

Organelle Relationships in Cultured 3T3-L1 Preadipocytes

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ABSTRACT In differentiating 3T3-L1 cells, lipid spheres, the endoplasmic reticulum (ER), microperoxisomes, and mitochondria form "constellations" that may reflect the interplay of lipid metabolizing enzymes in these organelles. ER cisternae are also situated very close to "rosettes," plasmalemmal specializations found in mature adipocytes *in vivo*. As in hepatocytes and absorptive cells of the intestine, this spatial relationship of ER and plasmalemma suggests a role for rosettes in the uptake of exogenous lipid precursors. The morphological differentiation of 3T3-L1 preadipocytes includes the loss of "stress fibers" and the appearance of microfilament like structures that encase, in a complex manner, the cytosolic lipid spheres that appear during differentiation. Other features described for the first time in 3T3-L1 preadipocytes include: (a) the presence of an extensive acid phosphatase (AcPase) positive GERL from which coated vesicles apparently arise (these coated vesicles display AcPase activity and are much smaller and far more numerous than the coated vesicles that seem to arise from the plasmalemmal coated pits); (b) the abundance of AcPase-positive autophagic vacuoles; and (c) a high level of α -naphthyl-acetate-esterase activity which, by light microscopy cytochemistry, appears to be localized in the cytosol.

Green and colleagues (20–23) established several cloned lines from disaggregated Swiss mouse embryos (80) that are capable of differentiating into adipocytelike cells (see reference 19 for review). During the course of differentiation, activities of enzymes involved in triacylglycerol biosynthesis increased 50- to 100-fold (8, 34, 39), and the cells became responsive to β -adrenergic agonists, ACTH (72), and insulin (22, 71). For these reasons, this cell line is being used as a model for investigations on mammalian adipogenesis and the structure and function of adipocyte organelles.

The cytology of the adipocyte has been reviewed often; relatively recent reviews are those of Slavin (77) and of Heindl et al. (27). A major problem in studying the mature adipocyte has been the difficulty of maintaining the structural integrity of cell organelles during fixation and subsequent processing for microscopy. Cultured 3T3-L1 cells possess many of the biochemical characteristics of mature adipocytes and are eminently suited for both light and electron microscope studies because they are easily preserved and because enzyme cytochemistry permits visualization of cell organelles that may be involved in the movement and metabolism of lipid. The observations presented in this communication document the cytological features of cultured 3T3-L1 preadipocytes and provide a basis from which one can begin to study the biochemical functions of adipocyte organelles.

MATERIALS AND METHODS

Culture conditions for the growth of 3T3-L1 cells in our laboratory have been previously described (72). Cells were originally provided by Dr. Howard Green. Differentiation into adipocytelike cells was stimulated by 48-h treatment with 0.25 μ M dexamethasone and 0.5 mM methylisobutylxanthine (71). As assessed by phase-contrast microscopy, differentiating cells (4–5 d posttreatment) grown on moderately carbon-coated No. 2 coverslips (sterilized by dry heat [200°C overnight]) were morphologically indistinguishable from cells grown in the usual manner on 100-mm Falcon culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). All observations were made on cells grown on such coverslips.

After culturing, coverslips were transferred with flattened forceps to solutions contained in Columbia staining jars (VWR Scientific Inc., San Francisco, Calif.). They were first rinsed briefly in Earle's modified Eagle's Minimal Essential Medium without serum, at room temperature, and then transferred to fixatives as follows.

For Light Microscopy Only

Cells were fixed in 4% formaldehyde (freshly prepared from paraformaldehyde)-0.1 M cacodylate buffer, pH 7.4, containing 5% sucrose, for 10 min at room temperature for the periodic acid-Schiff (PAS)-diastase procedure for glycogen (37) and for 6 min at 4°C for the nucleoside diphosphate (NDPase) activity procedure used to visualize the Golgi apparatus (48).

For staining of lipid spheres by Oil red O in 60% triethylphosphate cells were fixed in Karnovsky's aldehyde mixture (32), as modified by Miller and Herzog (43) (2% formaldehyde, 2.5% glutaraldehyde, and 0.025% CaCl₂ in 0.09 M cacodylate buffer, pH 7.2, containing 5% sucrose) at 4°C for 60 min at room temperature (17). For the determination of α -naphthyl-acetate-esterase activity

by the procedure described by Barka and Anderson (4) fixation was for 6 min at 37°C.

For Unincubated Cells for Electron Microscopy Only

Cells were fixed in a mixture of glutaraldehyde and OsO₄ at 4°C, as prepared by Hirsch and Fedorko (28), for 30 min. After rinsing in 7.5% sucrose at 4°C, the coverslips were treated in uranyl acetate at pH 5.0 (12, 33) for 30 min and processed as described by Robbins and Jentsch (68). After polymerization, the BEEM capsules (Better Equipment for Electron Microscopy, Bronx, N. Y.) were snapped off the coverslips. Thin sections were prepared from the Epon blocks with diamond knives (E. I. DuPont de Nemours & Co., Wilmington, Del.) on the LKB Ultratome II (LKB Instruments, Inc., Rockville, Md.), some through the depth of the cells and others parallel to the carbon surface. Only lead staining of thin sections (67) was employed. Microscopy was performed with the Philips 300 electron microscope with a 30- μ m objective aperture.

When measurements were being made, the electron microscope was calibrated with a germanium-shadowed carbon-replica diffraction grating with 54,864 lines/inch (Ladd Research Industries, Inc., Burlington, Vt.).

For Light and Electron Microscopic Enzyme Cytochemistry

Fixation was carried out in Miller and Herzog fixative at 4°C for 6 min. After rinsing in 7.5% sucrose at 4°C, the coverslips were incubated in the following media at 37°C: (a) for NDPase activity, as described by Novikoff and Goldfischer (48); (b) for AcPase activity by the method of Novikoff (46) utilizing cytidine-5'-monophosphate as described elsewhere (52); and (c) for catalase by the procedure of Novikoff et al. (53).

The coverslips used for electron microscopy were then rinsed in sucrose, treated in uranyl acetate, processed, and studied as described above.

RESULTS AND DISCUSSION

Most observations were made on differentiating cells, either 4 or 5 d after the 48-h treatment with dexamethasone and methylisobutylxanthine (71). At this stage, the cells appear rounded and possess numerous large cytosolic lipid spheres as revealed by Oil red O staining (Fig. 1 *b*). The small size of lipid spheres in the undifferentiated cells is illustrated in Fig. 1 *a*.

The lipid spheres of the differentiating cells are generally electron lucent with the procedures we have used for electron microscopy (Figs. 2, 3, 6–11, 13, 14, and 19–21). Occasionally, part of the lipid may appear electron opaque (Fig. 5). At higher magnifications the limits of the lipid spheres are sometimes electron opaque (Figs. 8, 11, 19, and 20).

Cytosolic Lipid Spheres, Endoplasmic Reticulum (ER), and Related Organelles

Incubation of differentiating 3T3-L1 cells to reveal sites of NDPase activity (similar localizations are obtained with TPP or IDP as substrate) makes the ER unmistakable (Figs. 2, 3, and 13). Once this identification is made in incubated cells, recognition by fine structure alone is possible in unincubated cells (Figs. 8–11 and 19–21) or in cells incubated for sites of catalase (Figs. 6 and 7). In the differentiating cells, it is possible to see ribosomes clearly. Thus, rough ER and smooth ER can be discriminated. In some instances, individual glycogen particles may be confused with ribosomes. Generally, however, the distinction is clear. Ribosomes possess a higher electron opacity and are better delineated than glycogen particles. Ribosomes are most easily seen when the glycogen is either not present or not preserved, as in Fig. 11.

The ER encases each lipid sphere of the differentiating cell over most of its surface. Because the cells have been fixed, it is not known whether the lipid surface is separated from the ER

in intact cells. Where osmium tetroxide–glutaraldehyde was the primary fixative (Figs. 8–11 and 19–21), an area of cytosol, ~160 Å thick, separates lipid surface and ER. Microfilamentlike structures are often seen between the ER and lipid surface (see below).

