THE FINE STRUCTURE OF TESTICULAR INTERSTITIAL CELLS IN GUINEA PIGS

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ABSTRACT

In guinea pig testes perfused with either glutaraldehyde or osmium tetroxide fixative, the cytoplasm of the interstitial cells contains an exceptionally abundant agranular endoplasmic reticulum. The reticulum in central regions of the cell is a network of interconnected tubules, but in extensive peripheral areas the reticulum is commonly organized into closely packed, flattened cisternae which are fenestrated. Occasional small patches of the granular reticulum occur in the cytoplasm and connect freely with the agranular reticulum. The mitochondria have a dense matrix and contain cristae and some tubules. The Golgi complex is disperse and shows no evidence of secretory material. The cytoplasm also contains lipid droplets. Lipofuscin pigment granules are probably polymorphic residual bodies and contain three components: (1) a dense material which at high magnification shows a 75-A periodicity; (2) a medium-sized lipid droplet; and (3) a cap-like structure. In glutaraldehyde-perfused testis the interstitial cell cytoplasm appears to have the same density from cell to cell, and the agranular reticulum is tubular or cisternal but not in the form of empty vesicles. Thus the "dark" and "light" cells and the vesicular agranular reticulum sometimes encountered in other fixations may be artifacts. Biochemical results from other laboratories, correlated with the present findings, indicate that the membranes of the agranular endoplasmic reticulum in guinea pig interstitial cells are the site of at least two enzymes of androgen biosynthesis, the 17-hydroxylase and the 17-desmolase.

It is now well established in laboratory mammals that the interstitial cells are the main source of testicular androgens.[1] In addition, some progress has been made in determining where within the interstitial cell the biosynthesis takes place. Christensen and Fawcett (8) found a rich agranular endoplasmic reticulum in the cytoplasm of opossum interstitital cells. By correlating this fine structure with biochemical work carried out on cell fractions in other laboratories, these authors proposed that the membranes of the agranular reticulum are the site of at least some enzymes acting in steroid biosynthesis. However, the biochemistry had been done in guinea pigs and rats, and there was some uncertainty in correlating it with fine structure from opossums.

[1] Recent direct evidence, both histochemical and biochemical, has established the interstitial cells as the main site of androgen production in the testes of laboratory mammals. The 3β-hydroxysteroid dehydrogenase histochemical reaction, localizing a step in androgen biosynthesis, is positive in the interstitial cells but negligible in the seminiferous tubules of several species (57, 30, 33), including guinea pigs (33, 29).

In a biochemical approach to the problem, Christensen and Mason (9) separated the seminiferous tubules from the interstitial tissue of rat testes and incubated the components separately with labeled progesterone, an androgen precursor. Analysis of the resulting steroids showed that the interstitial tissue is the predominant source of androgens, although the tubules are capable of some production.

The present study overcomes this difficulty by elucidating the fine structure of interstitial cells in guinea pigs, a species used for some of the pertinent biochemical work (32, 22).

MATERIALS AND METHODS

Testis material from English short-haired guinea pigs (500 to 900 gm) was fixed for electron microscopy by perfusion or by the usual dicing method. Of several fixatives that were tried, 2.5 per cent glutaraldehyde (46) buffered with dichromate (11), and 1 per cent osmium tetroxide buffered with phosphate (34) proved to be most useful for this material. Non-perfused tissue fixed with osmium tetroxide for about an hour was rapidly dehydrated with ethanol and then embedded in Epon (31); non-perfused tissue fixed in glutaraldehyde about 4 hours was left overnight in phosphate buffer, postfixed for 3 hours in phosphate-buffered osmium tetroxide fixative, and then dehydrated and embedded. Light gold sections were stained with uranyl acetate (56) and lead citrate (42), and were viewed in an RCA EMU-3F electron microscope.

The perfusion method was based on that of Palay *et al.* (38), but was somewhat less elaborate (Fig. 1). A 500-ml separatory funnel served as a perfusion vessel and was fitted with several inches of rubber tubing closed off by a pinch clamp. The perfusion line consisted of 8 feet of polyethylene tubing (Clay-Adams "Intramedic," PE 60), with the shaft of a 20-gauge needle mounted in one end and the shaft of a 26-gauge needle mounted in the other end by means of a short length of PE 20 tubing. Animals anesthetized with Nembutal were injected intraperitoneally with 1 ml of 1 per cent sodium nitrite, and a few minutes were allowed for dilation of the blood vessels. About 20 ml of balanced saline (38) was poured into the separatory funnel (placed some 4 feet above the preparation), air was removed from the stem by pressing the rubber tubing, and the 20-gauge needle of the perfusion line was pushed through the rubber tubing to begin the flow of saline. In early perfusions the testicular artery was cannulated with the 26-gauge needle in the abdominal cavity, but it later proved simpler to place a ligature and cannulate this artery as it passed over the surface of the testis, after which the spermatic cord could be severed and the testis removed from the animal and placed in a beaker of saline (see Fig. 1). After 1 or 2 minutes of perfusion, the saline in the separatory funnel was drained down to the level of the stopcock by opening the pinch clamp, and about 50 ml of fixative was poured into the funnel. Perfusion of fixative was begun by briefly opening the pinch clamp to drain off the remaining saline. The temperature of the perfusion fluid was controlled by having the last part of the line pass in coils through a pan of water of appropriate temperature—for the saline perfusion and beginning of fixation the perfusion fluid was lukewarm, and thereafter it was cooled by an ice bath. After all the fixative had perfused through the testis (1 to 2 hours), the preparation was allowed to stand for about an hour more, and the testis was then cut up with razor blades and the pieces of testis were either placed in cold buffer (glutaraldehyde-perfused material) or dehydrated (OsO_4-perfused material). The subsequent steps were the same as for the non-perfused tissue.

OBSERVATIONS

Fig. 2 shows the seminiferous tubules and interstitial tissue in guinea pig testis perfused with glutaraldehyde, postfixed in osmium tetroxide, embedded in Epon, then sectioned and stained for light microscopy. The normal structural relationships of the testis components are well maintained. The interstitial cells in the interstitial tissue occur either singly or in clusters and are polyhedral cells about 8 to 12 μ in diameter. The only structures visible in their cytoplasm at this magnification are lipid droplets, some of them partially extracted. The connective tissue space around the interstitial cells contains fibrocytes with slender processes, collagen strands, congealed interstitial fluid, and connective tissue matrix. The blood vessels are expanded and empty because of the perfusion. Macrophages are common among the interstitial cells, although they are often difficult to distinguish with the light microscope.

A field comparable to that in the small box at upper left in Fig. 2 is shown in a low-power electron micrograph in Fig. 3, which includes portions of four interstitial cells. The nuclei (*n*) assume various shapes but are often ovoid. The most striking feature of the interstitial cell cytoplasm is the agranular or smooth endoplasmic reticulum (*aer*), which fills all the cytoplasmic space not occupied by other organelles. There are also scattered small patches of granular or rough endoplasmic reticulum (*ger*). Mitochondria (*m*), Golgi elements (*g*), large lipid droplets (*lp*), and lipofuscin pigment granules (*p*) are also prominent in low power micrographs. The plasma membrane of the cells is often evaginated as slender filopodia (*f*), except where the membranes of adjacent cells lie next to one another. In such places, occasional poorly developed desmosomes may help maintain cell relationships in the cluster (Fig. 6).

The agranular endoplasmic reticulum reaches

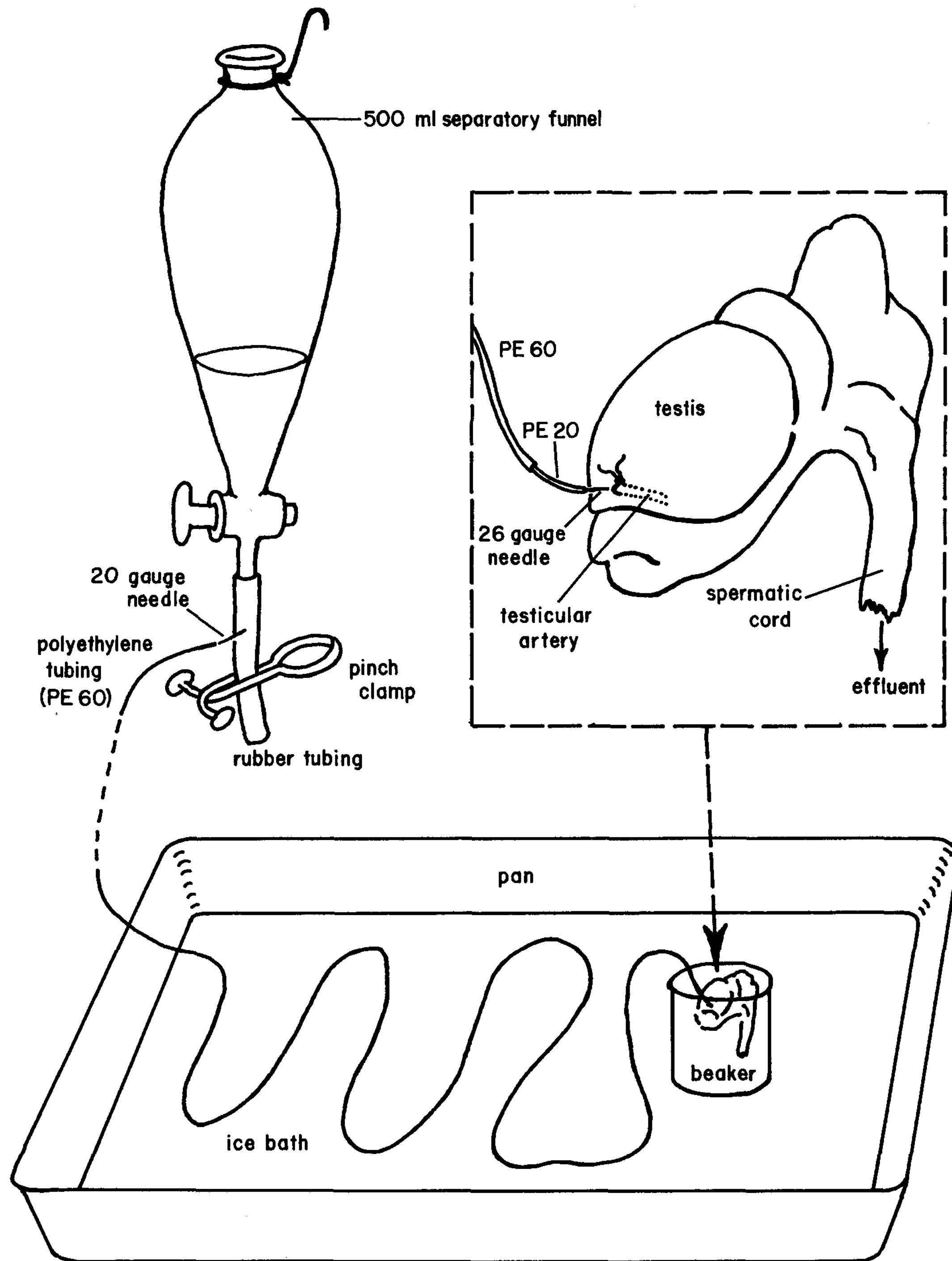


FIGURE 1 The perfusion apparatus used in this study. The fixative passes from the separatory funnel out through the polyethylene tubing to perfuse the isolated testis. An ice bath cools the fixative. For details, see Materials and Methods.

