ARTICLES

LOCALIZATION OF ACID PHOSPHATASE IN LIPOFUSCIN GRANULES AND POSSIBLE AUTOPHAGIC VACUOLES IN INTERSTITIAL CELLS OF THE GUINEA PIG TESTIS

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ABSTRACT

The intracellular localization of acid phosphatase in guinea pig testicular interstitial cells was investigated by incubating nonfrozen thick sections of glutaraldehyde-perfused testis in a modified Gomori medium and preparing the tissue for electron microscopy. Lipofuscin pigment granules in these cells contain dense pigment, granular matrix, and often a lipid droplet. Reaction product is seen in the matrix of the pigment granules, and they may therefore be called residual bodies. At least some of the dense pigment appears to be derived from myelin figures and membrane whorls, since suitable intermediates can be seen. Lipid droplets found free in the cytoplasm are another possible source of pigment. In both cases the chemical mechanism is presumed to be autoxidation of unsaturated lipid. Acid phosphatase is present in the inner cisterna of Golgi elements. Enzyme activity also appears in possible autophagic vacuoles bounded by double membranes; the reaction product lies between the membranes. Consideration of the enzyme as a tracer suggests that the autophagic vacuoles are derived from the Golgi complex. Possible stages in the formation of these vacuoles by the inner Golgi cisternae are observed.

INTRODUCTION

Lysosomal enzymes have been localized in the lipofuscin pigment granules of many cells by light microscope histochemistry (17). In 1961 Essner and Novikoff (15) first demonstrated acid phosphatase in the lipofuscin granules of liver by electron microscopy. Acid phosphatase has also been demonstrated at the electron microscope level in the lipofuscin of mouse mammary gland (29). The morphology of the pigment has been shown in electron micrographs of many cell types (2, 4, 18, 22, 23, 36, 41, 45), including human testicular interstitial cells (16). The cytochemical localization of acid phosphatase suggests that lipofuscin is a residue of lysosomal digestion. However, biochemical studies have failed to show acid phosphatase in lipofuscin, even though a few other hydrolytic enzymes have been demonstrated (see reference 19).

The prominent lipofuscin in steroid-secreting cells (4, 16, 45) (in unpublished observations on guinea pig corpus luteum by A. K. Christensen and on human corpus luteum by S. W. Gillim) has not yet been examined for lysosomal enzymes at the electron microscope level. Murakami (30) demon-

strated acid phosphatase in rat interstitial cells with the electron microscope, but these cells do not contain lipofuscin pigment. In a study of guinea pig testicular interstitial cells, Christensen (6) described lipofuscin granules that contain dense pigment, a granular matrix, and often a lipid droplet. They were called polymorphic residual bodies, on morphological grounds. In the present study we utilized recently developed methods in electron microscope cytochemistry to investigate acid phosphatase activity in guinea pig interstitial cells. Precise localization of enzyme was possible in the particularly well-organized lipofuscin granules of these cells. At the same time, we made observations on sites of enzyme activity in the Golgi complex and in possible early autophagic vacuoles.

MATERIALS AND METHODS

Testis material from English short-haired guinea pigs (550–1300 g) was prefixed by perfusion (6) with 1.4% distilled glutaraldehyde in 0.067 M cacodylate buffer (35) for ½–2½ hr. Whole testes were then left in fixative for an additional ½–1 hr. A solution of 25% glutaraldehyde was distilled at atmospheric pressure, as suggested by Smith and Farquhar (38), and a fraction collected at 101°–102°C was utilized for fixation without checking the pH. Tissue stored in the cold in 0.067 M cacodylate buffer for periods of up to 11 wk after perfusion showed no noticeable losses in enzyme content or structural integrity.

Thick sections of unfrozen testis material were then taken according to the method of Smith and Farquhar (37). Under optimal conditions we obtained sections varying from 50 to 100 μ although many thicker sections were incubated as well. The best sections were selected at various stages of the preparation, and this approach gave adequate results if appropriate rinses were used.