Constellations consisting of: (a) a cytosolic lipid sphere; (b) ER, which encases the sphere to varying degrees; (c) mitochondria; and (d) microperoxisomes are seen in the differentiating 3T3-L1 cells. These constellations are similar to those described by Novikoff (55) in hepatocytes, except that in these latter cells they contain peroxisomes instead of anucleoid microperoxisomes (51, 58).

As with the ER, once cytochemistry reveals the microperoxisomes (Figs. 1 *g* and 5–7) these organelles are readily identified by their morphology in unincubated cells (Figs. 8 and 19–21) or in NDPase-incubated cells (Figs. 2 and 3). These organelles are more numerous in differentiating 3T3-L1 cells than in any cell type thus far studied; the greatest number of microperoxisomes encountered is shown in Fig. 7. Because they are numerous and lie close to each other, superimposed microperoxisomes and superimposed reaction product when incubated in the alkaline 3,3'-diaminobenzidine (DAB) medium are included in a 2- μ m-thick section (Fig. 1 *g*).

In differentiating 3T3-L1 cells, as in other cells (51), microperoxisomes lack nucleoids, are small (they range from 0.05 to 0.25 μ m in length and are ~0.1 μ m wide in the 3T3-L1 cells), and show contiguities (probably continuities) with the ER (Figs. 6 and 7). Most of them are elongated, but horse-shoe-shaped microperoxisomes are seen fairly frequently (Fig. 6).

Mitochondria are recognized by their well-known fine structure, including an outer tripartite membrane and the shelflike extensions (cristae) of the inner membrane (Figs. 2, 3, 5–9, 11, 13, and 19–21). They lie close to the lipid spheres, as was demonstrated by Palade in 1959 (61).

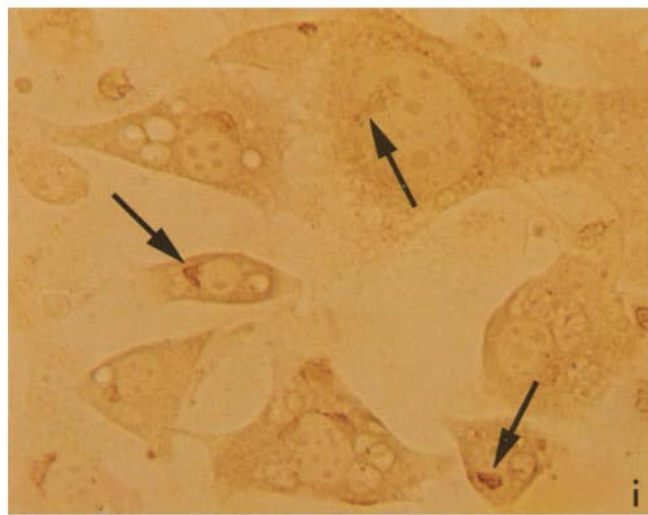
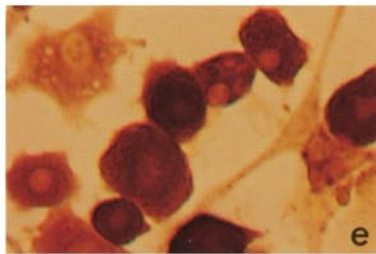
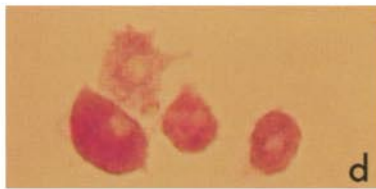
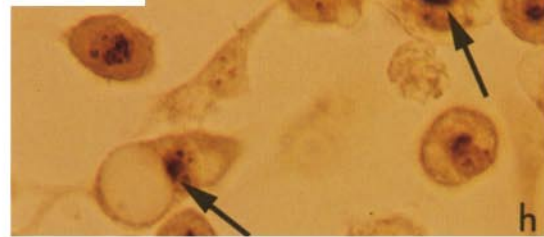
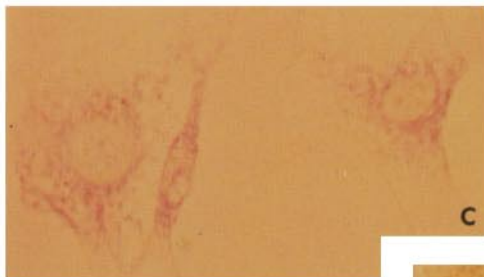
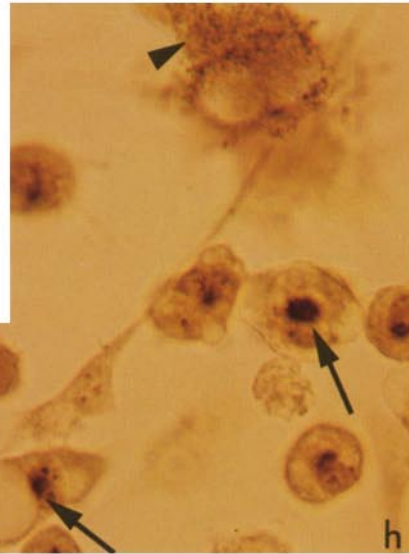
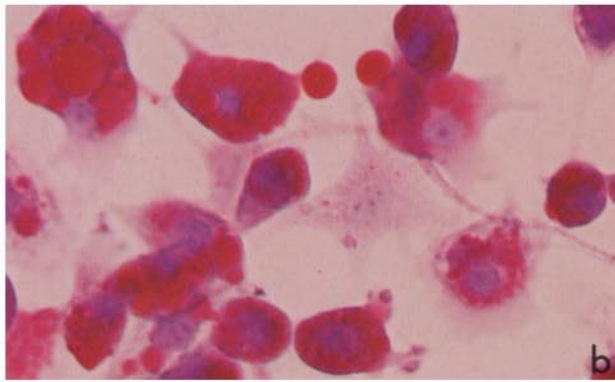
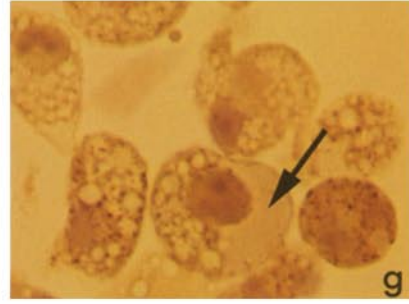
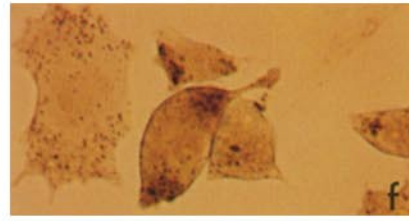
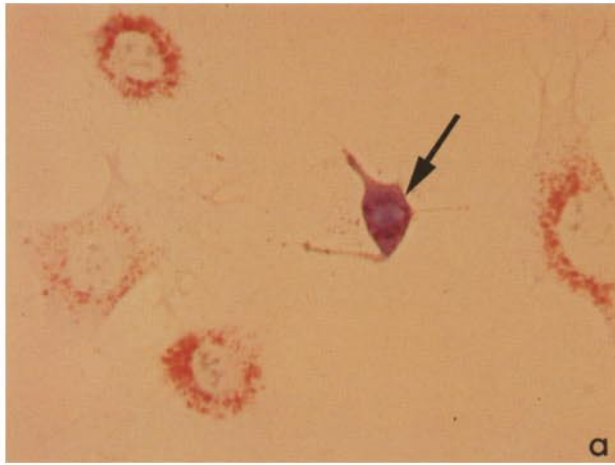
Plasmalemmal Rosettes and ER; is the ER Polarized in its Distribution?

Differentiating 3T3-L1 cells possess numerous rosettes, specialized structures characteristic of adipocyte *in vivo*, first seen in 1962 by Sheldon et al. (74) and described more fully and named by Williamson in 1964 (81). These rosettes are complex three-dimensional arrangements of vesicles, ~700 Å in diameter, opening to invaginations of the plasmalemma (Figs. 2–4 and 9). Their three-dimensional structure is best suggested by Fig. 4 (to the right of the rosette marked with an *x*). The plasmalemma invaginations are best seen in Fig. 3.

In the preceding section, the intimate spatial relationship between the ER cisternae and the cytosolic lipid spheres was described. The ER cisternae are also closely spatially related to the plasmalemmal rosettes (Figs. 3, 4, 9, and 11). The ribosome appearances suggest that “degranulation” (50) of rough ER, to become smooth ER, occurs at the ends of the cisternae adjacent to the rosettes and those adjacent to the cytosolic lipid spheres.