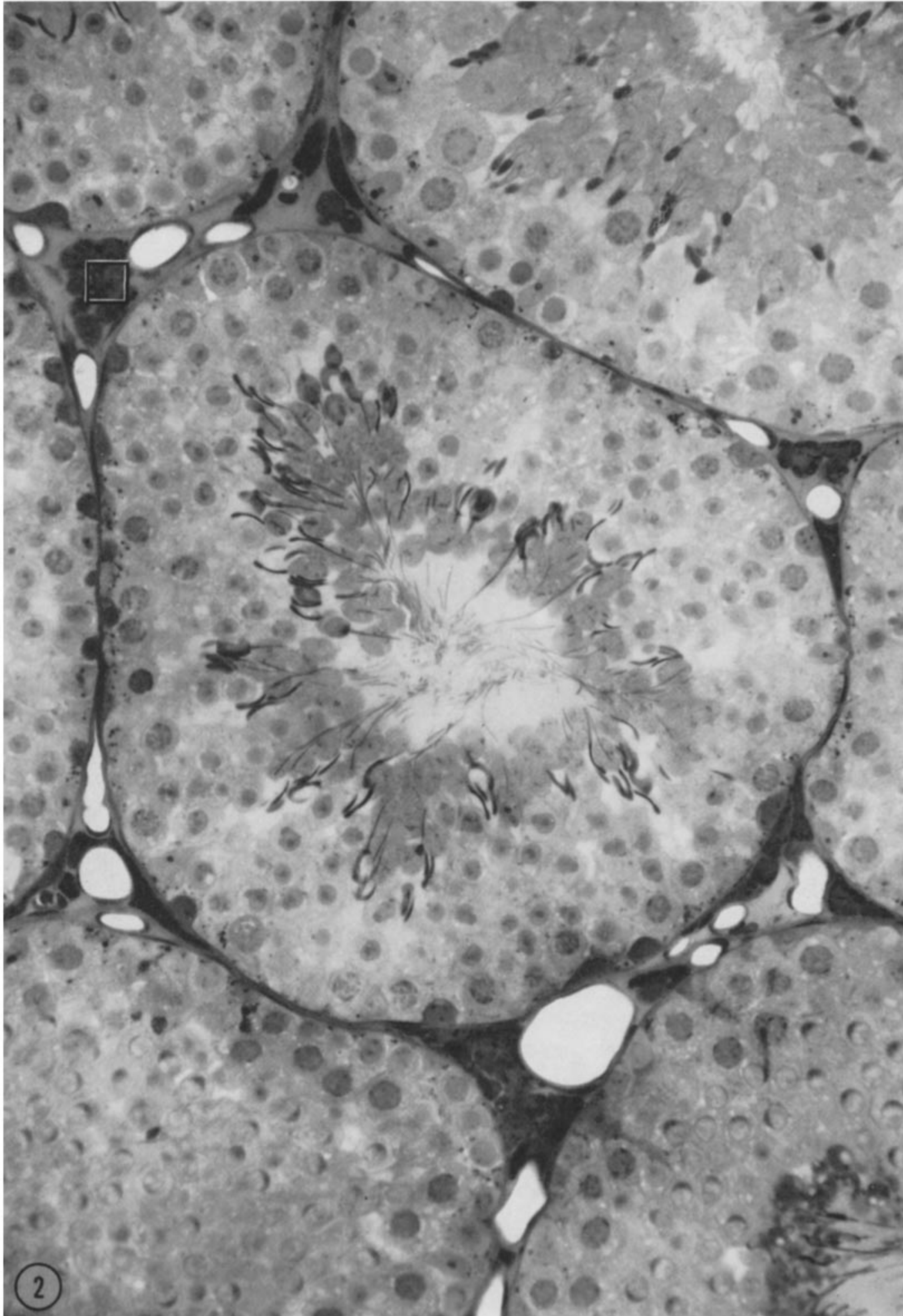
an unusual degree of development in guinea pig interstitial cells (Figs. 3 to 11). In central regions of the cell, where most of the mitochondria and other organelles occur, the reticulum is present in the usual form of randomly disposed interconnecting tubules averaging about 450 A in diameter (Figs. 3 (*t*), 4, 5, and 10). In some cells, most of the reticulum is in this tubular form. However, in the majority of interstitial cells the agranular reticulum in the peripheral areas of the cytoplasm is organized into closely packed, fenestrated cisternae which are 300 to 400 A in thickness and are 150 to 200 A apart (Figs. 3 (*c*), 6 to 8). The fenestrations are commonly about 400 A in diameter and can be seen to better advantage in surface views of the cisternae (Figs. 7 to 9, arrows). The agranular cisternae usually lie parallel with the cell surface (Figs. 3 and 6) and sometimes are arranged in concentric whorls around lipid droplets or other formed elements (Figs. 6 and 8). Upon rare occasions, cisternae are observed that show few fenestrations (Fig. 11); this is a configuration more characteristic of the granular endoplasmic reticulum.

Forms intermediate between the tubules and cisternae of the agranular reticulum are often seen, which suggests that the two are freely interconvertible. Possible stages in such transformations are presented diagrammatically in Fig. 15. The usual network of randomly oriented tubules (Fig. 15 *A*, compare Fig. 3, (*t*)) may reorganize first into flattened sheets of tubules[2] (Fig. 15 *B*, compare Fig. 3, (*ts*)). As the tubules are further flattened and the space between adjacent tubules is diminished, each sheet becomes a flattened cisterna and what were spaces between tubules become fenestrae—giving rise to a fenestrated cisterna (Fig. 15 *C*, compare Fig. 3, (*c*)). The reversion of cisternae into tubules would proceed in reverse order.

The agranular endoplasmic reticulum of steroid-secreting cells is difficult to preserve for electron microscopy. In the present study, the results of a given fixation method were often variable, and even in the same sections some interstitial cells might be well fixed according to current criteria, while other cells nearby might be poorly preserved. Thus, with any of the methods used it is possible to find cells that are well fixed, but their comparative number varies with the method. In the best glutaraldehyde perfusions, virtually all the interstitial cells are well preserved, with the agranular reticulum in the tubular or cisternal form as described above. However, when fixed by other means, the agranular reticulum in a variable percentage of the cells shows a tendency to become dilated into empty-appearing vesicles (Fig. 16) which either can remain interconnected by narrow portions of the tubules or may separate off into isolated vesicles. This tendency toward vesiculation of the agranular reticulum is virtually absent in material perfused with glutaraldehyde; it is minimal in interstitial cells fixed with glutaraldehyde without perfusion, is somewhat greater after osmium tetroxide perfusion, and is most pronounced after fixation with osmium tetroxide without perfusion. The size of the vesicles varies from cell to cell, in some cases averaging about 1500 A (Fig. 16), but in others the vesicles are larger and the cells appear swollen and seem near dissolution. In such swollen cells the vesicles are

[2] Sheets of tubules reported in the ciliary epithelium (53) were originally interpreted as agranular reticulum, but were later found to be artifacts of OsO_4 fixation resulting from a breakdown of interdigitating invaginations of the plasma membranes of adjacent cells. In the present study, however, the tubule sheets are present in glutaraldehyde-fixed material, are clearly agranular reticulum, and are reasonable intermediates between the tubular and cisternal forms of the reticulum.

FIGURE 2 Light micrograph of guinea pig testis that has been perfused with glutaraldehyde fixative. The seminiferous tubules are maintained in their normal relationship to one another. In the interstitial tissue between the tubules are numerous single or clustered interstitial cells whose dense cytoplasm contains partially extracted lipid droplets, but little else can be distinguished at this magnification. The interstitial cells are surrounded by interstitial fluid and connective tissue matrix, and by dilated blood vessels that are empty because of the perfusion. The area in the small box at upper left is approximately comparable to that shown in Fig. 3. Epon section about 2 μ thick, stained according to Richardson *et al.* (43). $\times$ 430.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often widely separated and clearly isolated from one another without interconnections.

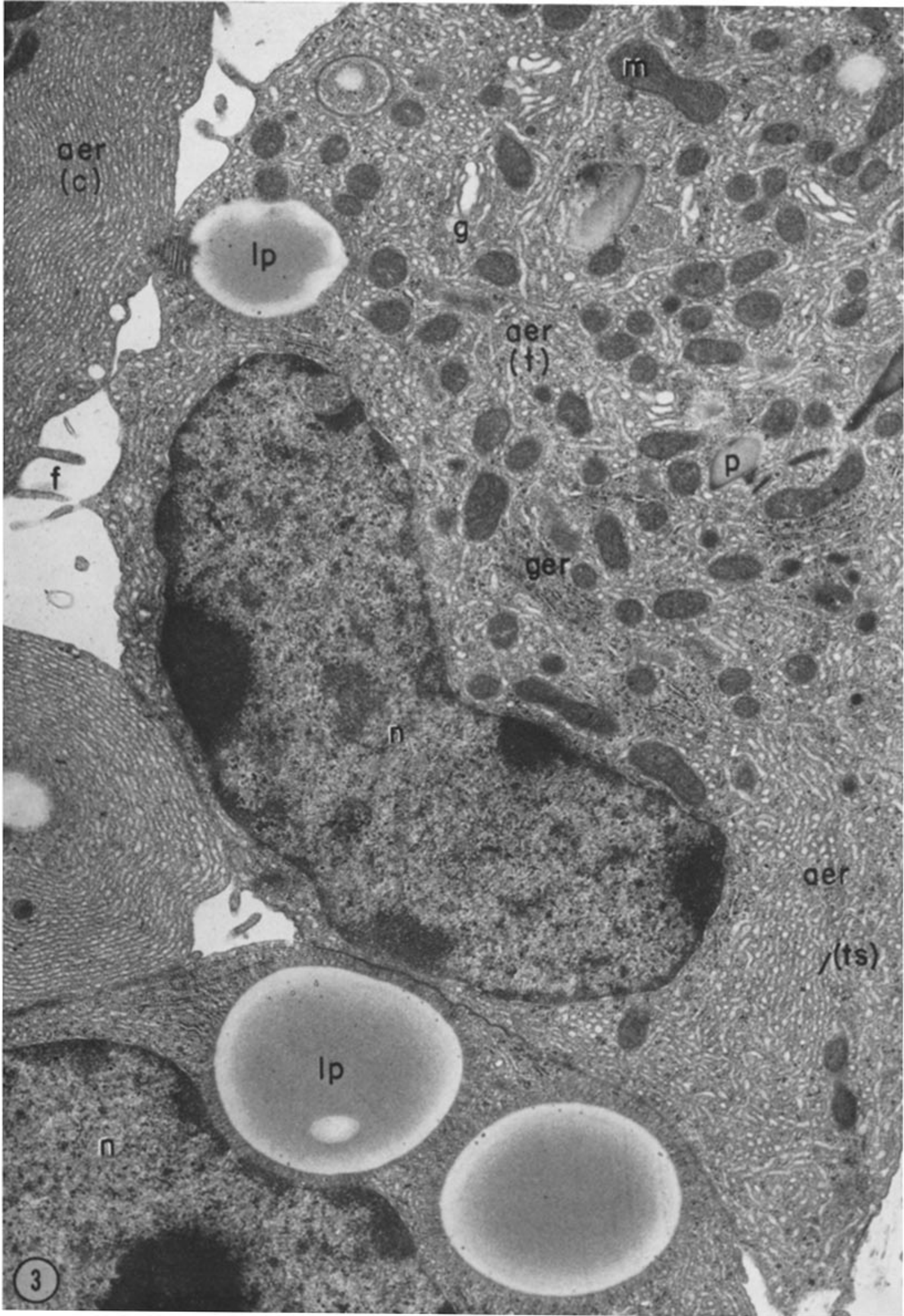
In cells in which the agranular reticulum is vesicular, there are clusters of very fine interconnecting tubules (Fig. 16, *ft*) about 200 A in diameter which appear in places to be continuous with the agranular reticulum (Fig. 16, arrows). Similar clusters have been described in rabbit interstitial cells (10). In the present study, no comparable structures were observed in cells with tubular or cisternal reticulum (here considered better preserved), and this may suggest that the fine tubules arise from certain portions of the reticulum as the rest of the reticulum dilates into vesicles. It is unlikely that the fine tubules derive from Golgi elements, since intact elements are often visible in the same cells as the clusters and no expected intermediates are observed. The significance of these fine tubule clusters is obscure, although they may indicate that the membranes of the agranular reticulum are not entirely homogeneous in their chemical and possibly functional organization.

In testes fixed in glutaraldehyde with or without perfusion, the cytoplasm of the interstitial cells shows no appreciable difference in density from one cell to the next. However, in tissue fixed in OsO_4, especially without perfusion, there is a considerable difference in density between cells, giving rise to "dark" and "light" cells resembling those that have been described for interstitial cells in other species (8, 27, 10), and in other tissues. If glutaraldehyde fixation is considered to give a more faithful preservation than OsO_4 used alone, then these results suggest that the difference between "dark" and "light" interstitial cells is an artifact of fixation.