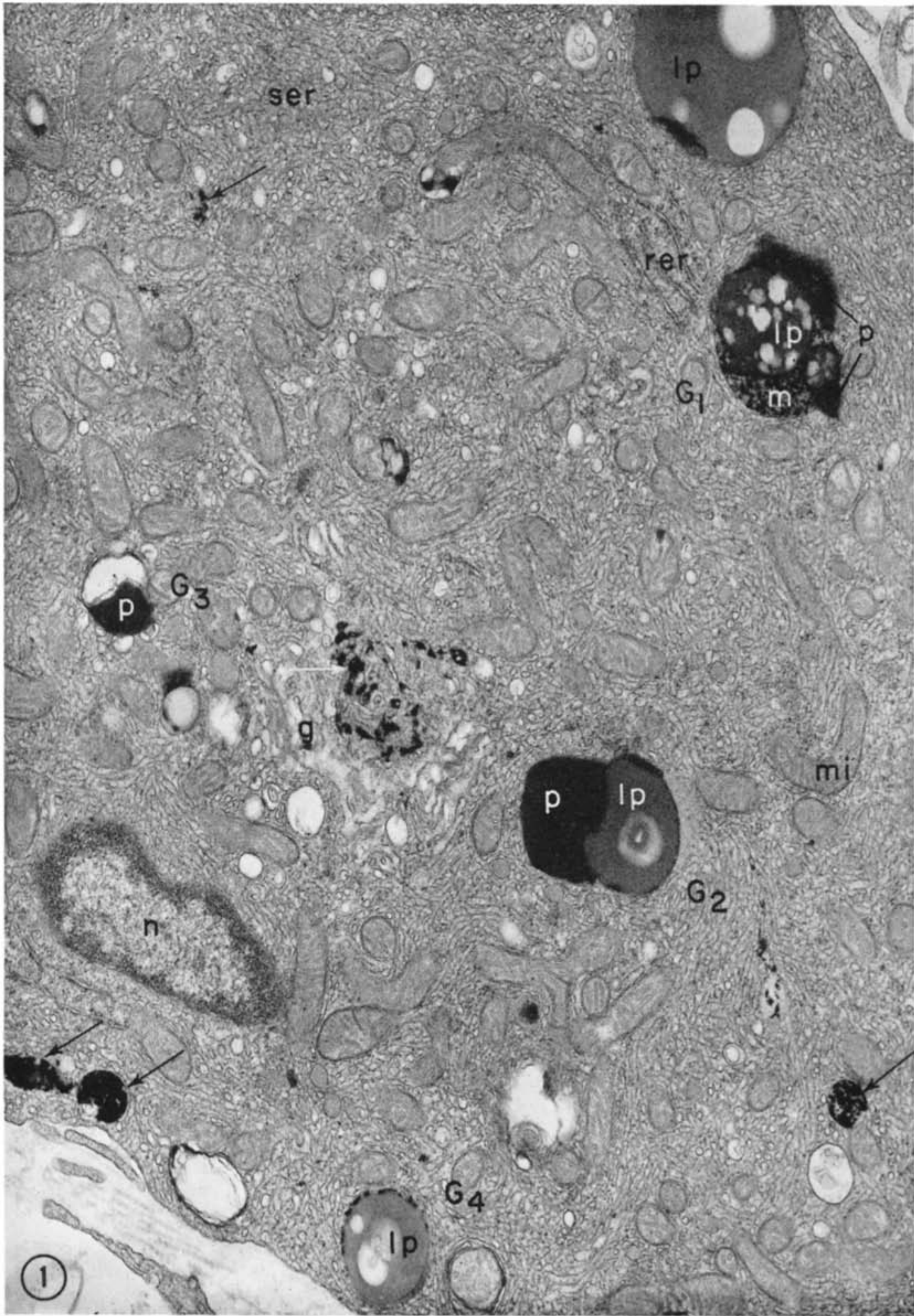
Sections were incubated 20 min–2 hr in Gomori's acid phosphatase medium, as modified by Barka and Anderson (1), prepared fresh each time. Control media were prepared by omitting substrate or by adding 0.01 M NaF to the incubation medium.

In accordance with the general procedure of Smith and Farquhar (38), incubated sections were washed three times in 0.05 M acetate–Veronal with 7% sucrose added (pH 7.4), postfixed in acetate–Veronal buffered osmium tetroxide (32) for 1 hr, placed in 0.5% uranyl acetate in Michaelis acetate–Veronal buffer (pH 5–6) for 1–2 hr, dehydrated, and embedded in Epon. Presumably the uranyl acetate staining solution also functioned as an acid rinse in removing nonspecific precipitate. In earlier, less satisfactory experiments, a collidine–sucrose rinse and collidine–buffered osmium tetroxide were used, with no uranyl acetate treatment in block (Figs. 8, 9 and 19).

Sections that had been stained in block with uranyl acetate were not stained further; others were stained with uranyl acetate (44) and lead citrate (43). Sections were viewed in an RCA EMU-3F electron microscope.

For histochemical identification of the pigment granules in interstitial cells, paraffin sections of glutaraldehyde-perfused testis were utilized for Sudan black B staining (24) and the Schmorl reaction (33) or ferric-ferricyanide reaction (24). Fresh-frozen sections of testis mounted in water were examined for fluorescence.

FIGURE 1 An area of cytoplasm of a guinea pig interstitial cell, incubated to show the location of acid phosphatase. A small portion of the nucleus (*n*) and a few cisternae of rough endoplasmic reticulum (*rer*) can be seen, although mitochondria (*mi*) and tubules of the smooth endoplasmic reticulum (*ser*) fill most of the cytoplasm. This figure shows deposition of reaction product in the lipofuscin pigment granules and in the Golgi complex, the two commonest sites of enzyme localization in these cells. One lipofuscin granule (G_1) has all three components that can appear in these bodies: lipid (*lp*), pigment (*p*), and granular matrix (*m*), which shows reaction product. Another granule (G_2) has only lipid and pigment. Some pigment appears alone (G_3). A lipid droplet shows a thin rim of reaction product (G_4) and is probably a section through a lipofuscin granule like that of Fig. 2. Several small bodies (black arrows) show reaction product. They may represent sections of lipofuscin granules or possibly lysosomes of another type. An oblique section of the Golgi complex (*g*) demonstrates the presence of acid phosphatase in this organelle (white arrow). × 21,000.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OBSERVATIONS

The fine structure of guinea pig interstitial cells has already been described (6). The lipofuscin granules in this study are like those in the earlier work except that the pale staining of the pigment after glutaraldehyde perfusion was not encountered here. In section, the lipofuscin granules can be seen as any combination of three components: lipid, dense pigment, and granular matrix (Figs. 1–4 and 6). All these images can be interpreted as various sections through a three-component granule, although granules with one or two components may exist. In this work, sections of granules showing only matrix and pigment (Fig. 3) appear a good deal more frequently than other possible sections; we suspect that granules lacking lipid occur commonly.

The use of a cytochemical procedure to demonstrate acid phosphatase in these cells did not adversely affect preservation of structure in the best experiments. No precipitate was found in cells that had been incubated in control media.

Acid phosphatase activity is present in the granular matrix of the lipofusion granules in guinea pig interstitial cells. In Figs 1–4 and 6, reaction product can be seen in nearly all the granules containing matrix. Some granules lack reaction product, even when adjacent structures show enzyme activity (Fig. 6). Possible explanations for this have been discussed by others (see reference 20). When reaction product is present in the granules, however, it is invariably located only in the matrix.