Evidence was sought for a polarized arrangement of the ER, as is found, e.g., in hepatocytes and absorptive intestinal cells of mammals. NDPase-incubated cells were sectioned through their depth (i.e., perpendicular to the cells on the coverslip), parallel to their surface, and at random. No evidence for such polarity is evident in any plane of section.

The widespread distribution of the ER cisternae in the cytoplasm and the relations of these cisternae to other cell organelles have been little appreciated in adipocytes. As late as



1974 Hales et al. (26) had to call attention to the fact that "the cytoplasm of the rat isolated fat cell contains a highly organized, interconnected system of smooth endoplasmic reticulum having close association with the central lipid mass, mitochondria, and cytoplasmic lipid droplets".¹ However, these authors paid little attention to the plasmalemmal vesicles. Undifferentiated 3T3-L1 cells show plasmalemmal vesicles (Fig. 17) similar to those in many cultured mammalian cells. These include endothelial cells (e.g., reference 76 and personal communication), smooth muscle cells (e.g., reference 70), and fibroblasts (e.g., reference 69; but see reference 6). In contrast, the plasmalemmal vesicles of the differentiating 3T3-L1 cells are arranged in a complex three-dimensional manner around invaginations of the plasmalemma, as in the rosettes of adipocytes. Madison et al. (40) describe similar structures in mesothelial cells of mouse omentum. Williamson (81), who coined the term rosettes for the complex structural arrangement of plasmalemmal vesicles of adipocytes, considered it likely that the rosettes arose when fatty acids, complexed with protein, left the adipocyte; he did not, however, rule out a role for rosettes in pinocytosis of lipid moieties. Jarett and Smith (31) reported electron microscope observations on the binding of ferritin-insulin complexes to the plasmalemma of the rosettes, or what they prefer to call "surface connected vesicles." They concluded that the vesicles are "contiguous with, and do not separate from, the plasma membrane and are certainly not moved back and forth across the cytoplasm." Our observations on the 3T3-L1 cells are consistent with their conclusion. There is no suggestion, in our numerous micrographs, of small vesicles moving away from or toward the plasmalemma. However, our observations should be reinforced by exposing the cells to media containing electron-opaque tracers such as those used by De Bruyn et al. (10) and Farquhar (11).

The demonstration that cisternae of ER are intimately related spatially, both to the plasmalemmal surface and to the surface of the cytosolic lipid spheres, suggests that the ER of

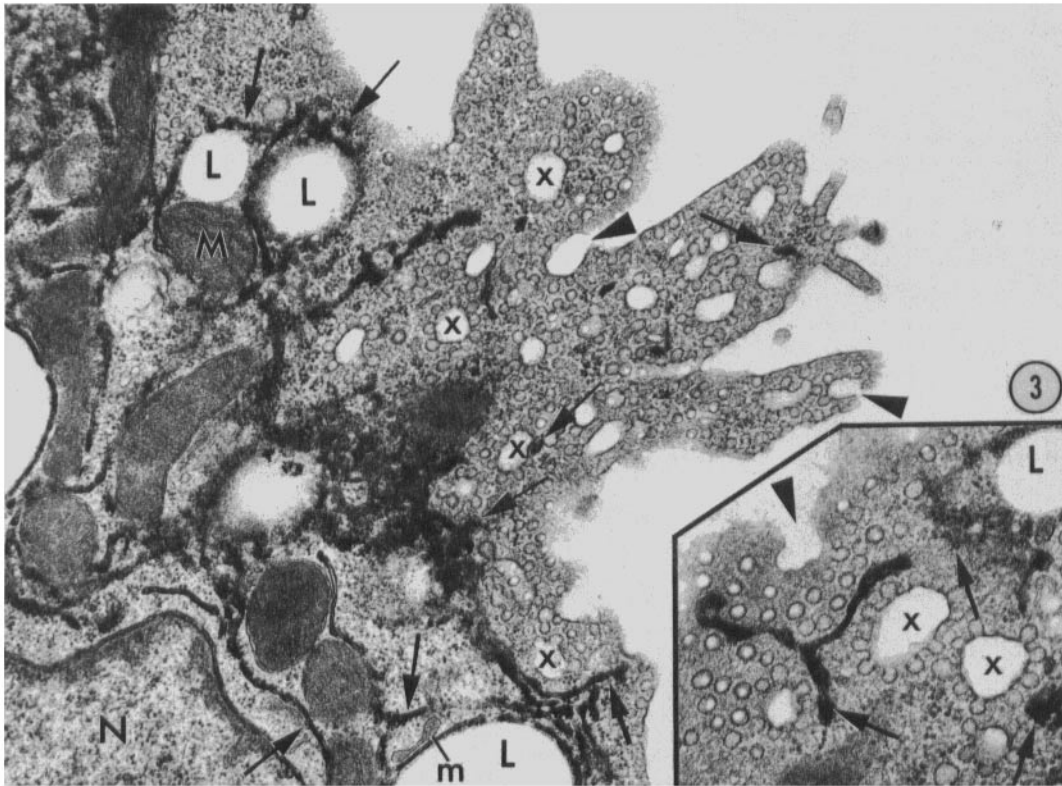
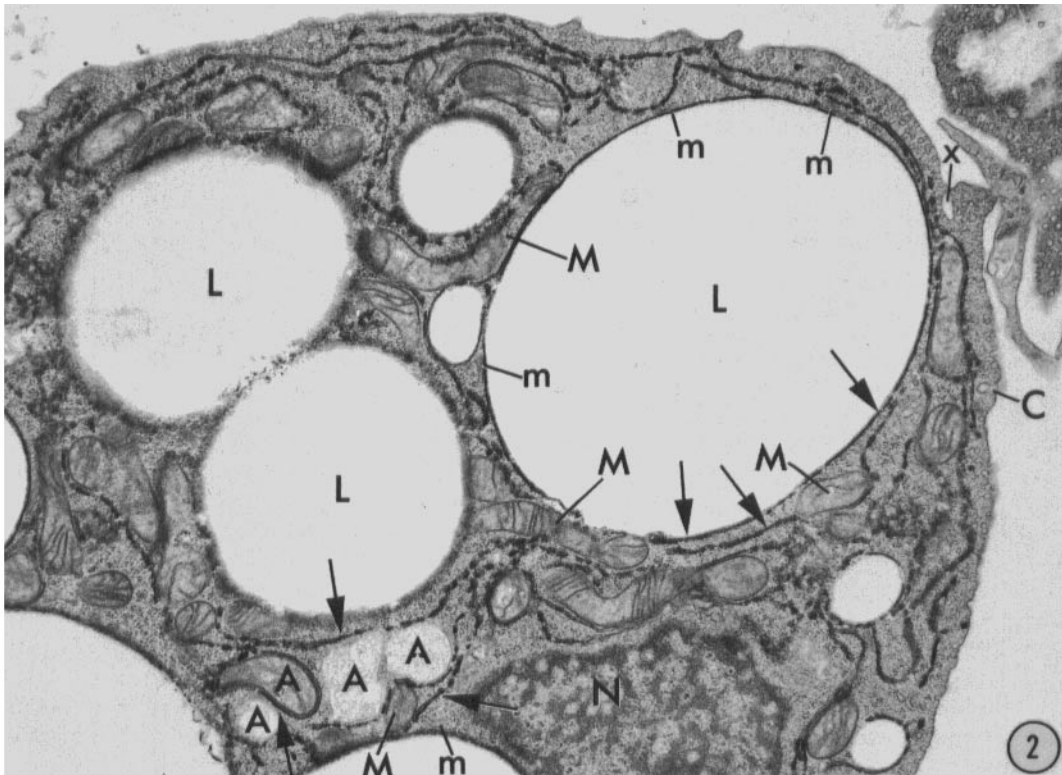
¹ It is likely that microperoxisomes were overlooked because DAB incubations for catalase were not performed (Dr. L. Herman, personal communication). In brown fat, mitochondria and catalase-positive microperoxisomes have been described on the surfaces of lipid spheres (63).

differentiating 3T3-L1 cells may function in lipid metabolism and transport as it does in mammalian hepatocytes. The evidence for the uptake of fatty acids at the hepatocyte plasmalemma and their movement into the ER, where they are esterified, is reviewed by Novikoff and Edelman (56). This study demonstrated the morphologic relation of the ER to cytosolic lipid spheres as the spheres are either deposited (fatty liver formation) or removed (reversal of fatty liver); and also the constellations of cytosolic lipid spheres, ER cisternae, mitochondria, and peroxisomes.