In addition to the agranular reticulum, there are scattered small patches of granular endoplasmic reticulum (Figs. 3 to 5, 10, and 12), composed of ribosome-studded cisternae whose membranes connect freely with those of the surrounding agranular reticulum (Figs. 10 and 12, arrows). The cisternae of the granular reticulum exhibit no fenestrations, even where they are continuous with fenestrated cisternae of the agranular reticulum (Fig. 12). Even very small samples of granular reticulum seem to be in cisternal form (Fig. 4, *ger*). This strong tendency toward cisternal form without fenestrations and the propensity for adsorbing ribosomes suggest that the membranes of the granular reticulum in the interstitial cells differ, in their chemical constitution or in some material on their surface, from the membranes of the agranular reticulum. Free ribosomes are common in regions of the tubular agranular reticulum, where they lie at random between the tubules (Figs. 4 and 5). In areas of cisternal agranular reticulum the sparse free ribosomes are often situated in the middle of the fenestrations.

The mitochondria (Figs. 4 and 5) are moderately abundant in the interstitial cells, except in the peripheral cytoplasm in which the cisternal reticulum occurs. The mitochondria are generally rod-shaped, with a diameter that is variable but may be up to 0.5 μ. The matrix appears dense, and the internal structure includes lamellar cristae and some tubules, although the tubules are generally less common than have been described in the mitochondria of interstitial cells of rabbits (10), humans (20), and mice (17). There are few mitochondrial granules and no large granules such as occur in the mitochondria of opossum interstitial cells (8). In one of the animals used in the present study, the mitochondria of most of the interstitial cells had bizarre shapes and interlocking processes similar to those that Sheridan and Belt found in

FIGURE 3 Electron micrograph of portions of four interstitial cells, from an area roughly comparable to that shown in the small box at upper left in Fig. 2. The most abundant organelle in the interstitial cell cytoplasm is the agranular endoplasmic reticulum (*aer*), which in central regions of the cell is in the form of randomly disposed, interconnecting tubules (*t*), but in peripheral areas may be in the form of sheets of tubules (*ts*) or more highly organized flattened, fenestrated cisternae (*c*) (see Fig. 15). Small patches of the granular endoplasmic reticulum (*ger*) connect freely with the agranular reticulum. Mitochondria (*m*), Golgi elements (*g*), lipid droplets (*lp*), and lipofuscin pigment granules (*p*) are also present in the cytoplasm, and slender filopodia (*f*) extend from the surface of the cells. The nuclei (*n*) assume various shapes, but are often ovoid. Glutaraldehyde perfusion. × 14,000.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adrenocortical cells of occasional guinea pigs (49, see their Fig. 9).

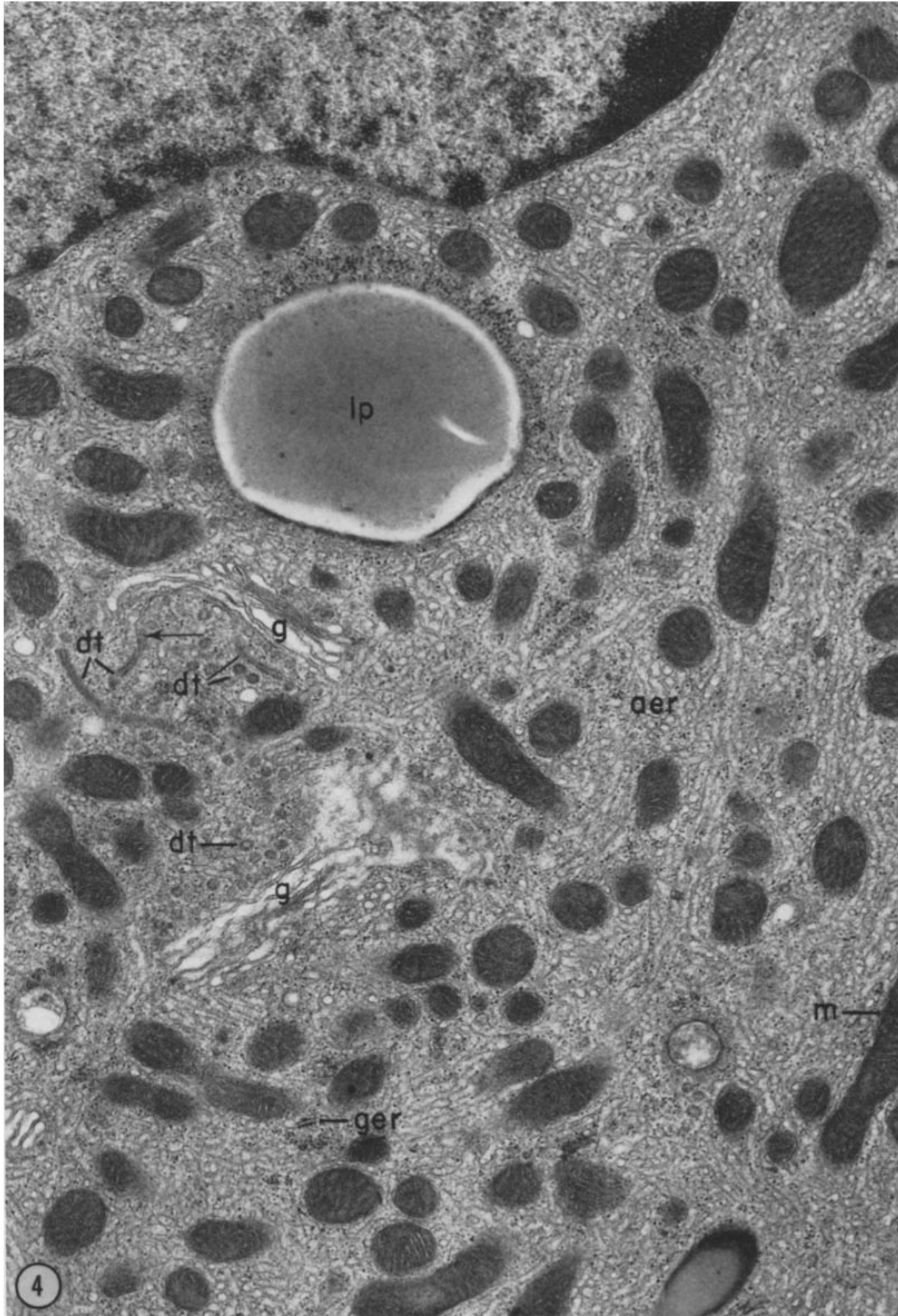
The elements of the Golgi complex are dispersed in the cytoplasm, rather than being concentrated at one pole of the nucleus. Each element is made up of several flattened, closely packed cisternae and associated small vesicles (Fig. 4). Although the cisternae are sometimes swollen, there is no evidence of any accumulation of secretory product within the elements. Associated with the Golgi elements in some cases are unusual tubules about 550 A in diameter that contain and are also surrounded by some sort of dense material (Fig. 4, *dt*). These dense tubules seem to connect with the Golgi cisternae (Fig. 4, arrow). Similar profiles have been described in rabbit interstitial cells (10) and in cardiac muscle cells (25). The two centrioles, oriented at right angles to one another, are seen in favorable sections (Fig. 13). Cell division is rare in mature interstitial cells.

The interstitial cells contain many large lipid droplets (Figs. 2 to 4, 6, and 8). In cells that are well preserved it is difficult to ascertain whether the lipid droplets are membrane bounded. However, in damaged cells the lipid is sometimes entirely extracted and the droplets are separated from adjacent structures. In such cases there appears to be a bounding membrane.

The pigment granules in the interstitial cells are presumably lipofuscin, which has been described widely in steroid-secreting cells, as well as in other tissues (see 39). The fine structure of such granules has been elucidated in the interstitial cells of some species (16, 20, 27), and in other tissues, such as cardiac muscle (25). In guinea pig interstitial cells, the lipofuscin granules generally contain three components (see Figs. 17, 18, and 20): (1) a dense component, here interpreted as the pigment proper, *p*; (2) a lipid droplet, *lp*, about 1 μ in diameter; and (3) a cap-like structure, *cap*, which has a granular matrix and contains linear patterns in small patches. A majority of the lipofuscin granules observed in this study can be interpreted as various sections through a complex of this kind (Figs. 21 and 22), although the components may possibly occur separately. The pigment appears dense and homogeneous in OsO_4-fixed material (Figs. 17, 20, and 22), but is less dense after prefixation in glutaraldehyde (Figs. 3 and 18). In favorable sections at higher magnification (Fig. 19), a 75-A periodicity is evident in the pigment, suggesting a crystalline structure. The pigment usually lies at the surface of the lipid droplet, and the variation in pigment size suggests that it may arise there (compare Figs. 18, 20, and 17). The cap is the component that most resembles lysosomal material, for example in Fig. 21, and the existence of what appear to be myelin figures in the cap in some cases (Fig. 22) implies that at least this component may constitute part of a residual body. A bounding membrane occurs on the outside of both the pigment (Figs. 19, 20, and 22, arrows) and the cap (Figs. 18 and 21, arrows), and it seems likely that this membrane also extends over the lipid droplet to enclose the whole lipofuscin granule, which may tentatively be considered a polymorphic residual body. It is not known whether any of the components contains acid phosphatase, usually taken as a diagnostic character for lysosomes or residual bodies. The detailed histochemical characteristics of the lipofuscin granules at both the light and electron microscope levels will be the subject of a separate study.

Nicander (36) found little glycogen in guinea pig interstitial cells studied with the light microscope after the periodic acid–Schiff reaction. Similarly, in the present study glycogen particles are not evident, with the electron microscope, in the interstitial cells of most guinea pigs. However, in occasional adult animals the cytoplasm of the majority of interstitial cells contains conspicuous particles (Fig. 10) whose morphology is that now taken to indicate glycogen (41). The particles are larger (250 to 400 A) and more dense than nearby

FIGURE 4 More detailed view of interstitial cell cytoplasm. The agranular reticulum (*aer*) is in the tubular configuration. A bit of the granular reticulum (*ger*) appears, despite its small size, still to be in the characteristic form of a flattened cisterna. Mitochondria (*m*) have a dense matrix and contain lamellar cristae and occasional tubules. Associated with two Golgi elements (*g*) are dense tubules (*dt*), seen in longitudinal and cross-section, which appear to connect with the Golgi sacs (arrow). The lipid droplet (*lp*) is somewhat extracted around the edges. Glutaraldehyde perfusion. × 22,000.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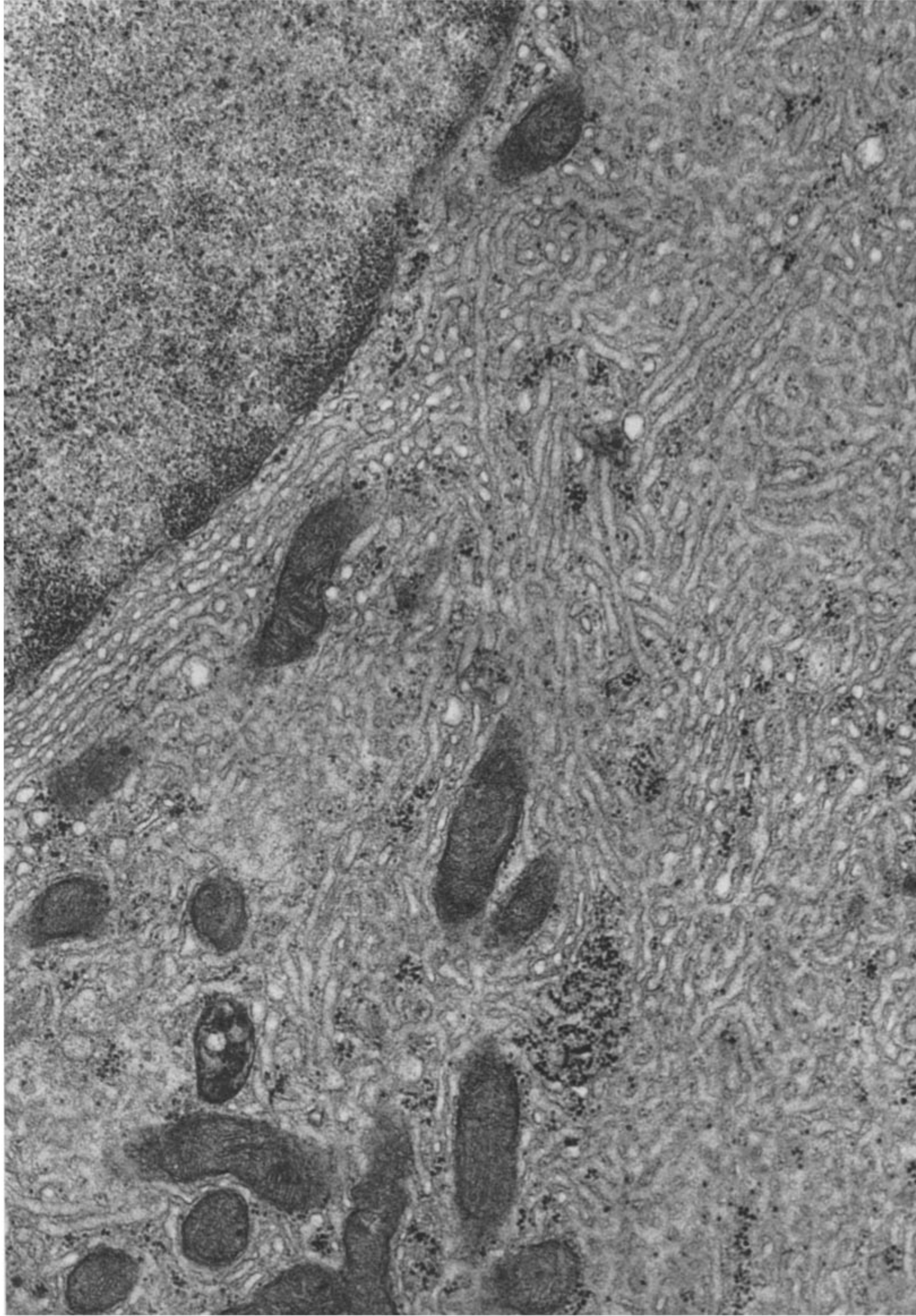