Membrane whorls and myelin figures are often seen in guinea pig interstitial cells and sometimes contain reaction product. They may appear alone in the cytoplasm or associated with a lipid droplet (Fig. 5). They also occur in the lipofuscin granules (Figs. 6, 7), and this may be the case even when it is not apparent in section.

Occasionally, dense granules are observed that appear to be conglomerates of compact membrane whorls (Fig. 8). They look less uniform than the dense pigment found in the more usual lipofuscin granules. However, portions of some of these compact whorls show a 70 A periodicity (Fig. 9), similar to that found in pigment of the three component granules: 75 A in the previous study (6) and 65 A as measured in the present study. Reaction product is often present but not prominent in the conglomerate granules. Again, it is possible that these conglomerate granules are merely special sections through three-component granules.

The inner cisterna on the concave face of Golgi elements frequently shows reaction product (Figs. 10, 11). Adjacent small vesicles, or sections of tubules or cisternae, are sometimes seen within the concavity of the Golgi elements and may contain reaction product (Fig. 11). In other cases the vesicles or the reaction product in them may be absent (Fig. 10).

On rare occasions the inner cisterna appears in section to be partially (Fig. 12, g_1) or completely (Figs. 12, g_2, and 13) surrounding a pocket of cytoplasm on the concave surface of the Golgi element. The double-membrane wall formed by the inner cisterna contains reaction product and may appear continuous (Fig. 12, g_1) or discontinuous (Fig. 13). The next innermost cisterna may show enzyme activity (Fig. 12) or darkened membranes (Fig. 13).

Acid phosphatase also occurs in infrequently seen double-walled structures that were not described in the earlier paper (6). An example in Fig. 14 shows a group of vesicles surrounded by a double membrane (arrow). Reaction product appears between the limiting membranes. Figs. 15 and 16 show similar double-walled bodies with complex contents. In one (Fig. 15), the double wall encloses a small pigment–matrix granule, while in the other (Fig. 16), there are two small bodies inside the large one that show the same double-walled structure (arrows) and enzyme localization. When these structures are seen in off-center sections, membranes are difficult to discern, although a heavy ring of reaction product is still noticeable (Fig. 17).

Without serial sections it is impossible to be certain whether the double-walled circular profiles seen in Figs. 12–17 actually represent closed vacuoles (see reference 27). The profiles in the region of the Golgi complex (Figs. 12 and 13) could represent unusual sections across a cup formed by the inner Golgi cisterna. This is a less likely interpretation of the circular profiles in Figs. 14–17, where no other part of the Golgi complex can be seen. If these circles were sections across an isolated cuplike structure, we would expect to see other possible sections of double-walled cups away from the Golgi complex. None were observed.

Occasionally, large (5–8 μ) inclusions showing reaction product on an area of what appears to be

cell debris are seen in sections of interstitial cells (Figs. 18–20). Interlocking cell processes are seen around each inclusion. The inclusion is separated from the interstitial cell cytoplasm by one or two membranes, or may appear to blend into the cytoplasm in some areas (Fig. 20), presumably because the section is oblique to the membrane in those regions.