Because there is now evidence that not only the ER and mitochondria but also the peroxisomes possess enzymes of lipid metabolism (16, 36, 41, 42), it is important that constellations similar to those found in hepatocytes are striking features of differentiating 3T3-L1 cells. Recent results of Hajra et al. (25) demonstrate that peroxisomes of rat liver participate in the biosynthetic pathways of lipids and suggest that the same may be true of the microperoxisomes in rat brain. Attention is being focussed on the peroxisomal membrane (30), and Appelkvist and Dallner (3) have speculated that a fatty acid-binding protein may be present in this membrane. The present study suggests that uptake of exogenous lipid precursors and their esterification in the ER may occur in 3T3-L1 cells, as in mammalian hepatocytes (78). Cardell et al. (7) discuss lipid uptake and transport by the ER in the absorptive cells of the mammalian intestine. In contrast to other cultured cells, differentiating 3T3-L1 cells can utilize external triacylglycerols. Their capacity to secrete lipoprotein lipase may be essential for efficient use of the exogenous lipid (82). Some aspects of lipid metabolism in isolated adipocytes are discussed by Schlossman and Bell (73).

Coleman et al. (8) have demonstrated that during 3T3-L1 differentiation there is a concomitant 30- to 100-fold increase in specific activities of four microsomal enzymes of triacylglycerol biosynthesis: fatty acid CoA ligase, glycerol-3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, and diacylglycerol acyltransferase. Similar findings have been reported by Grimaldi et al. (24). The results of Hajra et al. (25) raise the possibility that some of the microsomal enzyme activities are associated with microperoxisomes rather than with the ER.

FIGURE 1 Light micrographs of 3T3-L1 cells. The undifferentiated cells were used 1 d before confluence. Differentiating cells were used 4 or 5 d after their return to standard medium following drug treatment, except for *i*. (a) Undifferentiated cells. Fixed in cold glutaraldehyde-formaldehyde for 70 min; stained first with Oil red O (for lipid) and then with toluidine blue (for nuclei). Small lipid spheres are seen in all cells, including the one in mitosis (arrow). $\times 420$. (b) Differentiating cells. Stained at the same time and in the same manner as in *a*. Most cells are rounded and show numerous cytosolic lipid spheres, some very large. A few cells, probably slower in differentiating, possess small lipid spheres. $\times 420$. (c) Undifferentiated cells. Fixed in 4% formaldehyde at room temperature for 10 min. Stained for glycogen with the PAS reagent before diastase digestion. All cells show some staining. $\times 420$. (d) Differentiating cells. Stained at the same time and in the same manner as in *c*. The cells appear to stain more intensely. We do not know how much of this apparent increase in staining is caused by the rounding of the cells. $\times 420$. (e) Differentiating cells. Fixed in cold glutaraldehyde-formaldehyde for 3 min. Incubated for α -naphthyl-acetate-esterase activity for 15 min. Most cells show intense cytoplasmic staining. $\times 420$. (f) Differentiating cells. Fixed as in *e*. Incubated for AcPase activity for 40 min. The individual lysosomes are seen more clearly in the flat cell at the left than in the other cells in the field. Their size indicates that they are autophagic vacuoles; cf. Figs. 8-10, 12, and 14. $\times 420$. (g) Differentiating cells. Fixed as in *e*, incubated in alkaline DAB for 90 min, and processed for electron microscopy. A 2- μ m Epon section was prepared and stained with toluidine blue, which stains the nuclei and especially the nucleoli. At the periphery of the lucent lipid spheres dark structures are seen. Electron microscopy (Fig. 7) shows these to be microperoxisomes. They are visible by light microscopy because they are superimposed. The arrow indicates an extensive glycogen area, seen in occasional cells (cf. Fig. 9). $\times 620$. (h) Differentiating cells. Fixed for 5 min in cold 4% formaldehyde. incubated in IDP medium for 70 min. The intensely stained Golgi apparatus is seen in most cells (arrows). An arrowhead indicates a cell with intensely stained ER. $\times 630$. (i) Cells treated for 1 d, then returned to normal medium for 5 d. Incubated in TPP medium for 90 min. The Golgi apparatus is visualized and its network nature is apparent (arrows). $\times 630$.



Key to Symbols for Figs. 2-21

Figs. 2-21 are from differentiating 3T3-L1 cells, except for Figs. 16-18. Portions of the ER are indicated by arrows. Many rosettes are sectioned so that their continuities with the plasma membrane lie outside the plane of section; some of these are marked with an x. Some rosettes in which these continuities lie in the section plane are indicated by arrowheads. For fixatives and incubation media, see text.

Symbols: A, autophagic vacuole; C, coated pit or coated vesicle; G, glycogen; GE, GERL; GO, Golgi stack; L, cytosolic lipid sphere; M, mitochondrion; m, microperoxisome; N, nucleus. Some additional symbols are indicated in a few legends.

FIGURES 2 and 3 After incubation in NDPase medium for 60 min. Reaction product makes the ER visible. Cisternae of ER extend to both plasmalemmal rosettes and cytosolic lipid spheres. The constellations of lipid spheres, ER, mitochondria, and microperoxisomes are evident. In Fig. 2, four autophagic vacuoles are seen, all with ER nearby; the second from the left appears to be an early autophagic vacuole, with ER seen wrapped around the sequestered cytoplasm. In Fig. 3, the inset is from another cell. Fig. 2, $\times 16,000$; Fig. 3, $\times 30,000$; inset, $\times 45,000$.