FIGURE 5 Further detail of the agranular endoplasmic reticulum. The reticulum is mostly in the tubular form, but some profiles of flattened cisternae are seen along the nucleus at left, and a surface view of sheets of tubules or fenestrated cisternae is seen at lower right. Osmium tetroxide perfusion. × 33,000.

ribosomes (compare *gl* and *rb* in Fig. 10). It is unknown why glycogen appears only in occasional adult animals.

Microtubules about 250 A in diameter are common in the cytoplasm of glutaraldehyde-fixed interstitial cells (Figs. 6 and 12, *mt*). The microtubules occur singly and seemingly at random, and do not form large bundles like those of opossum interstitial cells (8).

The plasma membrane of the interstitial cells occasionally exhibits dense-walled invaginations or "coated vesicles" (Fig. 14, arrows) similar to those that have been described in other cell types and interpreted as sites of selective absorption of proteins and perhaps other materials into the cell (45). Similar structures have been reported in what are considered to be rat interstitial cells (12), although some of the micrographs in the paper show macrophages.

DISCUSSION

The fine structure of steroid-secreting cells has been studied in a number of mammalian species. Some papers may be listed on testicular interstitial cells: oppossum (8), human (16, 20), rat (27, 48, 7), mouse (6, 17), and rabbit (10); ovarian interstitial cells: mouse (35); lutein cells: rat (14, 15), mouse (61), mink and armadillo (14); and adrenocortical cells: rat (47, 55, 28, 1), guinea pig (49), and mouse (62). The most prominent organelle in these steroid-secreting cells has generally been the agranular endoplasmic reticulum, although the mitochondria are sometimes well developed, especially in the adrenal cortex (1). The guinea pig interstitial cells described in the present study have the most abundant and highly developed agranular reticulum that has yet been reported in steroid-secreting cells. In addition to having the usual tubular form of the agranular reticulum, the interstitial cells in this species characteristically have extensive systems of flattened cisternae, a form of the agranular reticulum which is uncommon in steroid-secreting cells of other species. Cisternal reticulum was occasionally seen in opossum interstitial cells (8) and membrane whorls occur in mouse interstitial cells (6, 17), but in guinea pigs the cisternae are a characteristic form of the reticulum in the peripheral cytoplasm of a majority of interstitial cells. The organization of these membrane systems resembles that of the well developed granular endoplasmic reticulum described by Ito (24) in pancreatic acinar cells of starved and re-fed bats, except that in the interstitial cells there are no ribosomes on the membranes and the cisternae are fenestrated.

In a living interstitial cell the agranular endoplasmic reticulum is a dynamic system of membranes, and probably undergoes continuous changes of form and disposition. The agranular reticulum is particularly difficult to preserve adequately for electron microscopy, and it is, therefore, hard to be sure which forms seen in electron micrographs are within the normal range of variation of the intact organelle, and which are artifacts produced during preservation for electron microscopy. In the present study, it has been assumed that the agranular reticulum is normally a network of interconnected tubules of essentially uniform diameter. In peripheral regions of the guinea pig interstitial cell, this abundant tubular reticulum can become reorganized into sheets of tubules and these can transform into closely packed fenestrated cisternae. On the other hand, the tubules can also develop dilations along their length, giving rise to numerous vesicles that are still interconnected. These may ultimately separate off into isolated vesicles. Both the interconnected and isolated vesicles are here considered a result of faulty preservation. Some of the basis for the above assumptions can now be considered.

In early studies of steroid-secreting cells, the agranular reticulum was reported, with rare exception (44), to be a system of isolated membrane vesicles. The possibility that the membranes might form a continuous system was seldom emphasized, in spite of earlier suggestions that in other tissues the vesicles of the agranular reticulum were interconnected (37). In my own early observations on rat interstitial cells (7) and in preliminary work on opossum interstitial cells with D. W. Fawcett, the techniques of the day usually revealed the agranular reticulum as a system of what seemed to be discontinuous vesicles. However, as techniques improved, the reticulum in the majority of opossum interstitial cells came to appear as a network of interconnecting tubules of more or less uniform diameter without local dilations (8). Similar results had been described earlier by Ross *et al.* (44) in human fetal adrenocortical cells. Yet, even in the best fixations there were still some cells that contained the vesicular agranular reticulum. The tubular form of the agranular reticulum, without vesicles, has now been described in a variety of steroid-secreting cells (8, 10, 14, 15, 17, 20, 27,

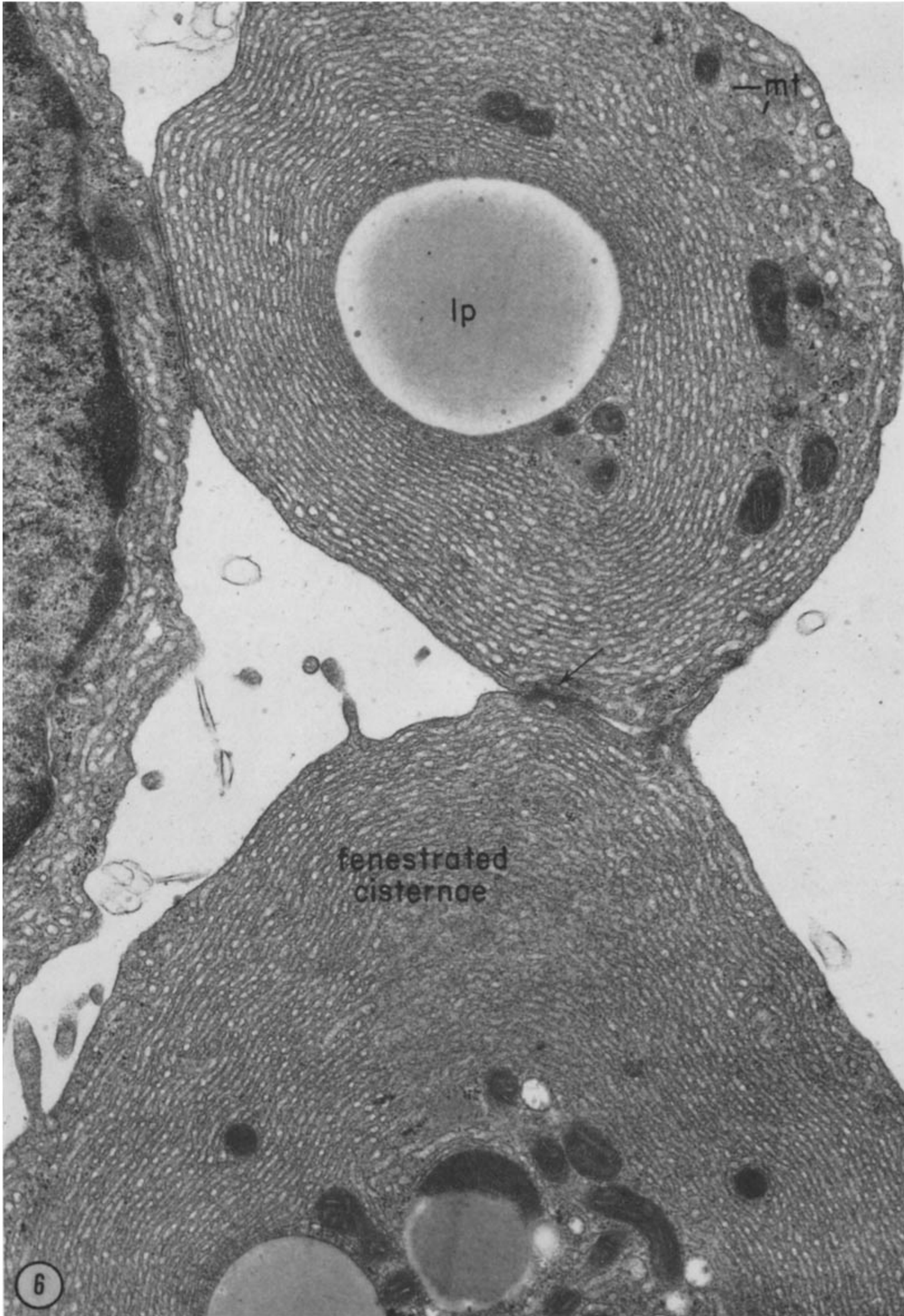
44, 55), but occasional recent papers still claim that isolated vesicles are the typical configuration in normal interstitial cells (48). The present study offers further evidence that the normal form of the agranular reticulum in interstitial cells is that of fairly uniform tubules, and not isolated vesicles or strings of vesicles. In guinea pig testis perfused with glutaraldehyde fixative, which, in current thinking, should be a method of choice, there are virtually no interstitial cells showing an agranular reticulum in the form of vesicles. It is, therefore, suggested that the vesicular agranular reticulum is an artifact of fixation resulting from dilation and eventual breakdown of the normal tubules of the agranular reticulum. There has been a similar shift of ideas in the study of gastric parietal cells, in which the agranular reticulum was early thought to be vesicular, but has since been shown by Ito (23) to be in the form of interconnecting tubules of fairly uniform diameter.

There is a possible source of concern regarding the above conclusion. It is conceivable that a very hypertonic fixative might dehydrate a cell to the point where membrane structures originally in the form of interconnected vesicles might become uniform tubules, or uniform tubules might become cisternae closely packed together. In this case the cisternae, or even the tubules, might, therefore, be artifacts. The dichromate-buffered 2.5 per cent glutaraldehyde fixative used in the present study was hypertonic (osmolarity 510 mOs/kg, compared to about 300 for mammalian serum), but the phosphate-buffered osmium tetroxide fixative was supposedly isotonic with mammalian serum (34), and in a majority of cells in perfused tissue it yielded the same sort of tubules (Fig. 5) and fenestrated cisternae (Fig. 8) that were seen in glutaraldehyde-perfused material. It is, therefore, considered unlikely that the tubules and cisternae are products of hypertonic fixation. However, fixation is a complex phenomenon, as the experiments of Wood and Luft (60) have recently emphasized, and it is possible that some details in the form or closeness of packing of the tubules or cisternae may result from fixation effects.

