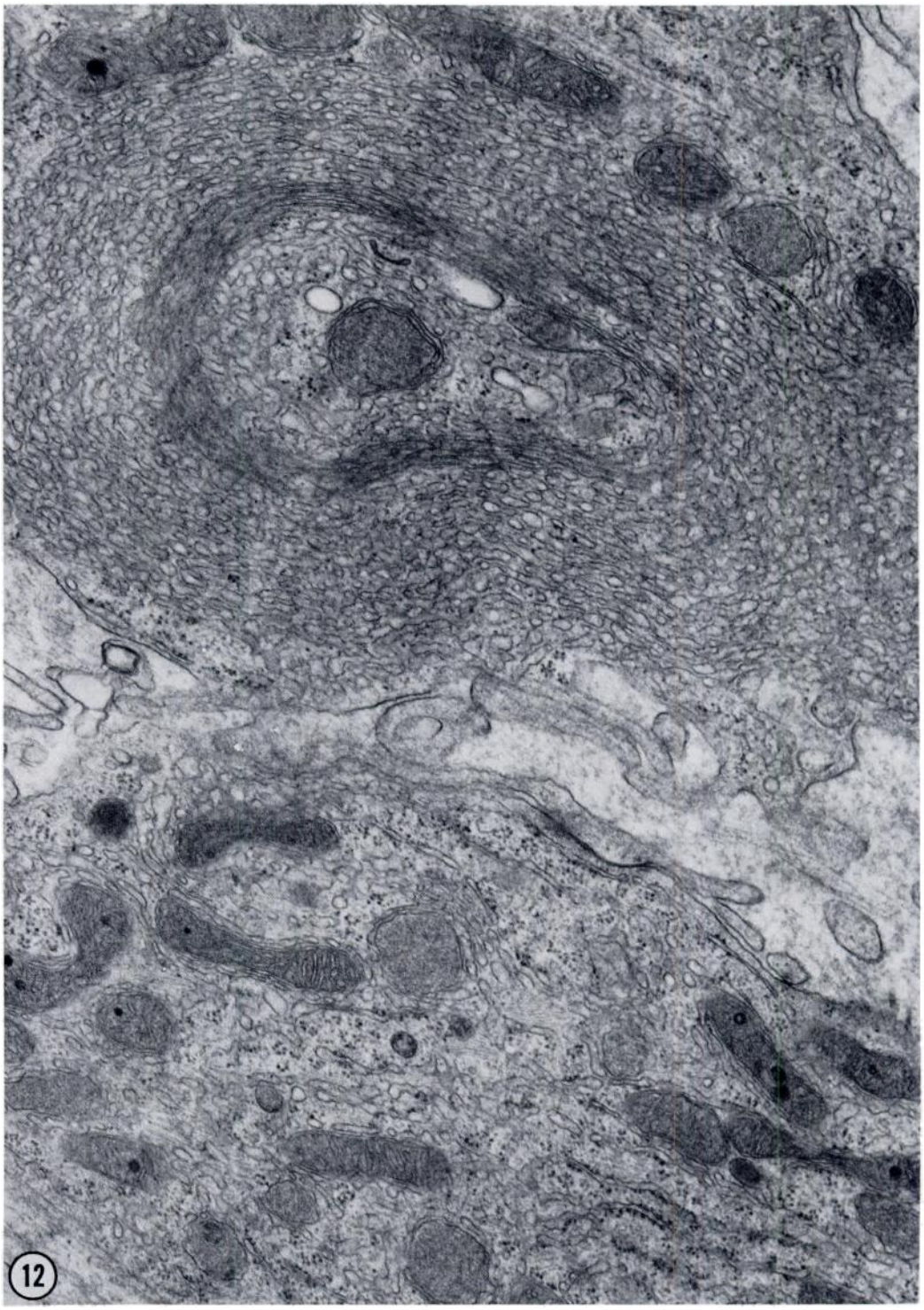


FIG. 12. Another example of a concentric array of hypertrophied endoplasmic reticulum in a Leydig cell near an implant of WIN 18446.

Golgi apparatus and a striking increase in smooth endoplasmic reticulum, including the appearance of large systems of concentric cisternae comparable to those described here. These changes were attributed to elevated LH levels, but in the light of the present observations, it is possible that they may have been due, in part, to local stimulation resulting from thermal damage to the tubules.