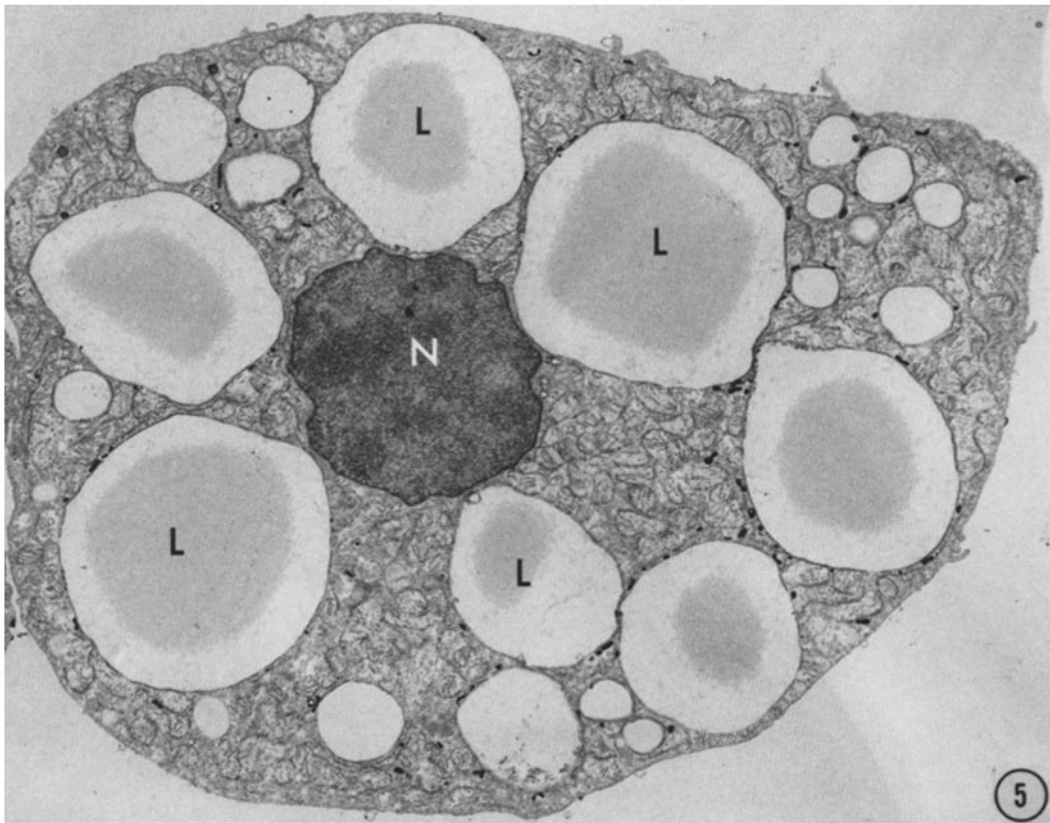
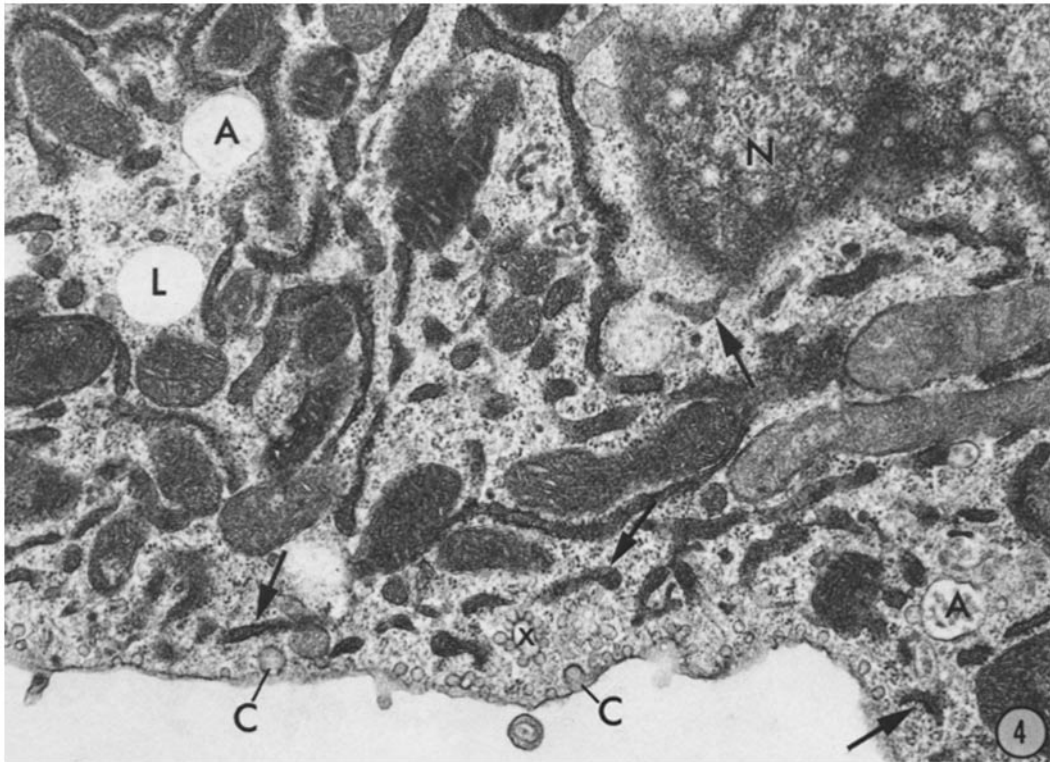
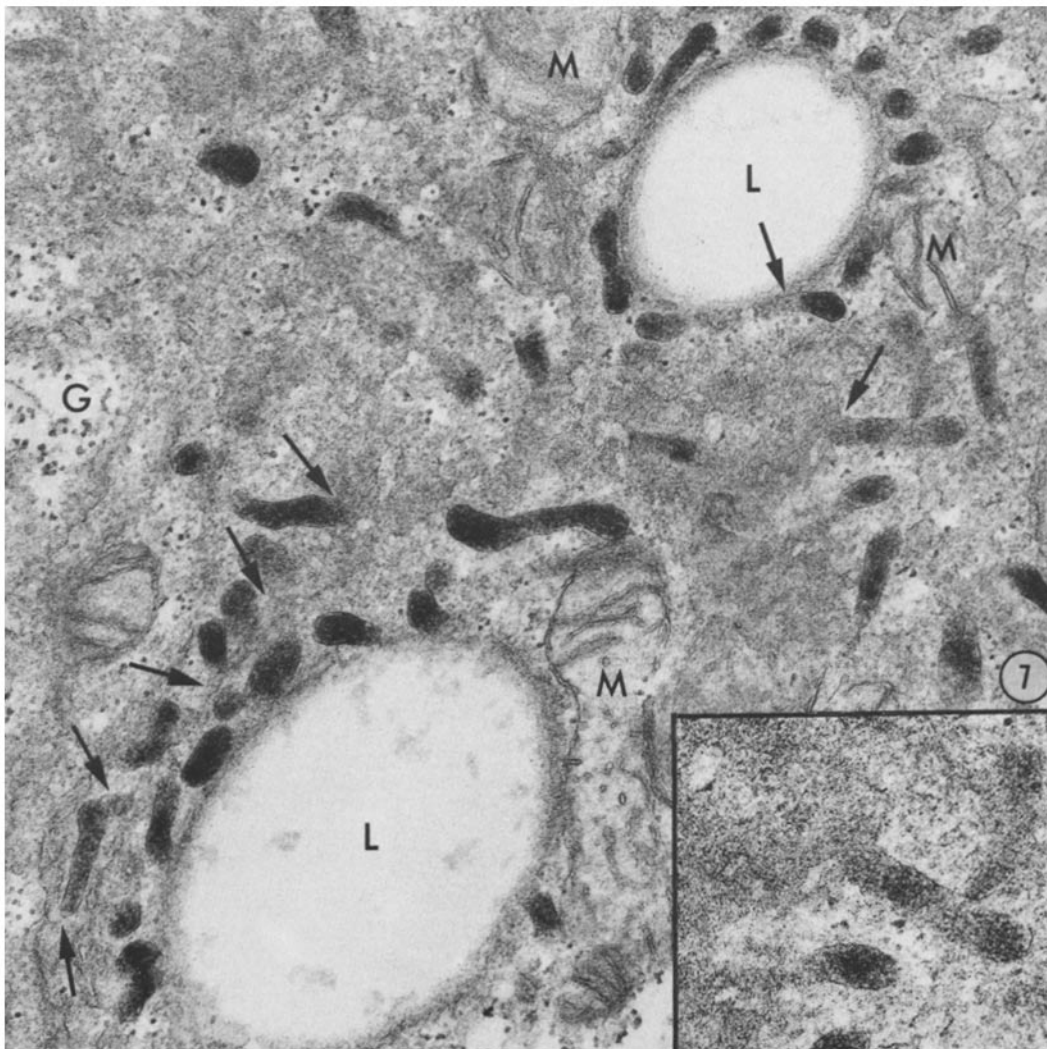
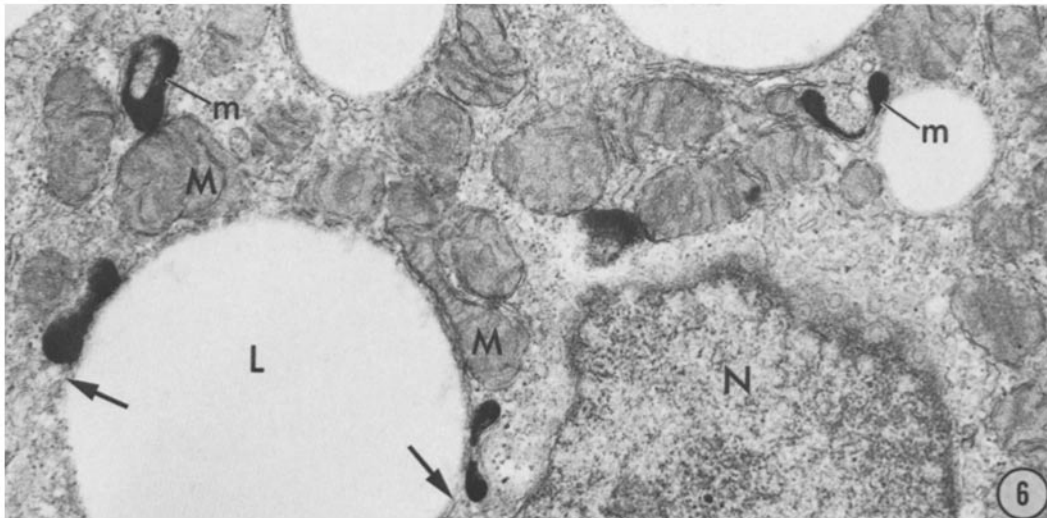


FIGURE 4 Although incubated as in Figs. 2 and 3, in this particular cell (such cells are encountered rarely among the differentiating cells) the ER shows little or no reaction product. The ER is dilated with a moderately electron opaque material. To the right of the rosette marked x another one is seen that displays its three-dimensional aspect more clearly. $\times 27,000$.