A major goal of cell biology is to understand the role of the various organelles in cellular phenomena and to elucidate the molecular basis of these functions. A good deal is known about the cell biology of some secretory cell types, especially those that secrete proteins (5). However, cells that secrete steroid hormones are still poorly understood. In view of the striking differences between steroids and other common cell products, with regard to chemical properties and biosynthetic pathways, there is no reason to assume that the pattern of organelle involvement in steroid secretion will follow that of better known cell types. The biosynthesis of steroid hormones has been worked out in considerable detail and a number of studies have been made on the fine structure of steroid-secreting cells, but there has been little progress so far in correlating data at the molecular level with organization at the cell level. An attempt will be made in the remainder of this discussion to correlate the fine structure of guinea pig interstitial cells with the available information from biochemistry. For this, it is necessary to turn to studies done in other laboratories on the biosynthesis of steroids and the location of the various enzymes in centrifugal fractions of testis homogenates.

The predominant pathway of androgen biosynthesis (Table I) is now well established (see 13). The main substrate is probably cholesterol, which is synthesized from acetate. The side chain of cholesterol is cleaved to produce pregnenolone, and this is then transformed successively to progesterone, 17-hydroxyprogesterone, Δ^4-androstene-3, 17-dione, and finally testosterone. The enzymes mediating the four steps from pregnenolone are 3β-hydroxysteroid dehydrogenase (and Δ^5-isomerase), 17-hydroxylase, 17-desmolase, and 17β-dehydrogenase. The localization in cell fractions of these and other enzymes of androgen biosynthesis in guinea pig and rat testes is shown in Table I. The 17-hydroxylase and the 17-desmolase are pre-

FIGURE 6 In the peripheral regions of these two cells the agranular endoplasmic reticulum is in the form of fenestrated cisternae. The cisternae are closely packed, lie parallel with the cell surface, and are sometimes organized around formed elements, such as the lipid droplet (*lp*) in the upper cell. Microtubules (*mt*), here seen in longitudinal section, are common in the interstitial cells. Occasional poorly developed desmosomes (arrow) may help maintain cell relationships. Glutaraldehyde perfusion. × 21,000.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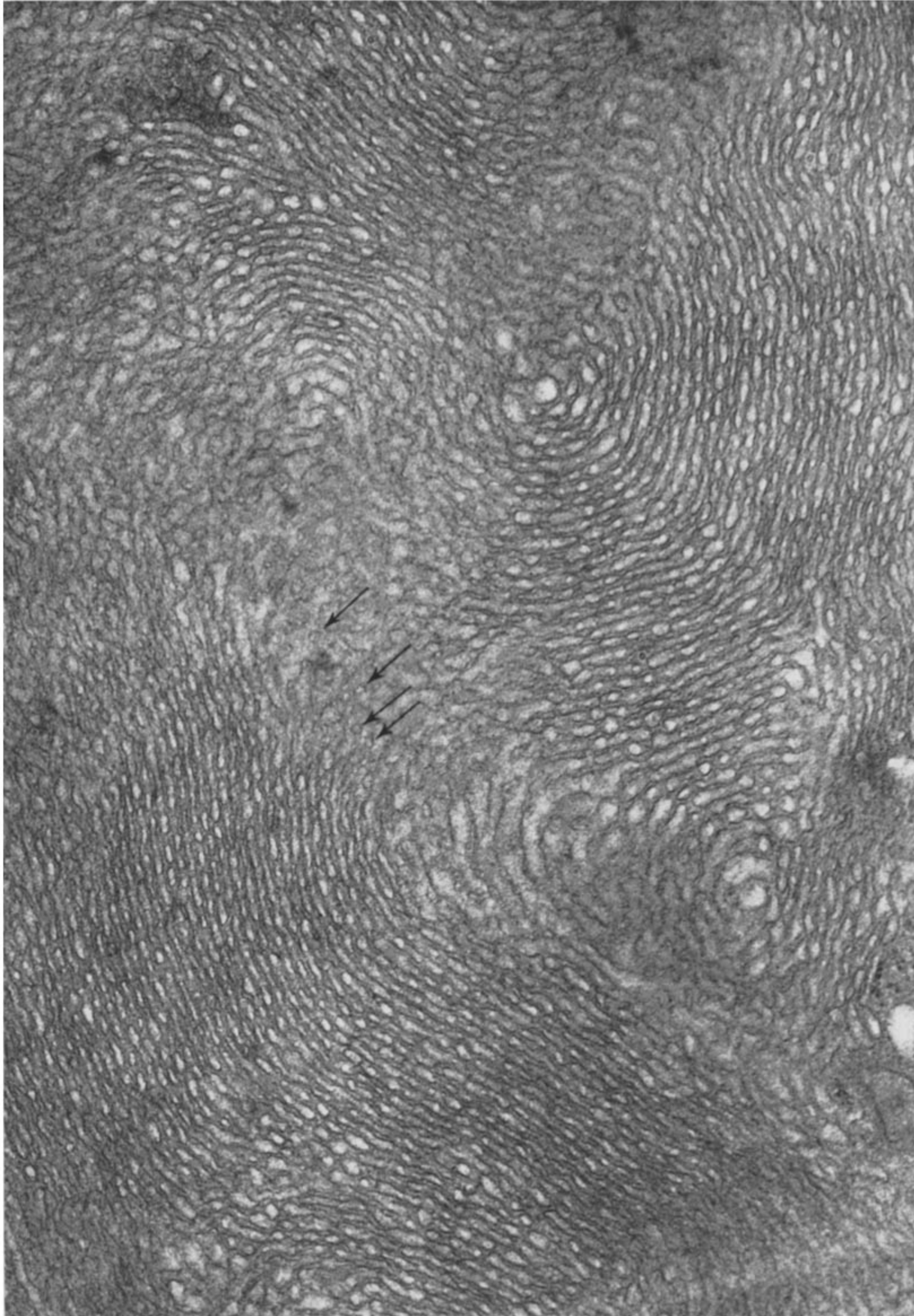


FIGURE 7 Region of cytoplasm in which the agranular reticulum is in the form of fenestrated cisternae. The cisternae are about 300 to 400 A thick and 150 to 200 A apart. In the center of the field, some of the cisternae are seen in surface view, and the fenestrations can, therefore, be seen to better advantage (arrows). Glutaraldehyde perfusion. × 33,000.

dominantly in the microsome fraction of guinea pig testis homogenates (32, 22) and are stable to ribonuclease (32), suggesting that these enzymes are associated with the membranes of the microsome fraction and not with the ribosomes. It is assumed, for the purposes of the present study, that this reflects the location of these enzymes in living cells, although there is always the danger in cell fractionation studies that during homogenization some enzymes may be adsorbed secondarily onto structures that they did not occupy when the cell was intact.

Microsome pellets of many tissues have been examined with the electron microscope and found to contain mostly membrane vesicles and attached ribosomes, and it is generally agreed that most of the vesicles arise during tissue homogenization by a fragmentation of the endoplasmic reticulum. It therefore seems reasonable to believe that during homogenization of guinea pig testes the abundant agranular reticulum of the interstitial cells breaks down into vesicles, and that these vesicles, with few attached ribosomes, are the main contribution of the interstitial cells to the microsome fraction. It would be difficult to check this by electron microscopy of microsomal pellets, since the great majority of vesicles in such preparations would be derived from the seminiferous tubules and those from the interstitial cells could not be distinguished among them. However, in the present study the agranular reticulum occasionally broke down into strings of vesicles or isolated vesicles under the stress of inadequate fixation (Fig. 16), and these changes may be analogous to those that occur during homogenization of the cells. In Fig. 16 the vesicles resemble those that are seen in microsome fractions, but in the present case they are seen *in situ*, and so are known to derive predominantly from the agranular reticulum of the interstitial cells.

Thus, the available biochemical evidence, correlated with the fine structure results of the present paper, indicates that the abundant agranular endoplasmic reticulum of guinea pig interstitial cells is the site of at least two enzymes of androgen biosynthesis, the 17-hydroxylase and the 17-desmolase.

Similar but more extensive results are available from biochemical work on rat testis homogenates (see Table I). Shikita and his associates (50, 51) studied enzyme activity in microsome fractions that had been shown by electron microscopy to be free of mitochondria or other contaminants. These authors found that the enzymes mediating testosterone biosynthesis from pregnenolone are firmly attached to the microsomes of rat testis homogenates.

The testes of both rats (54) and guinea pigs (21) are capable of making cholesterol from acetate. However, the most careful studies on this synthesis so far have been carried out in the rat liver (see 4), in which most of the enzymes occur in the microsome fraction, although some are in the soluble fraction. The microsomal enzymes are in the "heavy" microsome subfraction and are stable to ribonuclease, indicating that they are probably associated with the membranes of the endoplasmic reticulum rather than with the ribosomes. There is a great deal of current interest in cholesterol biosynthesis in the testis, and so far the meager data from fractionation studies suggest that, as in the liver, the enzymes occur in the microsome and soluble fractions (19, lanosterol to cholesterol). A further suggestion that the agranular reticulum is involved in cholesterol synthesis comes from a correlation of fine structure observations with biochemical data (58) on the extent of cholesterol synthesis in the testes and adrenals of rats and guinea pigs. The rat adrenal cortex makes almost none of its free cholesterol, absorbing it instead from the plasma; the adrenocortical cells in this species contain a very sparse endoplasmic reticulum (55). The guinea pig adrenal cortex makes about 40 per cent of its free cholesterol, and the agranular reticulum is moderately well developed (49). The rat testis makes about 60 per cent of its cholesterol, and its interstitial cells have a fairly abundant agranular reticulum (27). The guinea pig testis makes 87 per cent of its cholesterol, and its interstitial cells have an exceedingly abundant agranular reticulum, as described in the present study. In these cases, therefore, the amount of agranular reticulum in the cells seems to correlate well with the extent of cholesterol production. It is clear from the electron micrographs in the present study that any soluble enzymes of cholesterol biosynthesis cannot be very far from the endoplasmic reticulum, since the cytoplasmic space between the tubules or cisternae is limited.

In addition to their probable role as an energy source, the mitochondria of rat interstitial cells are the site of the enzymes that cleave the side chain of cholesterol to make pregnenolone (52). Since the enzymes that precede this stage of androgen

TABLE I

Location of Enzymes of Androgen Biosynthesis in Centrifugal Fractions of Testis Homogenates