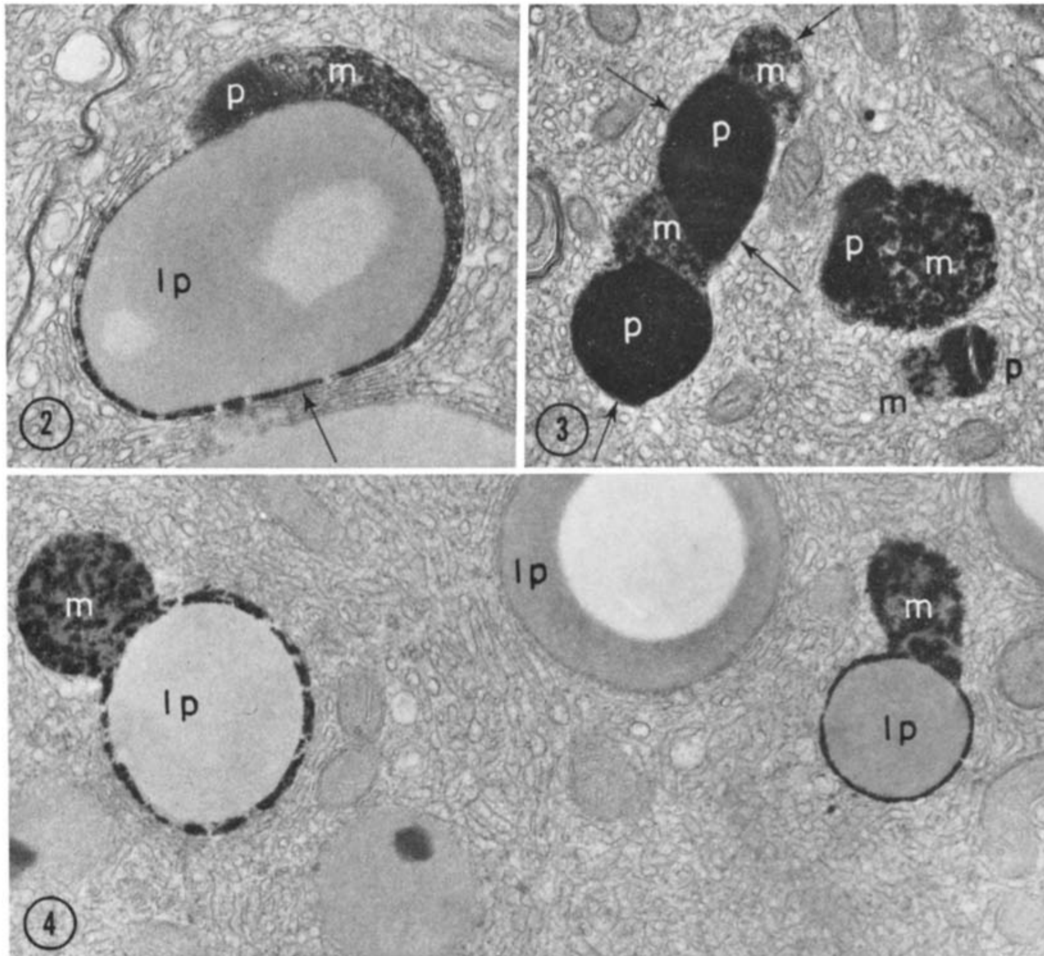


FIGURE 2 Another lipofuscin pigment granule with lipid (*lp*), pigment (*p*) and matrix (*m*) is shown here. The matrix, showing acid phosphatase activity, extends around the lipid droplet in a very thin layer (arrow). × 29,000.

FIGURE 3 Lipofuscin granules consisting of pigment (*p*) and matrix (*m*) only. Reaction product is seen in the matrix. The limiting membrane of the larger granule is seen at several points (arrows). This combination occurs so commonly in section that it is assumed that many pigment granules do not contain lipid. The pigment in the smallest granule has separated, showing the lamellar substructure (see Observations). × 22,000.

FIGURE 4 The granule on the right is made up of lipid (*lp*) and matrix (*m*) with reaction product. A free lipid droplet near the middle of the figure has been partially extracted. The granule on the left contains matrix showing enzyme activity and a light area that probably represents extracted lipid. In both granules, reaction product extends around the lipid droplet in a thin layer. × 28,000.

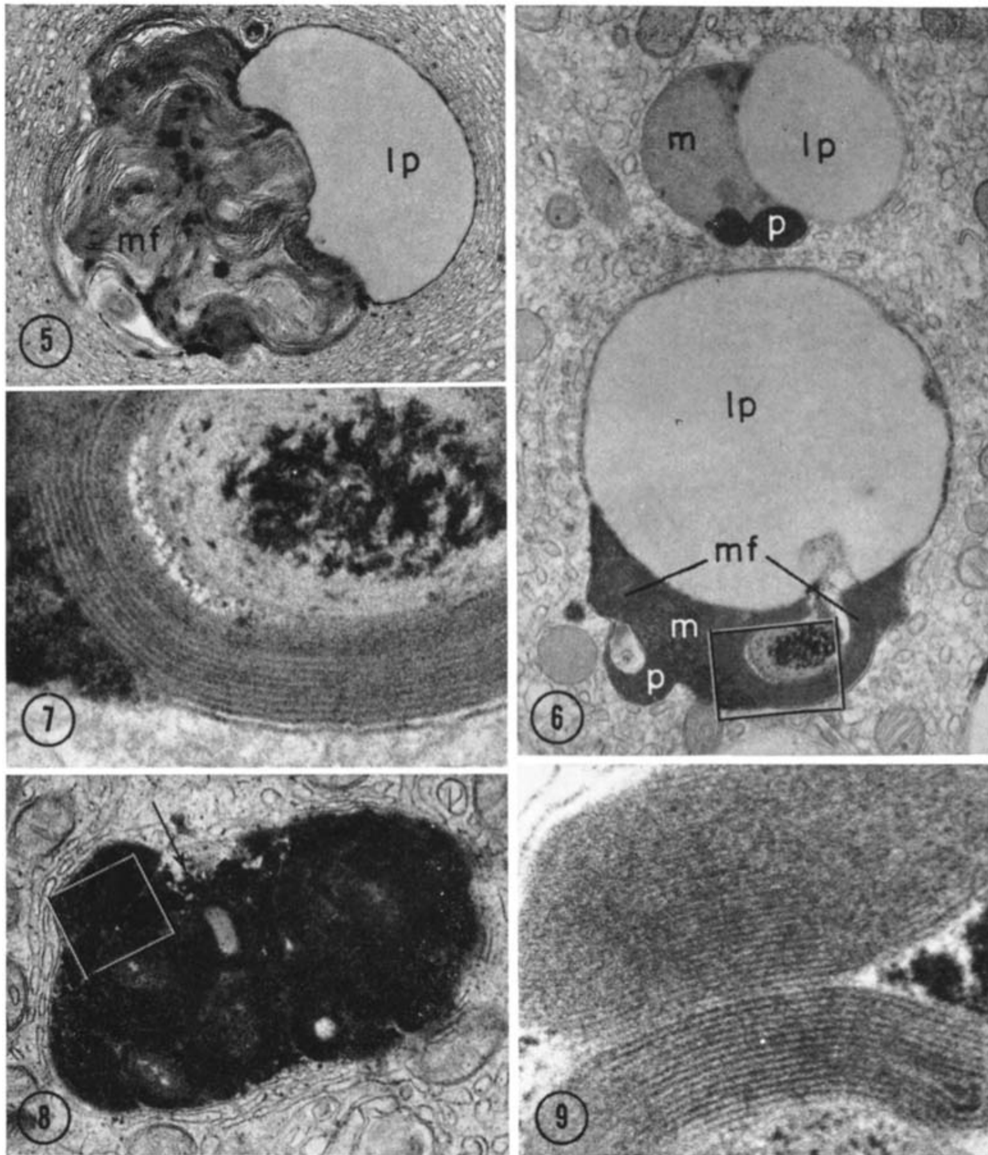


FIGURE 5 The large myelin figure (*mf*) or membrane whorl shown here is associated with a lipid droplet (*lp*). A small amount of reaction product is seen in the whorl. × 17,000.