FIGURE 5 After incubation in DAB medium for 90 min. Even at this low magnification ($\times 5,800$) the numerous electron-opaque microperoxisomes are evident on the surfaces of the lipid spheres. Cf. Fig. 1 g, the section thickness of which is ~ 40 times that used for the electron micrograph.



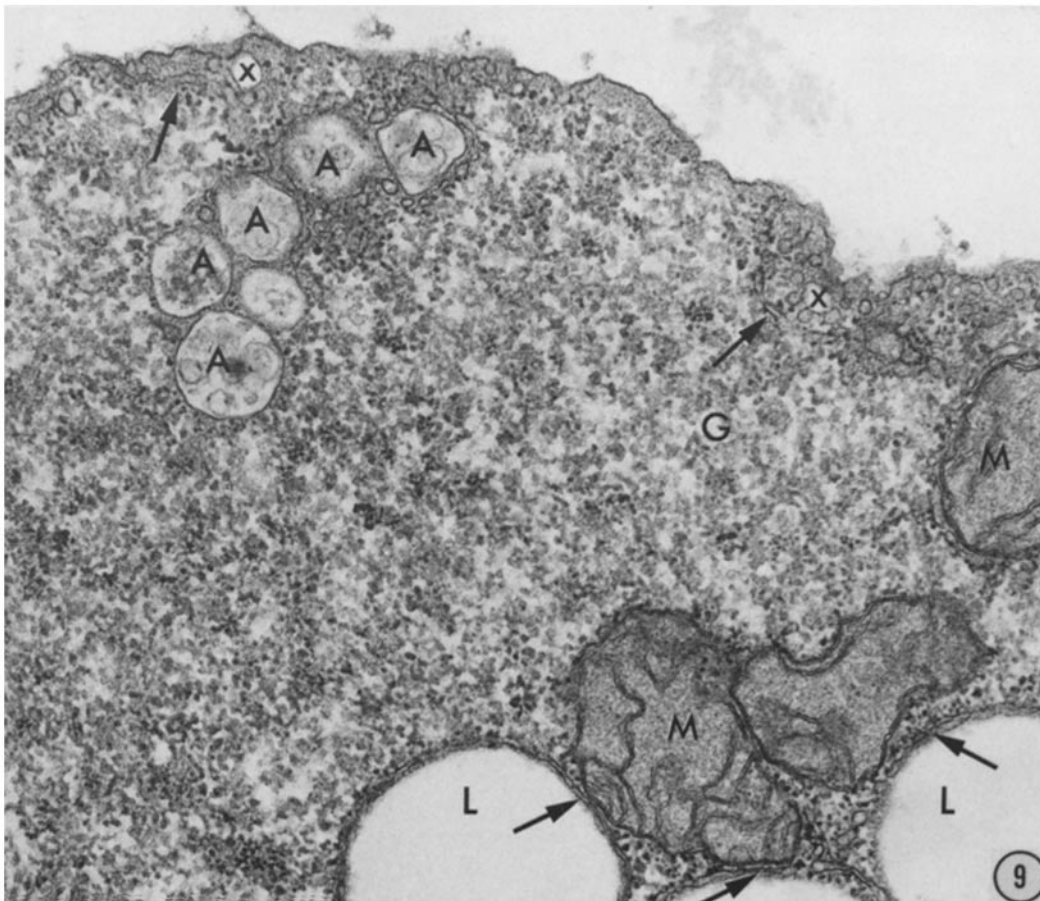
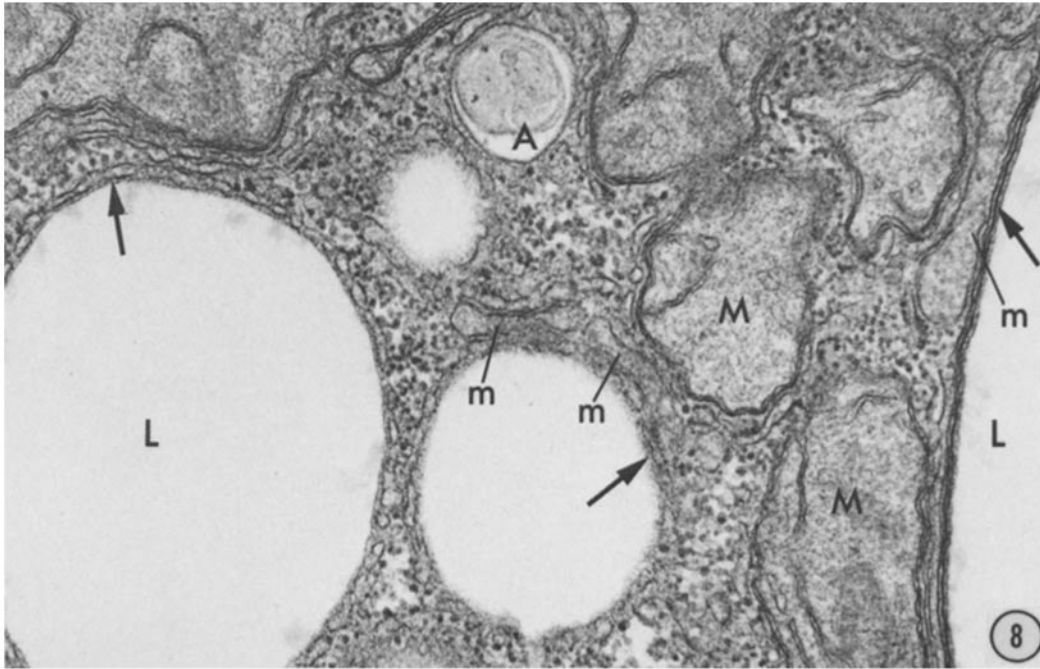
FIGURES 6 and 7 Incubated as in Fig. 5. The two microperoxisomes labeled *m* in Fig. 6 have shapes encountered fairly frequently in these cells. Although serial sections were not cut, it is likely that the shape at the left corresponds to a section passing across both arms of a microperoxisome, like the one at the right. The constellation of lipid spheres, ER, mitochondria, and microperoxisomes are more readily discerned in Fig. 6 than in Fig. 7. The cell in Fig. 7 possesses more microperoxisomes than any other encountered in this study. The *inset* is an enlargement of the area of Fig. 7 that includes the arrow farthest to the right. This area includes what is interpreted as a glancing section of ER (with fenestrations) with which the microperoxisome is continuous. In both Figs. 6 and 7, the arrows indicate regions where contiguities, possibly continuities, between microperoxisomes and ER are present. Fig. 6, $\times 22,000$; Fig. 7, $\times 33,000$; *Inset*, $\times 45,000$.

Centrosphere Region

The present study of the juxtannuclear centrosphere region in differentiating 3T3-L1 cells has concentrated upon the Golgi

apparatus, GERL, and coated vesicles that appear to arise from GERL.

The three-dimensional aspect of the Golgi apparatus is best appreciated by light microscopy (Fig. 1*h* and *i*). Electron



FIGURES 8 and 9 Unincubated. In Fig. 8, a still recognizable mitochondrion is seen within the autophagic vacuole. In contrast, in Fig. 9 the organelles sequestered in the autophagic vacuoles are no longer recognizable. In Fig. 8, note the electron-opaque limit of the lipid in the two spheres marked *L*. In Fig. 9, the glycogen area is unusually large; cf. Fig. 1 *g*. Such large glycogen areas are seen occasionally. Fig. 8, $\times 44,000$; Fig. 9, $\times 33,000$.