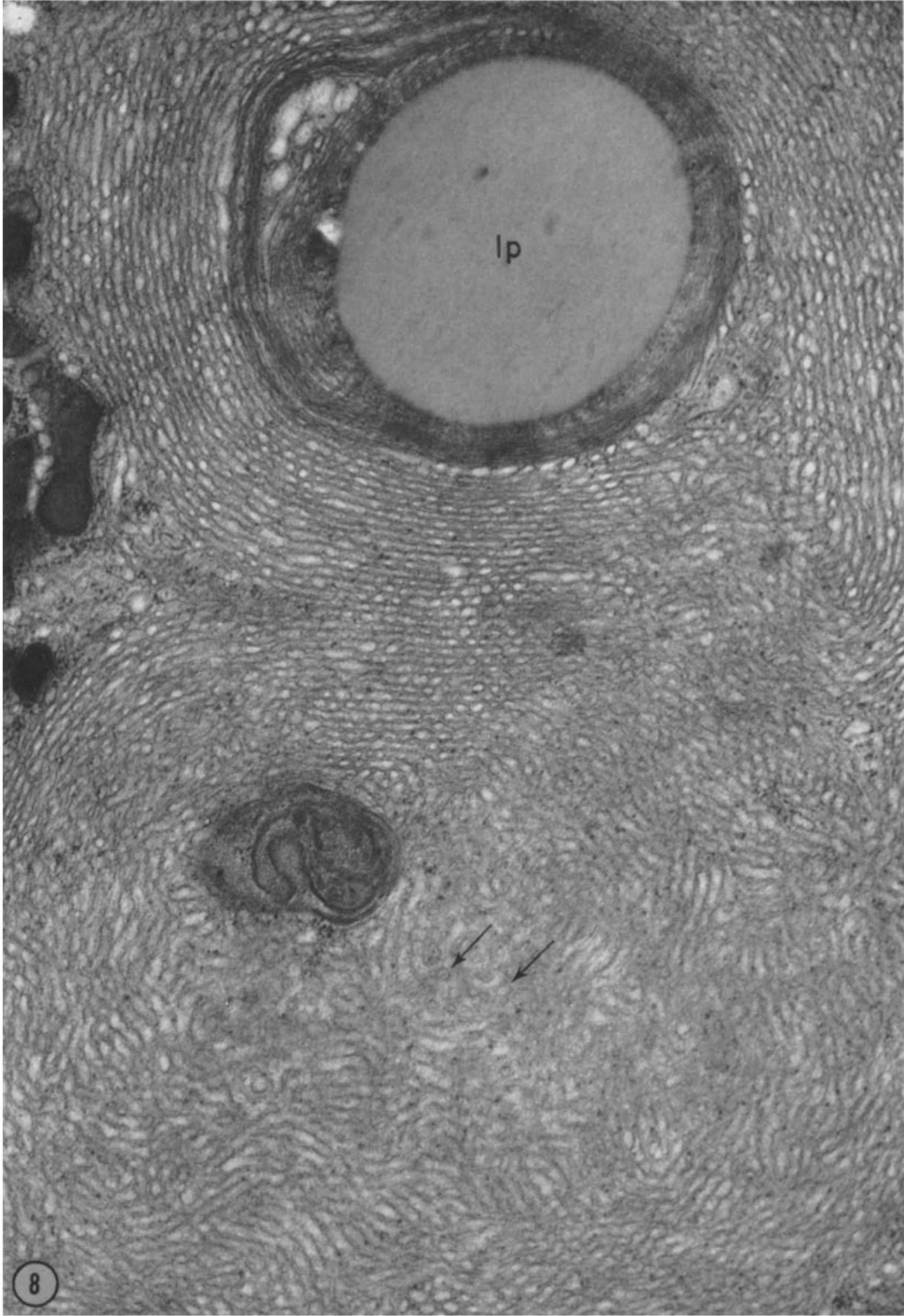
Synthesis	Enzymes	Centrifugal fractions
Acetate ↓ Cholesterol	(Numerous steps)	Microsomes and soluble: rat (19, lanosterol to cholesterol)
Cholesterol ↓ Pregnenolone	Side chain cleavage system	Mitochondria: rat (52)
Pregnenolone ↓ Progesterone	3β-Hydroxysteroid dehydrogenase (and Δ^5-isomerase)	Microsomes: rat (50)
Progesterone ↓ 17-Hydroxyprogesterone	17-Hydroxylase	Microsomes: guinea pig (32, 22), rat (50, 51, 32)
17-Hydroxyprogesterone ↓ Androstenedione	17-Desmolase	Microsomes: guinea pig (32, 22), rat (50, 51, 32)
Androstenedione ⇅ Testosterone	17β-Dehydrogenase	Microsomes: guinea pig (22), rat (50, 51) Soluble: guinea pig (32)

biosynthesis and those that follow it are predominantly in the microsome fraction, presumably cholesterol must pass from the vicinity of the endoplasmic reticulum to the mitochondria, and pregnenolone must return to the reticulum. If the cholesterol cleavage enzymes are inside the mitochondria, rather than on their surface, then there might be some accumulation of substrates within the mitochondria, which could explain the dense matrix that is a common feature of interstitial cell mitochondria as seen in electron micrographs. The mitochondria of interstitial cells are generally not so numerous or well developed as those of adrenocortical cells, in which the mitochondria are the site of 11-hydroxylation (3), a terminal step in the synthesis of both cortisol and corticosterone. In the adrenal cortex the final hormones may thus arise in the mitochondria (47), but this does not seem to be the case in the testis.

The results from biochemical studies of the rat testis, therefore, indicate that although the mitochondria are involved to some extent, the microsome fraction is the preponderant site of the enzymes involved in androgen biosynthesis. This agrees with the morphological finding that the agranular endoplasmic reticulum is the most prominent organelle in the interstitial cells of rats (27). In guinea pig interstitial cells the agranular reticulum is still more abundant, and although the biochemical evidence is limited at present, it seems likely that in guinea pigs also the agranular reticulum will be found to play a broad role in androgen biosynthesis.

Since a good histochemical method is available

FIGURE 8 Fenestrated cisternae in an interstitial cell fixed by perfusion with osmium tetroxide fixative. In the upper part of the micrograph the closely packed cisternae are organized around a large lipid droplet (*lp*). The membranes near the lipid droplet are arranged in especially tight whorls. In the lower third of the micrograph, the cisternae or sheets of tubules are cut in various planes of section, including a surface view in which fenestrations (arrows) are visible. A small area of general cytoplasm with dense mitochondria is seen at left. × 30,000.


lp
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for localizing 3β-hydroxysteroid dehydrogenase at the light microscope level (57, 30), it might be expected that a modification of the technique would allow localization of this enzyme at the electron microscope level. However, the histochemical reaction involves a diaphorase step interposed between the dehydrogenase and the reduction of the tetrazolium reagent. The final formazan deposit, therefore, marks the location of the diaphorase, which might be some distance from the dehydrogenase, perhaps even on a different organelle. A recent paper ignores this difficulty and reports a localization by electron microscopy of 3β-hydroxysteroid dehydrogenase in mitochondria of the rat adrenal cortex (2). There is biochemical evidence that this enzyme is actually in the endoplasmic reticulum of rat adrenocortical cells (26).

It is not known how androgens are transported from their site of synthesis in the guinea pig interstitial cell to the cell surface to be released into extracellular space. If the androgens are transported within the agranular reticulum in any concentration, they might be kept in solution by being bound to proteins produced by the patches of granular reticulum. There is no evidence that the pattern of androgen secretion resembles that described in protein-secreting cells (see 5), since in interstitial cells there is no indication that lipid droplets or other secretory inclusions arise in the Golgi complex or that lipid droplets are released from the cells. Although the droplets in interstitial cells contain cholesterol as well as neutral fats (see 59, pp. 460–474), it has not been established that androgens accumulate in the droplets. Lipid droplets do not seem to be essential in the secretion of steroid hormones, since they are rare in the interstitial cells of some species, such as opossums (8) and adult laboratory rats (although abundant in prepubertal rats) (7). It has been suggested by Fawcett and Christensen (17) that the extensive membranes of the agranular reticulum, in addition to providing sites for enzymes, may also act as a reservoir for the storage of cholesterol, since cholesterol is an important component of biological membranes (18).

The lipofuscins constitute a very heterogeneous group of lipid pigments that occur in many tissues (see 39). They have been described in a variety of steroid-secreting cells, and have been especially

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FIGURE 9 Fenestrated cisternae in surface view. Fenestrations occur throughout this field—some that are more clearly seen are indicated by arrows. The diameter of the fenestrations varies, but is commonly about 400 A. Glutaraldehyde perfusion. × 72,000.

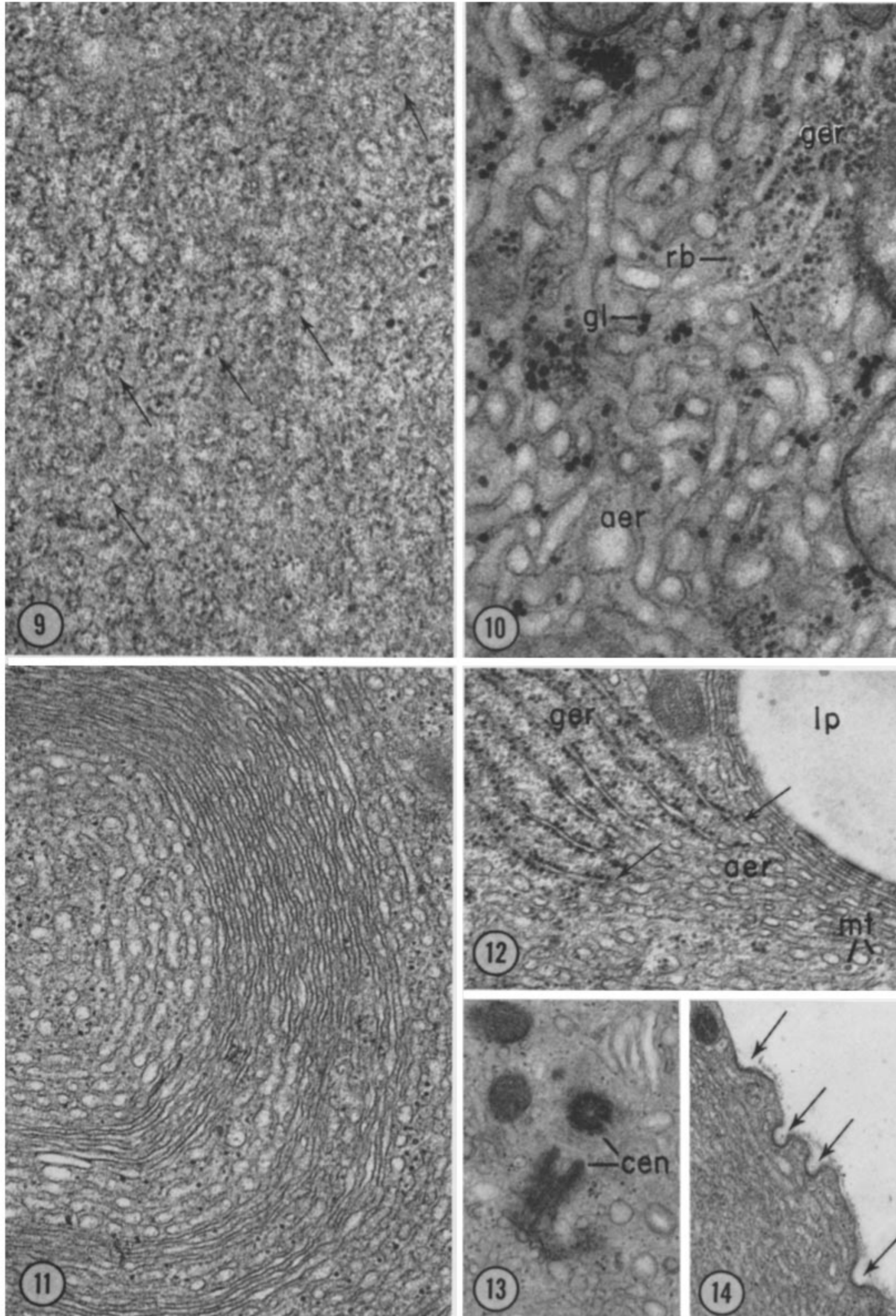
FIGURE 10 Particles presumed to be glycogen (*gl*) are present in the interstitial cells of occasional guinea pigs, although most animals lack them. When present, the particles are larger and stain more densely than ribosomes (*rb*) in adjacent patches of the granular reticulum. Note the continuity (arrow) between the membranes of the granular (*ger*) and the agranular (*aer*) reticulum. Osmium tetroxide, without perfusion. × 48,000.

FIGURE 11 In rare instances the agranular cisternae have few fenestrations. This form of the reticulum is more characteristic of the granular endoplasmic reticulum. Glutaraldehyde perfusion. × 32,000.

FIGURE 12 The granular endoplasmic reticulum (*ger*) connects freely (arrows) with the agranular reticulum (*aer*). The cisternae of the granular reticulum lack fenestrations, while the adjoining cisternae of the agranular reticulum are fenestrated. Two microtubules are seen in cross-section (*mt*). Glutaraldehyde perfusion. × 34,000.

FIGURE 13 The interstitial cells contain two centrioles (*cen*) at right angles to one another. Cell division is rare in mature interstitial cells. Glutaraldehyde perfusion. × 21,000.

FIGURE 14 Some cells show dense-walled invaginations of the plasma membrane (arrows). These "coated vesicles" (45) in some other cell types have been suggested as possible sites of protein absorption. Glutaraldehyde perfusion. × 23,000.