Despite the appearance of Leydig cell stimulation in Kerr's study and up to 4-fold elevation of circulating LH, the levels of serum testosterone remained significantly below normal. It has been postulated that there is a temperature sensitive biochemical step essential to synthesis of androgen (Levi and Spaziani, 1968). Thus, despite the hypertrophy of the cytological organelles involved in synthesis, the Leydig cells of the cryptorchid testes are apparently unable to produce elevated or even normal levels of androgen. On the other hand, the hypertrophied Leydig cells associated with tubular damage in the scrotal testis may retain the capacity for increased testosterone synthesis. This possibility may be deserving of further study.

It may be relevant in this connection to note that Neumann and coworkers (1975) reported that daily injections of cyproterone to rats caused an inhibition of spermatogenesis after 3 weeks, but after 6–9 weeks, there was full recovery of the germinal epithelium in spite of continuous treatment. The Leydig cells appeared overstimulated when spermatogenesis was impaired and when the germinal epithelium returned to normal, the interstitial cells also acquired a normal appearance.

Hasan et al. (1973) reported a rise in plasma testosterone levels and testicular weights in rats on cyproterone for 46 days while the accessory gland weights declined. It was concluded that cyproterone stimulated gonadotropin secretion which in turn enhanced plasma testosterone levels. We suggest that without direct evidence of enhanced gonadotropin secretion, one should consider the alternative interpretation that chemical suppression of androgen sensitive cells in the seminiferous tubules led to a local response resulting in hyperactivity of the Leydig cells without increased gonadotropin secretion.

It is one of the purposes of the present paper to alert investigators to the possibility that when the familiar long feedback mechanism between the testis and the hypophysis does not adequately account for their observations, a short feedback mechanism between the tubules and the Leydig cells should be considered. This possibility has previously been suggested by Neaves (1975).

The experiments carried out in the present study show that a continuous release of microquantities of various antifertility compounds implanted interstitially in the testis, arrests spermatogenesis in seminiferous tubules in the immediate vicinity of Silastic or silk implants. The local involution of the seminiferous tubules is accompanied by hyperplasia and hypertrophy of Leydig cells in the neighboring interstitial tissue, but not in those elsewhere in the same testis. In addition, electron micrographs of these Leydig cells showed nuclear changes, enlargement of the Golgi complex, pleomorphism of mitochondria and a remarkable hypertrophy of the smooth endoplasmic reticulum. These ultrastructural changes are characteristic of the stimulated state of these cells. Exactly similar alterations are displayed by Leydig cells upon stimulation with exogenous LH or hCG (Aoki, 1970; Aoki and Massa, 1972).

These local effects upon the volume and ultrastructure of the interstitial cells cannot be adequately explained by an imbalance in the normal feedback control upon the hypothalamo-hypophyseal axis and consequent stimulation of Leydig cells by elevated serum gonadotropin. A hormonal stimulation would be expressed in a general response of the interstitial tissue throughout the testes and not in a local response limited to the immediate vicinity of the damaged tubules. Instead, our results strongly encourage the speculation that a diffusable product of the seminiferous epithelium may normally act upon the Leydig cells to regulate their androgen synthesis or to modulate their sensitivity to circulating LH. Damage to the tubules by various drugs may interfere with production of this agent. The Leydig cells then being released from its control, hypertrophy and undergo the morphological changes associated with hyperactivity. Conversely, damaged seminiferous tubules may release a substance that is directly stimulatory to the neighboring interstitial tissue. At present, a rational choice between these alternative speculations cannot be made.

One possible candidate for such a local regulatory role is estrogen. It is produced in appreciable amounts in the testis,

probably by the seminiferous tubules (de Jong et al., 1974) and *in vitro* studies indicate that it is the Sertoli cells that are able to aromatize androgen to estrogen (Armstrong and Dorrington, 1976). Interstitial cells of rats have been shown to have a high concentration of specific estrogen receptors (Mulder et al., 1974) and tritium-labeled estradiol has been localized autoradiographically in the nuclei of Leydig cells of immature rat testes (Sar et al., 1975). There are reports in the literature that estradiol inhibits testosterone secretion in the rat prior to a detectable decrease in the blood levels of LH (Steinberger, 1973; Jones et al., 1975) and it is thought to inhibit the action of LH on the Leydig cells. Although this interpretation has recently been questioned (Van Beurden et al. 1977), evidence is nevertheless accumulating that estrogens act on Leydig cells and may serve as local regulators of Leydig cell sensitivity to LH (Hansson and Ritzen, 1977). The results reported here provide preliminary experimental evidence for the existence of a local regulatory mechanism, but do not directly implicate estrogens. Further studies may succeed in correlating the morphological observations with the suggestive endocrinological evidence of a local regulatory role for estrogen synthesized in the seminiferous tubules.

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