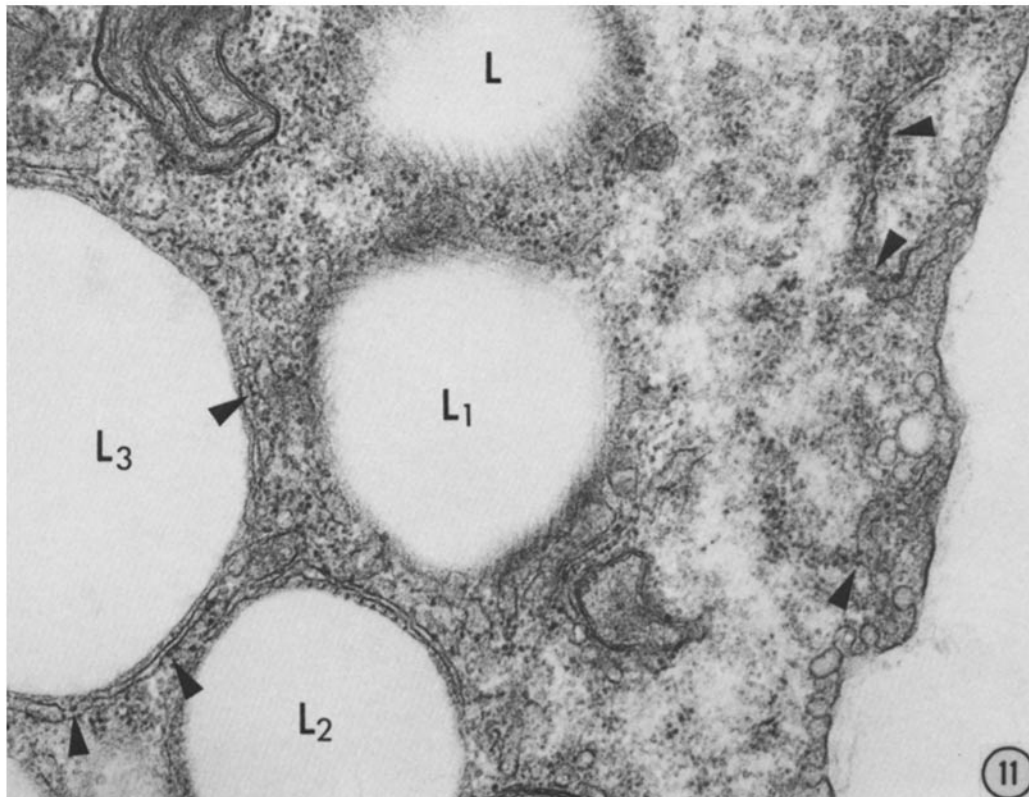
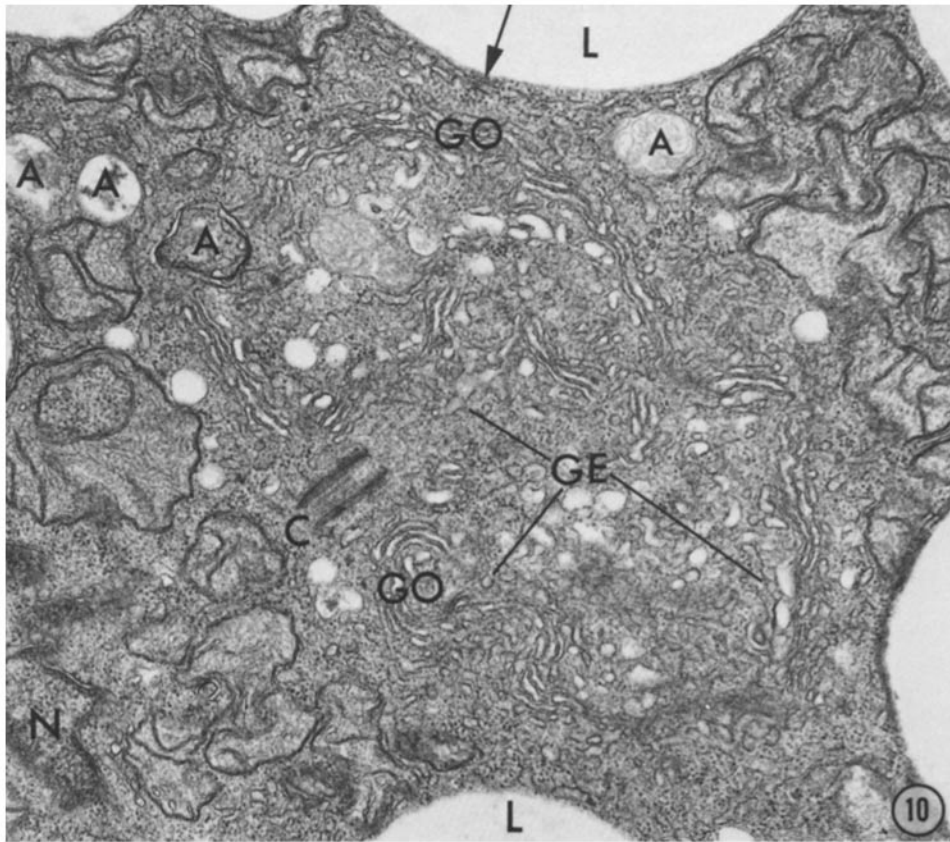


FIGURE 10 Unincubated. The section passes through the centrosphere region in which are seen a centriole (*C*), Golgi stacks, and GERL. The cytosolic lipid sphere, above, shows the adjacent ER. All four autophagic vacuoles show ER nearby. The third one from the left shows "compaction" (54) of the ER. Inside this vacuole, sequestered ER is recognizable. The uppermost autophagic vacuole (to the right) appears also to contain ER that is mostly digested. $\times 27,000$.

FIGURE 11 Unincubated. This section shows ribosomes well (arrows), in part because the glycogen particles are not preserved and, thus, the ribosomes are not obscured. Note that the ribosomes are present on the ER both near the rosettes, to the right, and near the lipid spheres, to the left. Adjacent to the spheres marked *L*, *L*₁, and *L*₂, microfilamentlike particles sectioned in different planes are evident; cf. Figs. 19–21. $\times 44,000$.

microscope study of thin sections passing perpendicularly through the Golgi apparatus shows "stacks" of three to four parallel elements of which the apparatus is composed (Figs. 10 and 12). The innermost, or "trans," Golgi elements have cy-

tochemically demonstrable NDPase activity (Fig. 13). As demonstrated unequivocally in earlier studies on neurons (57), thin sections can be misleading regarding the number of such NDPase-positive elements, and thick sections of differentiating