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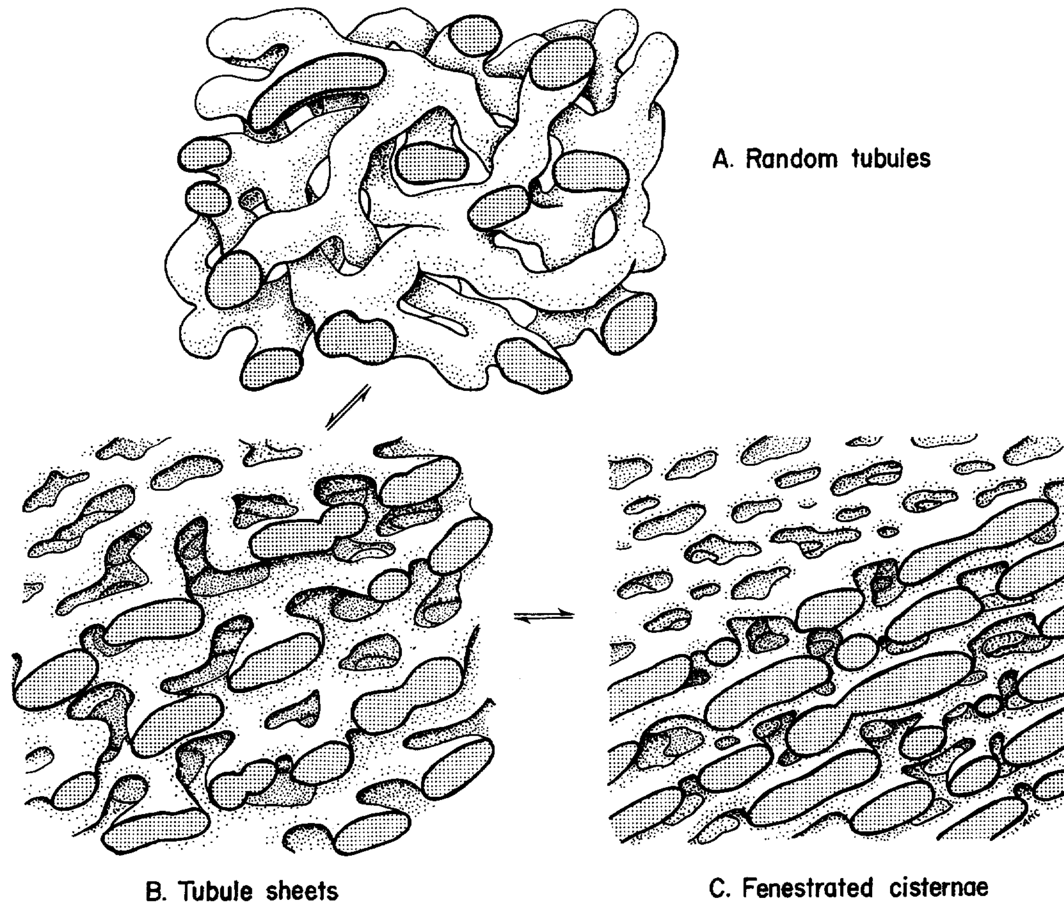
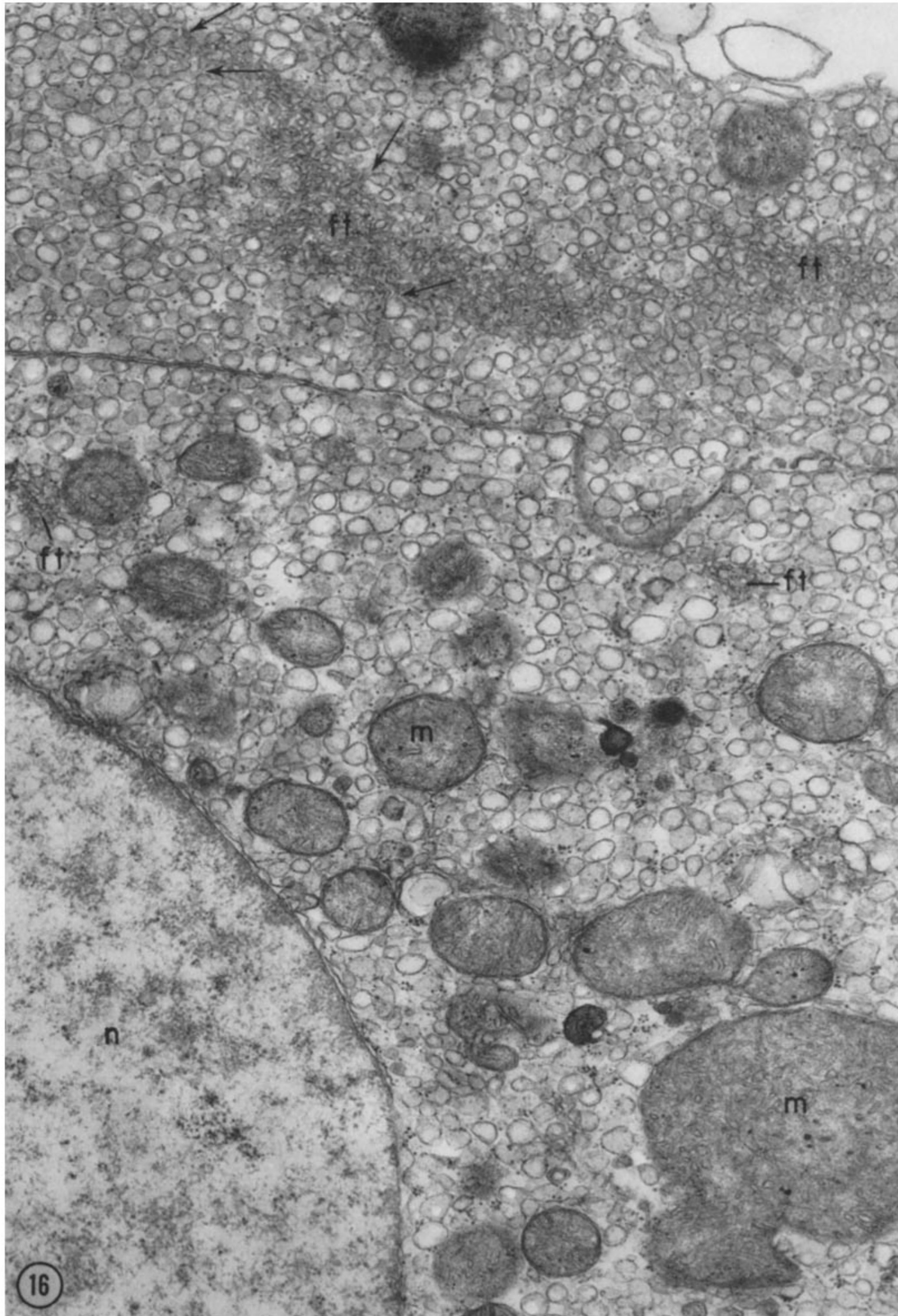


FIGURE 15 Diagram of various forms of the agranular endoplasmic reticulum seen in guinea pig interstitial cells. The possible interconversions of these configurations are indicated by arrows. *A*, Random network of interconnected tubules. This is the usual form of the agranular reticulum in the interstitial cells of other species and in the central region of guinea pig interstitial cells. *B*, Loose sheets of tubules. This is probably a transitional form. *C*, Fenestrated cisternae. This is a common form of the agranular reticulum at the periphery of guinea pig interstitial cells.

FIGURE 16 In tissue fixed by techniques other than glutaraldehyde perfusion, occasional cells are seen in which the agranular reticulum seems to have become dilated into vesicles which may remain interconnected or may separate into isolated vesicles. The vesicles in the cells shown in this micrograph average about 1500 A in diameter, but in some cases they are much larger. The virtual absence of cells containing vesicles in material fixed by glutaraldehyde perfusion suggests that the vesicles arise as an artifact by the dilation of tubules or cisternae during preservation. The mitochondria (*m*) seem relatively less affected, except that they are somewhat swollen and seem to contain more tubules.

In cells that contain vesicles, the cytoplasm also exhibits scattered clusters of fine tubules (*ft*) that average about 200 A in diameter and interconnect with each other and with the vesicles of the agranular reticulum (arrows). Their significance is unknown. Glutaraldehyde fixation, without perfusion. × 24,000.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16

well characterized in the adrenal cortex of several mammals, including guinea pigs (40). These pigments are generally thought to be polymerization products of oxidized unsaturated lipids, and it seems unlikely that they are directly related to hormone production.

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BIBLIOGRAPHY

1. BELT, W. D., and PEASE, D. C., Mitochondrial structure in sites of steroid secretion, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 369.
2. BRADBURY, S., and STEWARD, V. W., A note on the electron staining of diformazan deposits in tissue sections, *J. Roy. Micr. Soc.*, 1964, **83**, 467.
3. BROWNIE, A. C., and GRANT, J. K., The *in vitro* enzymic hydroxylation of steroid hormones. I. Factors influencing the enzymic 11β-hydroxylation of 11-deoxycorticosterone, *Biochem. J.*, 1954, **57**, 255.
4. BUCHER, N., Alterations of cholesterol biosynthesis in liver cell fractions from rats in various experimental conditions, *in* Biosynthesis of Terpenes and Sterols, CIBA Symposium (G. E. Wolstenholme and C. O'Connor, editors), Boston, Little, Brown and Co., 1959, 46.
5. CARO, L. G., and PALADE, G. E., Protein synthesis, storage, and discharge in the pancreatic

FIGURE 17 Lipofuscin pigment granules, presumably constituting polymorphic residual bodies, are usually made up of three components: the pigment proper (*p*), a lipid droplet (*lp*), and a cap-like structure (*cap*). The pigment (*p*) is homogeneous in appearance. The cap has a granular matrix and shows linear patterns in small patches. Part of a larger lipid droplet occurs at right (*lp*). The area of pigment in the box is shown at higher magnification in Fig. 19. Osmium tetroxide perfusion. × 33,000.

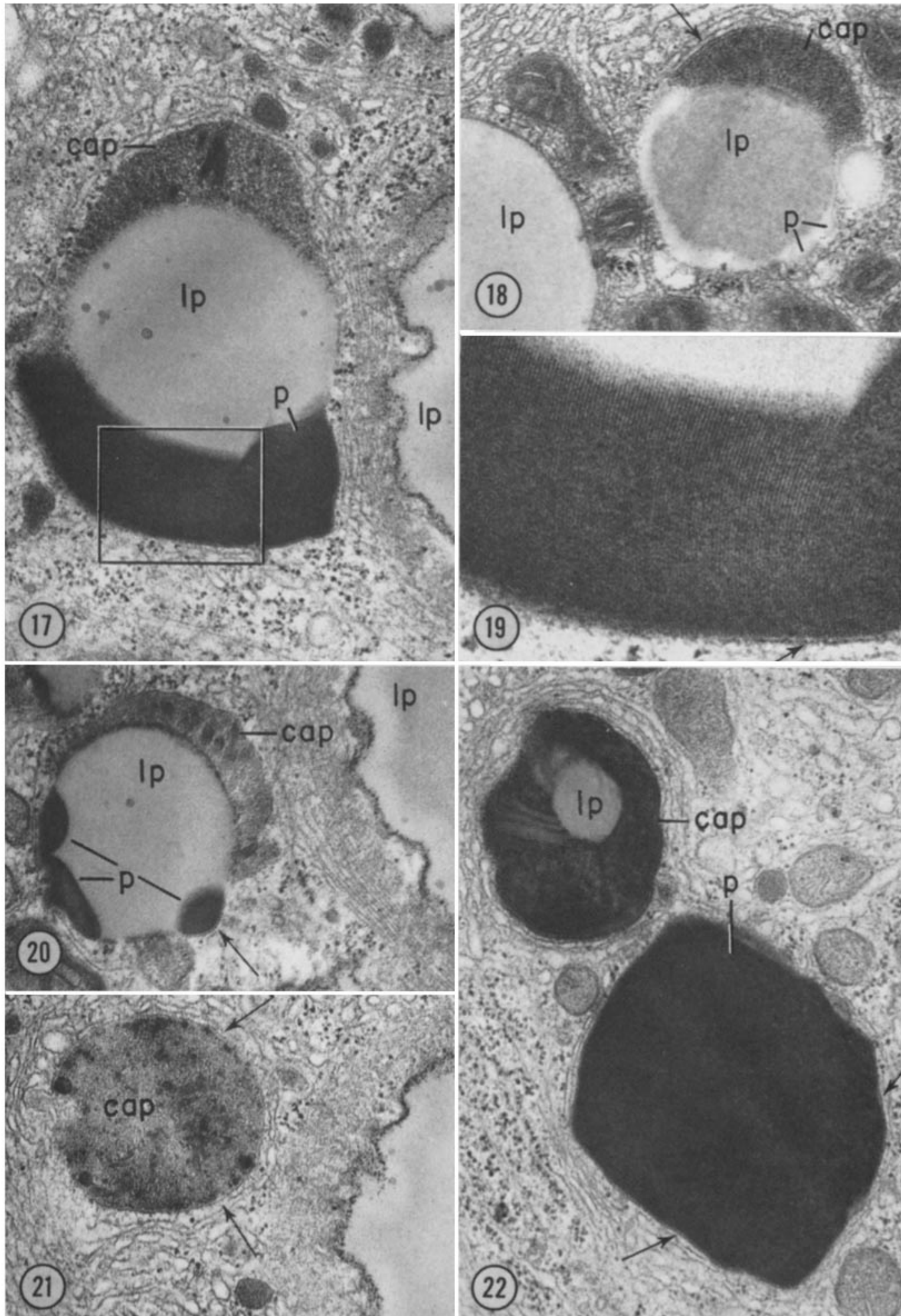
FIGURE 18 Another example of the complex granule formed by pigment (*p*), lipid (*lp*), and cap. The sparse pigment (possible early stage of formation) has a light density in this glutaraldehyde-fixed material. The cap is membrane-bounded (arrow). Another lipid droplet is shown at lower left (*lp*). Glutaraldehyde perfusion. × 33,000.