FIGURE 6 Three-component lipofuscin granules. The larger one has enzyme activity in the matrix (*m*), but the other shows no reaction product. Juxtaposition of reactive and unreactive bodies was commonly observed in these experiments. In addition to dense pigment (*p*) and lipid (*lp*), the larger granule has myelin figures (*mf*) in the matrix. The area in the rectangle is enlarged in Fig. 7. × 23,000.

FIGURE 7 An enlargement of the portion of myelin figure outlined in Fig. 6. The periodicity of 115 A is seen more clearly. × 98,000.

FIGURE 8 This granule appears to be a conglomerate of dense membrane whorls. The material at the arrow may be reaction product, but is not conspicuous or typical in appearance. Other granules of this type had more obvious enzyme activity. Some parts of this granule look quite homogeneous. One of these areas (rectangle) is enlarged in Fig. 9. × 30,000.

FIGURE 9 An enlargement of the outlined area in Fig. 8 shows that the homogeneous portions of the conglomerate granule have a 70 A periodicity, similar to that seen in the pigment of the three-component granules (6). × 149,000.

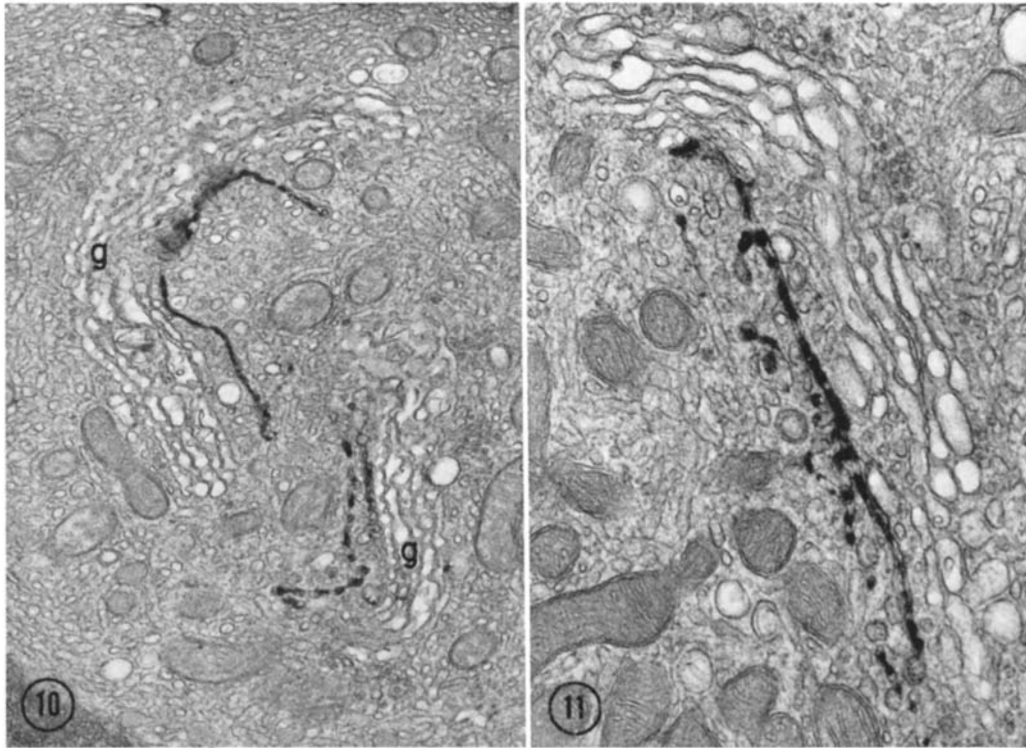


FIGURE 10 The two Golgi elements (g) in this figure contain enzyme activity in the inner cisterna. The smaller one also has enzyme in the next innermost cisterna. × 24,000.

FIGURE 11 This Golgi element at higher magnification shows reaction product in the inner cisterna. Some of the vesicles, or sections of tubules or cisternae, near the concave surface of the Golgi element also contain enzyme activity. × 30,000.

Under the light microscope, lipofuscin granules are difficult to see in frozen or paraffin embedded, unstained sections of glutaraldehyde-fixed material. Occasional light yellow granules are seen, but many granules must be colorless or very pale yellow. After Sudan black B staining of paraffin sections, from which the lipid has been extracted, sudanophilic granules can be observed in the interstitial cells. Neither the Schmorl nor ferric-ferricyanide reaction stain any granules in the testis, although colloid in rat thyroid, used here as a control, stains bright blue in the ferric-ferricyanide reaction. White to light-yellow fluorescent granules in interstitial cells can be seen in fresh frozen sections of testis mounted in water.

DISCUSSION

Lipofuscin is the term commonly used to describe a number of intracellular pigments that vary widely in histochemical characteristics and appear in both normal and pathological conditions in various tissues. Pearse (33) concludes from the histochemical data that the pigment results from the autoxidation of lipids, and that the variation arises from the different stages in this process. It has been known for some time that pigments similar to naturally occurring lipofuscin, which was isolated most recently by Bjorkerud (3), can be produced in vitro by the autoxidation of unsaturated lipids (5, 10). The process is thought to involve the formation of peroxy radicals which start a chain reaction that results in hydroperoxides, more radicals, and chain-terminating polymerization products (see reference 28). It is probable, therefore, that the production of the pigment in the cell is nonenzymatic, although it may be accelerated by hemoglobin, cytochrome C, or hemin (5, 39). Vitamin E prevents the peroxi-