FIGURE 19 Pigment at higher magnification. In this enlargement of the area shown in the box in Fig. 17, the pigment exhibits a 75 A periodicity, which suggests that it has a crystalline structure. The external surface of the granule seems to be membrane-bounded (arrow). × 87,000.

FIGURE 20 A further example of the pigment-lipid-cap complex, in which the pigment is intermediate in abundance between the complexes shown in Figs. 17 and 18. The pigment is bounded externally by a membrane (arrow), and in the cap the small patches of linear arrays are shown clearly. The edge of a larger lipid droplet is present at upper right (*lp*). Osmium tetroxide perfusion. × 33,000.

FIGURE 21 This cap is either separate or else is part of a complex but is sectioned horizontally so that the other components of the complex are not seen. It is membrane-bounded (arrows), and bears a morphological resemblance to a lysosome. Osmium tetroxide perfusion. × 33,000.

FIGURE 22 This cap is sectioned horizontally at a level which just grazes the small lipid droplet (*lp*) on which the cap lies. The cap is more dense than usual, and contains myelin figures, making it resemble a residual body. Nearby is a large pigment granule (*p*) that either may be lying free in the cytoplasm or may be part of another complex out of the plane of section (compare with Fig. 17). Osmium tetroxide perfusion. × 33,000.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exocrine cell. An autoradiographic study, *J. Cell Biol.*, 1964, **20,** 473.

6. Carr, I., and Carr, J., Membranous whorls in the testicular interstitial cell, *Anat. Rec.*, 1962, **144,** 143.
7. Christensen, A. K., The fine structure of interstitial tissue of the rat testis at various ages and after experimental treatment, *Anat. Rec.*, 1959, **133,** 367, abstract.
8. Christensen, A. K., and Fawcett, D. W., The normal fine structure of opossum testicular interstitial cells, *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 653.
9. Christensen, A. K., and Mason, N. R., The comparative ability of seminiferous tubules and interstitial tissue of rat testes to synthesize androgens from progesterone-4-^{14}C *in vitro*, *Endocrinology*, 1965, **76,** 646.
10. Crabo, B., Fine structure of the interstitial cells of the rabbit testes, *Z. Zellforsch.*, 1963, **61,** 587.
11. Dalton, A. J., A chrome-osmium fixation for electron microscopy, *Anat. Rec.*, 1955, **121,** 281, abstract.
12. Dessolle, N., Observation d'un corportement particulier de la cellule interstitielle testiculaire du rat, *Compt. rend. Acad. sc.*, 1964, **258,** 2893.
13. Dorfman, R. I., Forchielli, E., and Gut, M., Androgen biosynthesis and related studies, *Rec. Progr. Hormone Research*, 1963, **19,** 251.
14. Enders, A. C., Observations on the fine structure of lutein cells, *J. Cell Biol.*, 1962, **12,** 101.
15. Enders, A. C., and Lyons, W. R., Observations on the fine structure of lutein cells. II. The effect of hypophysectomy and mammotrophic hormone in the rat, *J. Cell Biol.*, 1964, **22,** 127.
16. Fawcett, D. W., and Burgos, M. H., Studies on the fine structure of the mammalian testis. II. The human interstitial tissue, *Am. J. Anat.*, 1960, **107,** 245.
17. Fawcett, D. W., and Christensen, A. K., The fine structure of testicular interstitial cells in mice, in preparation.
18. Finean, J. B., The nature and stability of the plasma membrane, *Circulation*, 1962, **26,** 1151.
19. Gaylor, J. L., and Tsai, S., Testicular sterols. II. Conversion of lanosterol to cholesterol and steroid hormones by cell-free preparations of rat testicular tissue, *Biochim. et Biophysica Acta*, 1964, **84,** 739.
20. Gordon, G. B., Miller, L. R., and Bensch, K. G., Electron microscopic observations of the gonad in the testicular feminization syndrome, *Lab. Invest.*, 1964, **13,** 152.
21. Gower, D. B., and Haslewood, G. A. D., Biosynthesis of androst-16-en-3α-ol from acetate by testicular slices, *J. Endocrinol.*, 1961, **23,** 253.
22. Hofmann, F. G., The inhibition by testis microsomes of corticosterone formation by adrenal mitochondria, *Biochim. et Biophysica Acta*, 1962, **65,** 511.
23. Ito, S., The endoplasmic reticulum of gastric parietal cells, *J. Biophysic. and Biochem. Cytol.*, 1961, **11,** 333.
24. Ito, S., Light and electron microscopic study of membranous cytoplasmic organelles, *in* The Interpretation of Ultrastructure, (R. J. C. Harris, editor), Symposium of the International Society of Cell Biology, New York, Academic Press, Inc., 1962, **1,** 129.
25. Jamieson, J. D., and Palade, G. E., Specific granules in atrial muscle cells, *J. Cell Biol.*, 1964, **23,** 151.
26. Koritz, S. B., The conversion of pregnenolone to progesterone by small particles from rat adrenal, *Biochemistry*, 1964, **3,** 1098.
27. Leeson, C. R., Observations on the fine structure of rat interstitial tissue, *Acta Anat.*, 1963, **52,** 34.
28. Lever, J. D., Electron microscopic observations on the adrenal cortex, *Am. J. Anat.*, 1955, **97,** 409.
29. Levy, H., Deane, H. W., and Rubin, B. L., Observations on steroid 3β-ol dehydrogenase activity in steroid-producing glands, *J. Histochem. and Cytochem.*, 1959, **7,** 320, abstract.
30. Levy, H., Deane, H. W., and Rubin, B. L., Visualization of steroid-3β-ol-dehydrogenase activity in tissues of intact and hypophysectomized rats, *Endocrinology*, 1959, **65,** 932.
31. Luft, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 409.
32. Lynn, W. S., Jr., and Brown, R. H., The conversion of progesterone to androgens by testes, *J. Biol. Chem.*, 1958, **232,** 1015.
33. Maeir, D. M., Species variation in testicular Δ^5-3β-hydroxysteroid dehydrogenase activity: Absence of activity in primate Leydig cells, *Endocrinology*, 1965, **76,** 463.
34. Millonig, G., Further observations on a phosphate buffer for osmium solutions, *in* 5th International Congress for Electron Microscopy, (S. S. Breeze, Jr., editor), New York, Academic Press, Inc., 1962, **2,** P-8.
35. Muta, T., The fine structure of the interstitial cell in the mouse ovary studied with electron microscope, *Kurume Med. J.*, 1958, **5,** 167.
36. Nicander, L., A histochemical study on glycogen in the testes of domestic and laboratory animals, with special reference to variations

during the spermatogenetic cycle, *Acta Morphol. Neerl.-scand.*, 1957, **1**, 233.

37. Palade, G. E., Studies on the endoplasmic reticulum. II. Simple disposition in cells *in situ*, *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 567.
38. Palay, S. L., McGee-Russell, S. M., Gordon, S., Jr., and Grillo, M. A., Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide, *J. Cell Biol.*, 1962, **12**, 385.
39. Pearse, A. G. E., Histochemistry, Theoretical and Applied, Boston, Little, Brown and Company, 2nd edition, 1960.
40. Planel, H., Guilhen, A., Soleilhavoup, J. P., and Tixador, R., Les pigments du cortex surrênal des Mammiefères, *Ann. d'Endocr.*, 1964, **25**, suppl., 93.
41. Revel, J. P., Electron microscopy of glycogen, *J. Histochem. and Cytochem.*, 1964, **12**, 104.
42. Reynolds, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.*, 1963, **17**, 208.
43. Richardson, K. C., Jarett, L., and Finke, E. H., Embedding in epoxyresins for ultrathin sectioning in electron microscopy, *Stain Technol.*, 1960, **35**, 313.
44. Ross, M. H., Pappas, G. D., Lanman, J. T., and Lind, J., Electron microscope observations on the endoplasmic reticulum in the human fetal adrenal, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 659.
45. Roth, T. F., and Porter, K. R., Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L., *J. Cell Biol.*, 1964, **20**, 313.
46. Sabatini, D. D., Bensch, K., and Barrnett, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation, *J. Cell Biol.*, 1963, **17**, 19.
47. Sabatini, D. D., de Robertis, E. D., and Bleichmar, H. B., Submicroscopic study of the pituitary action on the adrenocortex of the rat, *Endocrinology*, 1962, **70**, 390.
48. Schwarz, W., and Merker, H. J., Die Hodenzwischenzellen der Ratte nach Hypophysektomie und nach Behandlung mit Choriongonadotropin und Amphenon B, *Z. Zellforsch.*, 1965, **65**, 272.
49. Sheridan, M. N., and Belt, W. D., Fine structure of the guinea pig adrenal cortex, *Anat. Rec.*, 1964, **149**, 73.
50. Shikita, M., Kakizaki, H., and Tamaoki, B., The pathway of formation of testosterone from 3β-hydroxypregn-5-en-20-one by rat testicular microsomes, *Steroids*, 1964, **4**, 521.
51. Shikita, M., and Tamaoki, B., Testosterone formation by subcellular particles of rat testes, *Endocrinology*, 1965, **76**, 563.
52. Toren, D., Menon, K. M. J., Forchielli, E., and Dorfman, R. I. *In vitro* enzymatic cleavage of the cholesterol side chain in rat testis preparations, *Steroids*, 1964, **3**, 381.
53. Tormey, J. M., Differences in membrane configuration between osmium tetroxide-fixed and glutaraldehyde-fixed ciliary epithelium, *J. Cell Biol.*, 1964, **23**, 658.
54. Tsai, S., Ying, B., and Gaylor, J. L., Testicular sterols. I. Incorporation of mevalonate and acetate into sterols by testicular tissue from rats, *Arch. Biochem. and Biophys.*, 1964, **105**, 329.
55. Volk, T. L., and Scarpelli, D. G., Alterations of fine structure of the rat adrenal cortex after the administration of triparanol, *Lab. Invest.*, 1964, **13**, 1205.
56. Watson, M. L., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 475.
57. Wattenberg, L. W., Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections, *J. Histochem. and Cytochem.*, 1958, **6**, 225.
58. Werbin, H., and Chaikoff, I. L., Utilization of adrenal gland cholesterol for synthesis of cortisol by the intact normal and the ACTH-treated guinea pig, *Arch. Biochem. and Biophys.*, 1961, **93**, 476.
59. Wolman, M., Histochemistry of lipids in pathology, *in* Handbuch der Histochemie, (W. Graumann and K. Neumann, editors), Stuttgart, Gustav Fischer, 1964, **5**, part 2.
60. Wood, R. L., and Luft, J. H., The influence of buffer systems on fixation with osmium tetroxide, *J. Ultrastruct. Research*, 1965, **12**, 22.
61. Yamada, E., and Ishikawa, T. M., The fine structure of the corpus luteum in the mouse ovary as revealed by electron microscopy, *Kyushu J. Med. Sc.*, 1960, **11**, 235.
62. Zelander, T., Ultrastructure of the mouse adrenal cortex. An electron microscopical study in intact and hydrocortisone-treated male adults, *J. Ultrastruct. Research*, suppl. 2, 1959.