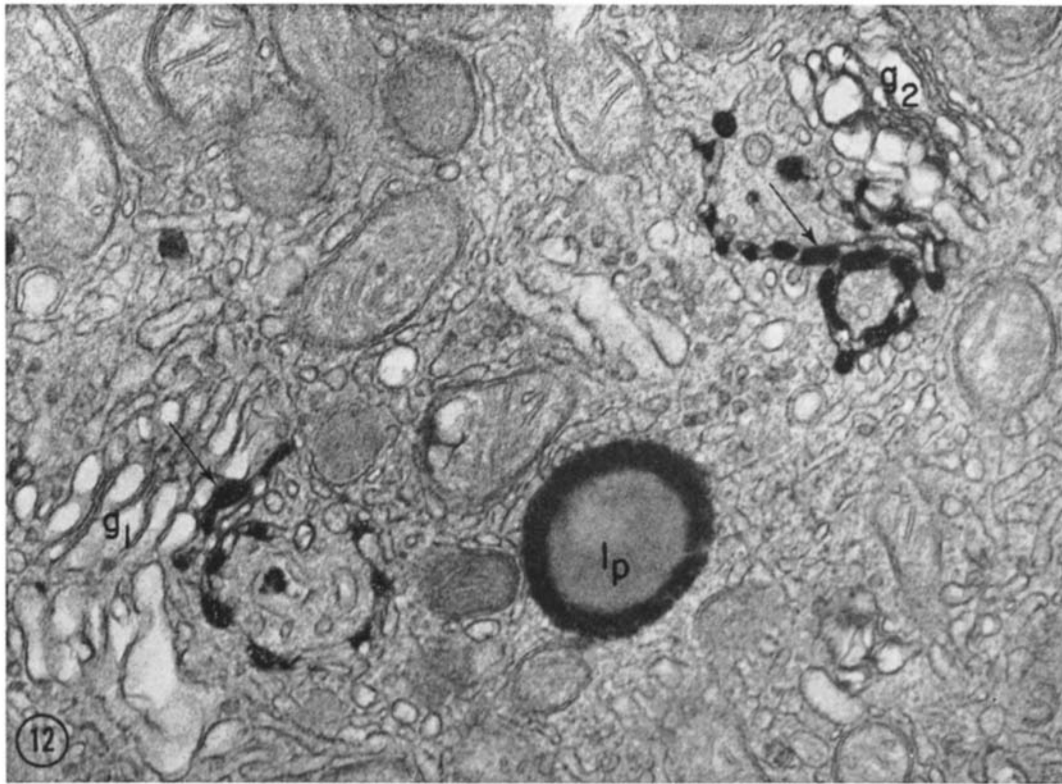


FIGURE 12 The inner cisterna of the two Golgi elements shown in this interstitial cell contain reaction product and appear to be partially (g_1) or completely (g_2) surrounding pockets of cytoplasm on the concave surfaces of the Golgi elements. The next innermost cisterna contains enzyme as well (arrows). Note that in g_2 this cisterna lies very close to the innermost. Also shown here is a lipid droplet with a rim of reaction product, perhaps an off-center section of the type of granule seen in Fig. 2. × 34,000.

dation of unsaturated lipids (see reference 40), and animals deprived of vitamin E develop a lipofuscin pigment in many tissues (25, 26).

Before the significance of lipofuscin can be completely understood, its composition and the reactions and conditions that produce it must be established. Further, it will be necessary to determine the origin of the unsaturated lipid, the location of the reactions, the manner in which the cell handles the pigment during and after its production, and the effects of the pigment on the cell's other functions.

Cytochemical studies at the light (17) and electron microscope (15, 29) levels have shown lipofuscin to be associated with acid phosphatase and other lysosomal enzymes. This had led to a lysosomal theory of the production, handling, and lipid source of the pigment. The present study confirms that the pigment occurs in membrane-bounded, acid phosphatase–positive bodies, or lysosomes. If myelin figures, pigment, lipid droplets, or all of these are considered to be residues of intracellular digestion, the lipofuscin granules may be called residual bodies. In addition, we have given further support to Essner and Novikoff's demonstration in the liver (15) that the enzyme is not in the dense pigment itself, but is restricted to the accompanying matrix. If this is true in other cell types, it would tend to explain the dissociation of the pigment from the enzyme in biochemical fractionation studies (see reference 19).

According to de Duve and Wattiaux (8), residual bodies containing pigment result from the digestion of cell constituents that have been engulfed into an autophagic vacuole. Because of the short supply or slow action of lipolytic enzymes in

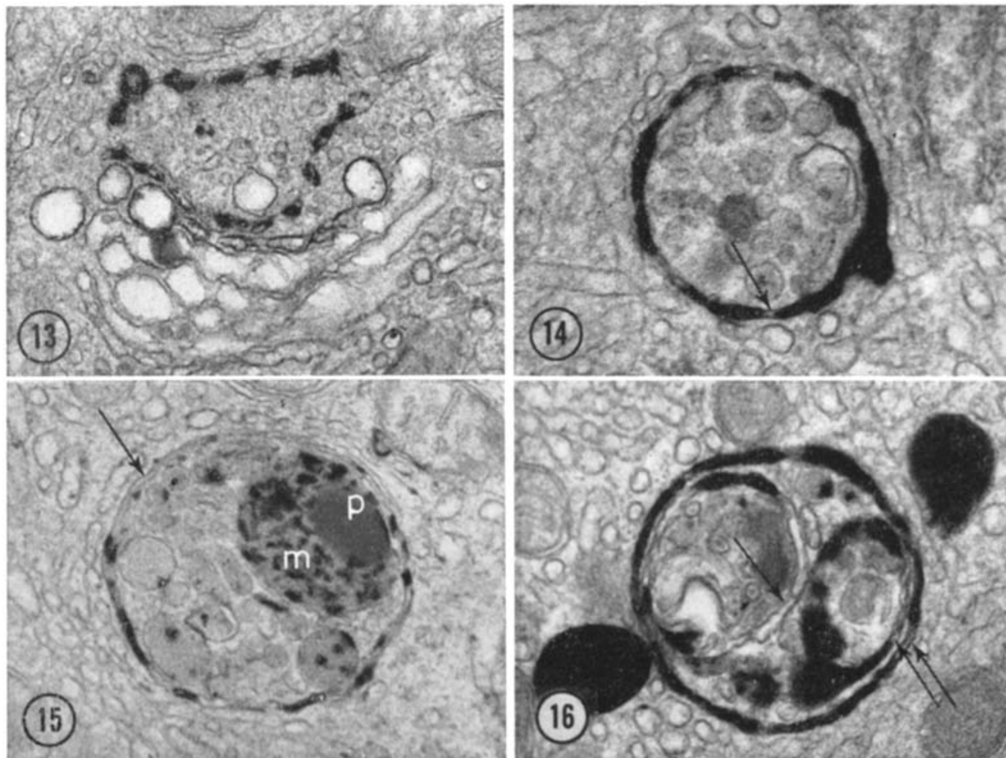


FIGURE 13 The inner cisterna appears to have formed a wall around a portion of cytoplasm on the concave surface of this Golgi element. However, the wall does not look continuous. The next innermost cisterna has darkened membranes. × 41,000.

Figs. 14–17 show double-membrane-bounded bodies that look like early autophagic vacuoles. Such bodies could result from the engulfment of an area of cytoplasm by the inner Golgi cisterna.

FIGURE 14 This vacuole, containing smaller structures, is bounded by two membranes (arrow) that have reaction product between them. × 50,000.

FIGURE 15 A double membrane (arrow) encloses a lipofuscin granule with pigment (*p*) and matrix (*m*) as well as vesicles of various sizes. The double-membrane wall, the matrix material, and several small vesicles all show reaction product. × 45,000.

FIGURE 16 In this case, a double membrane forms a vacuole that has two similar but smaller double-walled structures within it. All the double membranes (arrows) show reaction product. × 39,000.

lysosomes, lipid material accumulates, and there is a progressive change of the lipid into pigment substance which merges to form the visible granule. Peroxidation of cell lipids has been found to be an early event in some kinds of cell injury (7, 34, 40), and so the early reactions in pigment formation may occur before autophagy and hinder lysosomal digestion.

The lipids that de Duve and Wattiaux implicate as the source of lipofuscin are thought to be found in the membranous arrays and whorls commonly seen in the residual bodies of cells that contain lipofuscin. In guinea pig interstitial cells such membranous arrays do appear, sometimes in residual bodies. The conglomerate granules look as if they are derived from membrane whorls. It is possible that these granules are a stage in the formation of the more typical homogeneous pigment, particularly considering the 65–75 A periodicity that can be found in both places. Thus, these ob-

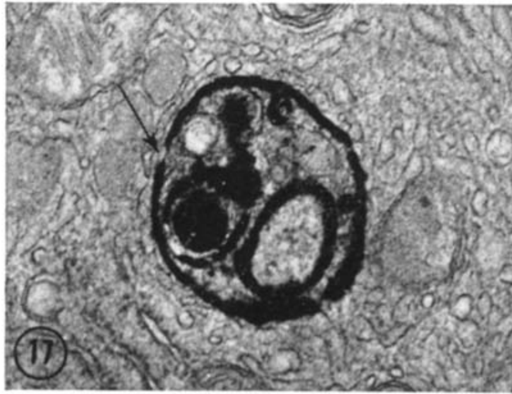


FIGURE 17 A vacuole like the one in Fig. 15 in somewhat off-center section. Double membranes are seen at one point (arrow). × 45,000.

servations would be consistent with de Duve and Wattiaux's scheme.

The frequent presence of lipid droplets within the lipofuscin pigment granules suggests the additional possibility that pigment is forming from unsaturated lipid in the droplet. Clearly, one source of lipofuscin does not preclude the other. Other workers have also shown lipofuscin granules containing lipid droplets (4, 29, 36, 41, 45). Endicott and Lillie (11) observed ceroid, now considered a form of lipofuscin, at the edges of coarse lipid droplets that arose in the livers of rats with dietary cirrhosis. They suggested that at least part of the pigment derived from the lipid droplets. In vitro, the pigment arises at the lipid–water interface of small lipid droplets in emulsions (5, 10). This might also be expected to occur inside the cell. In pigment derived from lipid droplets we would suppose any periodicity to be characteristic of the pigment itself rather than a remnant of membrane structure.

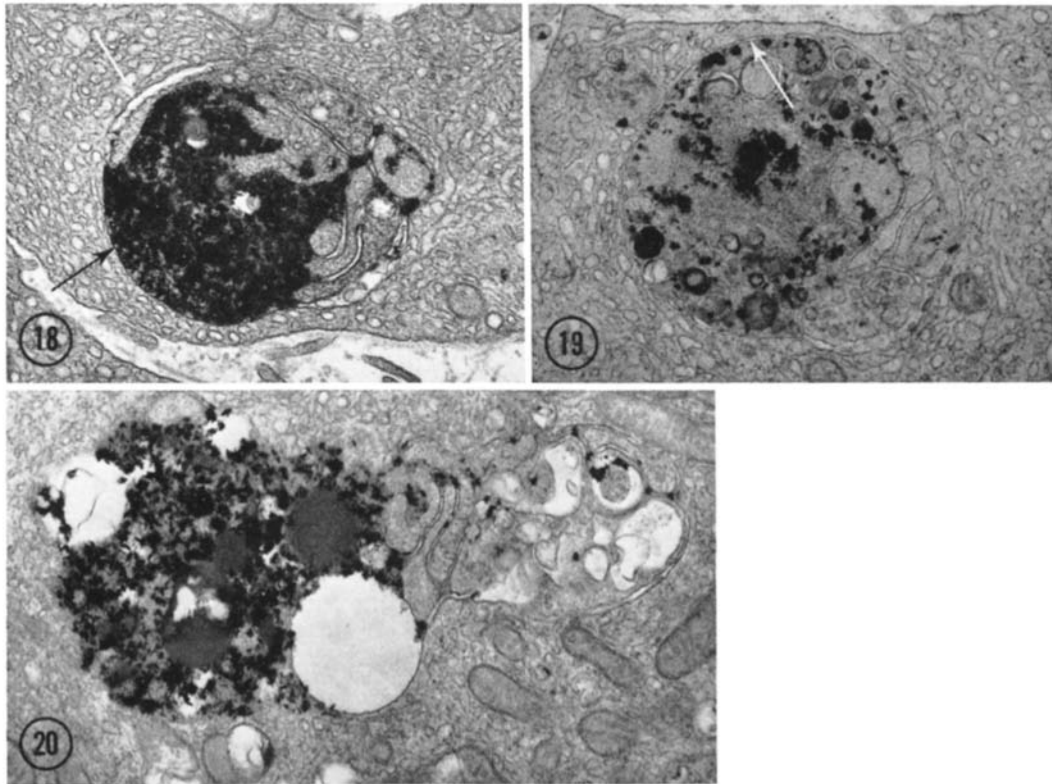
In guinea pig interstitial cells the lipid droplets in lipofuscin granules could be remnants of membrane digestion in autophagic vacuoles. We consider it more likely, however, that they result from autophagy of the lipid droplets that appear free in the cytoplasm, since the phospholipids from degenerating membranes are usually thought to form myelin figures. Peroxides and pigment may appear before or after the droplet is engulfed. Perhaps autophagy protects the cell from damaging peroxides or pigment that arise in the free lipid droplets.

The great majority of the acid phosphatase-positive bodies seen in this study can be interpreted as sections through the lipofuscin granules. The only significant exception we have observed were the double-walled vacuoles. These might represent an early stage in the autophagic and digestive process that presumably gives rise to the numerous residual bodies. Although the problem of sectioning artifact (see Observations) precludes any final conclusions about these early autophagic vacuoles or their mode of formation, our observations suggest one possibility.

In guinea pig interstitial cells the inner cisterna on the concave face of Golgi elements appears to engulf portions of the cytoplasm and to form a vacuole surrounded by a double membrane. Figs. 12 and 13 show what could well be stages in such a process. The autophagic vacuoles that would result are shown in Figs. 14–17. Presumably the small vesicles (Fig. 14), pigment granules (Fig. 15) or other contents of the autophagic vacuoles represent portions of cytoplasm that were near the inner cisterna. In Fig. 12 (g_2), the second innermost cisterna could subsequently from an autophagic vacuole enclosing the one already formed by the inner cisterna. This would result in a vacuole resembling the ones shown in Figs. 16 and 17 that enclose smaller autophagic vacuoles.

The inner Golgi cisterna often contains reaction product in these cells, and reaction product can also be seen in the "engulfing" cisternae and in autophagic vacuoles. The presence of the enzyme identifies the double-walled vacuoles as a kind of lysosome and, viewed as a tracer, supports the idea that these bodies derive from the Golgi complex. Dissolution of the inner membrane of the autophagic vacuole would release lysosomal enzymes into the vesicle.

Early autophagic vacuoles, or cytosegresomes, with double membranes have been frequently observed by other authors (12, 14, 27, 31, 42; also see reference 8), but the origin of the membranes is still in doubt. Several authors have included the Golgi membranes among the logical candidates (14, 31). There are indications that the smooth or rough endoplasmic reticulum sequesters areas of cytoplasm in some cell types, and several other mechanisms have been proposed as well (see reference 8). The rounding up of inner Golgi cisternae in the proximal tubule cells of the kidney was mentioned by Ericsson and Trump (13). A similar configuration of double membranes very close to a Golgi element was recently observed in rat nodosal ganglion cells (Fig. 29 in reference 21),



FIGURES 18–20 These figures show three examples of the large inclusions occasionally found in interstitial cells. They show reaction product (especially abundant in Fig. 18) and appear to be made up of cell debris partially surrounded by cell processes. They are separated from the interstitial cell cytoplasm by one (black arrows) or two (white arrows) membranes, or may appear to blend into the cytoplasm in some areas (Fig. 20). The nature and significance of these inclusions are unknown. Fig. 18, × 23,000; Fig. 19, × 17,000; Fig. 20, × 21,000.

and the membranes were interpreted as smooth endoplasmic reticulum. In our micrographs, the acid phosphatase–positive cisternae in Figs. 10–13 appear to be Golgi cisternae by the usual morphological criteria, and we see no reason in this case to suppose that they are smooth endoplasmic reticulum.

In the tubules of mouse testes, Dietert showed groups of acid phosphatase–positive Golgi cisternae forming autophagic vacuoles in the residual cytoplasmic bodies (9). We feel that the findings we have presented here furnish additional evidence of direct participation of the Golgi complex, especially the inner cisterna, in the autophagic processes of cells.

The large inclusions seen occasionally (Figs. 18–20) are difficult to interpret. They could be intrusions of macrophage cytoplasm indenting the interstitial cells. Macrophages show heavy and diffuse reaction product with the incubation times used in this study. This would be an attractive possibility if the inclusions were always bounded by two membranes. However, the frequent presence of single membranes suggests that the material may actually be extracellular.

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