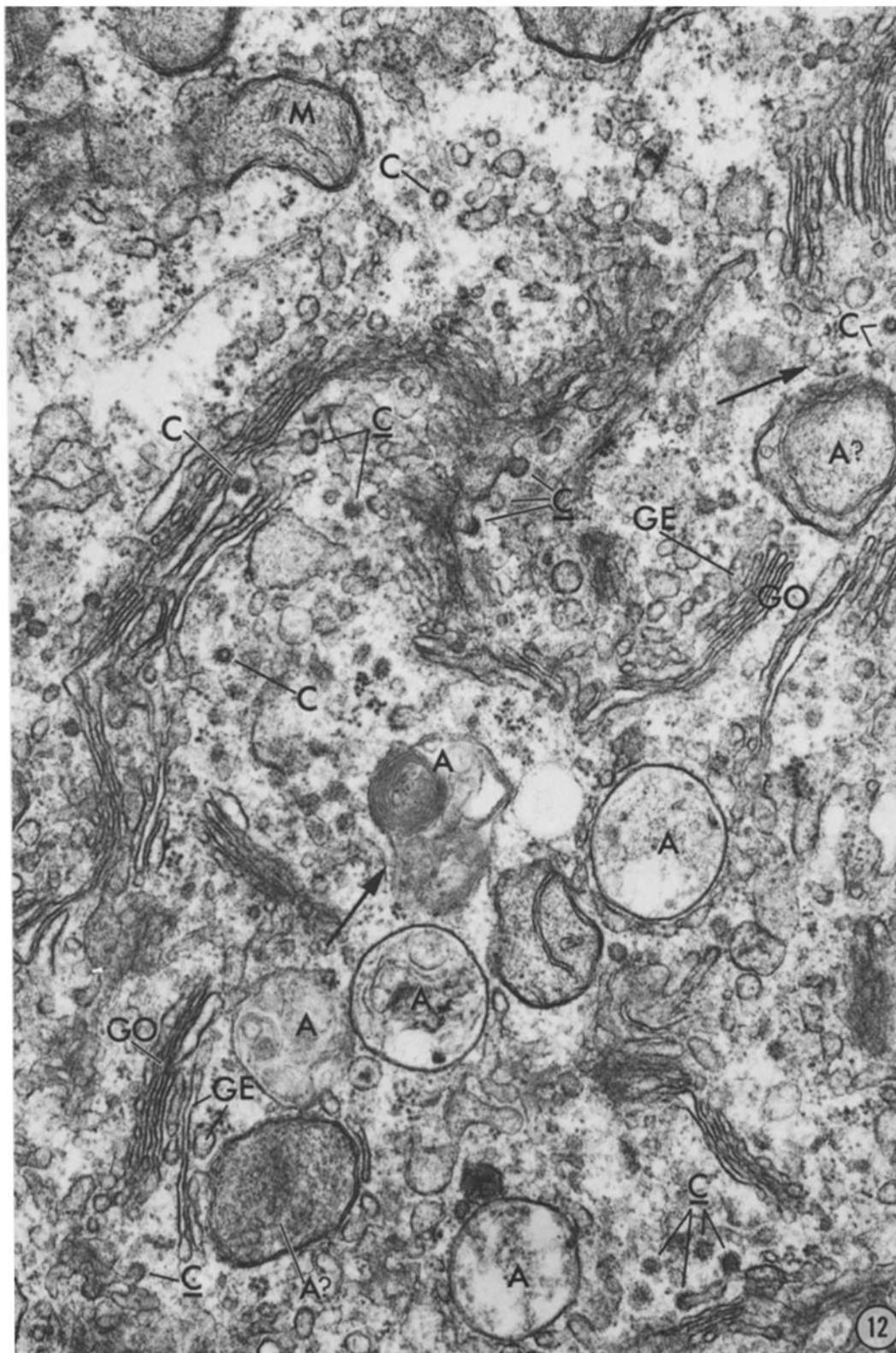


FIGURE 12 Unincubated. Section passing through the centrosphere region. Numerous coated vesicles (C) are present in the region. Those that appear to be arising from GERL are underlined (C). Autophagic vacuoles are numerous in these cells; see Results and Discussion for probable significance. The arrows indicate ER nearby. Possible early stages in autophagic vacuole formation are indicated by A?. X 42,000.

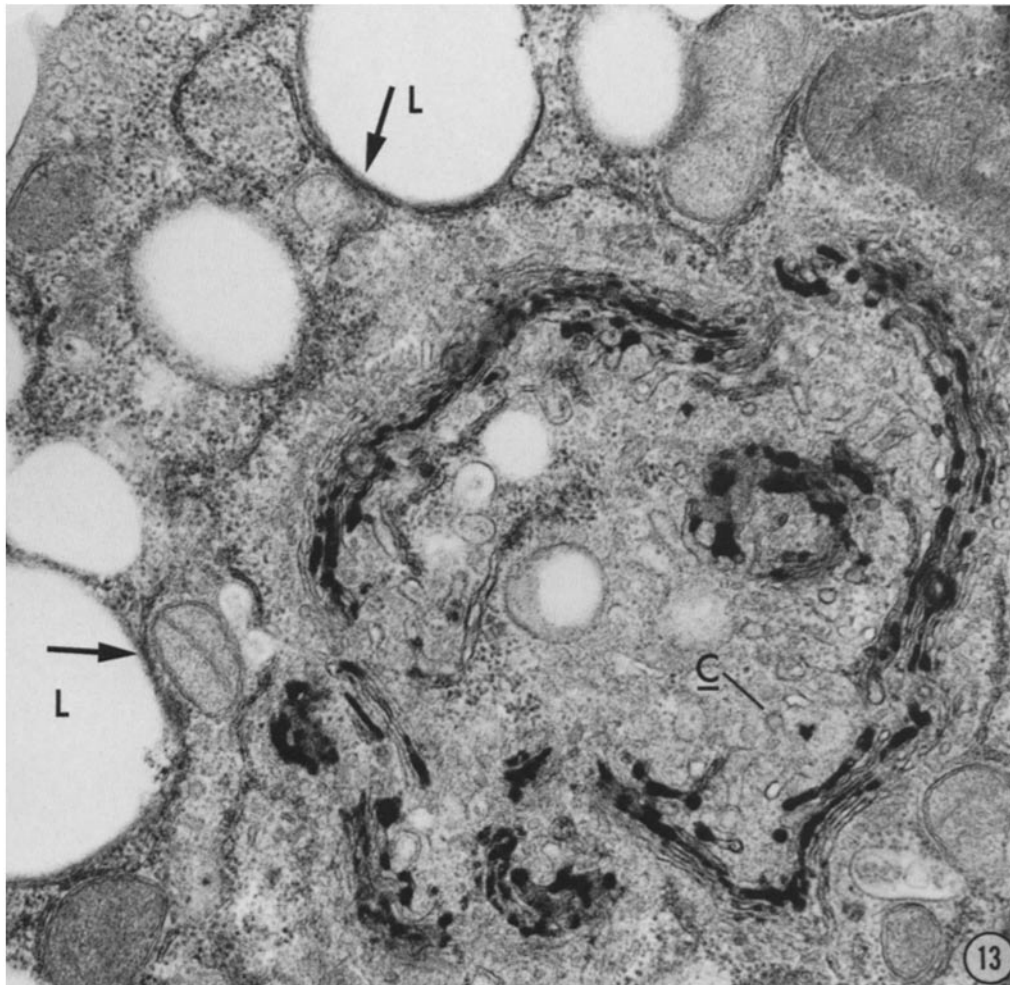


FIGURE 13 After incubation in NDPase medium for 60 min. Reaction product makes the ER visible, as seen at the arrows adjacent to the cytosolic lipid spheres; cf. Figs. 2 and 3. In addition, because the centrosphere region of the cell is included in this field, reaction product makes the inner (trans) elements of the Golgi apparatus visible (see Results and Discussion). The area enclosed by the Golgi apparatus includes parts of GERL (cf. Figs. 12 and 15), lacking reaction product. A coated region, as if separating from GERL, is indicated by C. $\times 40,000$.

3T3-L1 cells have yet to be studied. The Golgi apparatus of these cells lack the two features associated with secretion, e.g., in hepatocytes (56, 59, 60), visible material within its elements and lateral dilatation of the elements.

GERL of differentiating 3T3-L1 cells is too small to be recognizable by light microscopy of AcPase preparations (Fig. 1f). By electron microscopy, GERL is seen to be extensive and anastomosing (Figs. 12–15) and to possess cytochemically demonstrable AcPase activity. The most impressive feature of GERL in these cells is the large number of AcPase-positive coated vesicles associated with it (Figs. 14 and 15). Using the criteria of Palade and Bruns (62), in which flasklike shapes and elongated necks are considered to be characteristic of vesicles when separating from a membrane surface, the vesicles, probably coated, appear as if separating from GERL rather than fusing with it (Figs. 12, 13, and 15). The inner diameter of these vesicles is ~ 500 Å, considerably less than that of the coated pits or coated vesicles ($\sim 1,800$ Å) associated with the cell surface.² Their greater number, smaller size, and possession of

cytochemically demonstrable AcPase activity are features that should aid in resolving those vesicles apparently derived from GERL from the coated vesicles that seem to derive from the plasmalemma. Data acquired from fractions enriched in GERL-derived coated vesicles should show the presence of clathrin (64, 65) and related proteins (66, 85), they might also bring support for the suggestion from the work of Blitz et al. (5) that coated vesicles are not only involved in the internalization of surface receptors but also function in transporting biologically active molecules from one cell organelle to another.

Autophagy

No mammalian cell of which we are aware (29, 49) shows a greater number of autophagic vacuoles than do differentiating 3T3-L1 cells (Figs. 2, 4, 8–10, 12, and 14). Cisternae of ER are always very close to these vacuoles, but “compaction” (54) of the ER membranes is suggested only infrequently (Figs. 2 and 10). We speculate that this compaction occurs more frequently

² In 1967 Friend and Farquhar (15) described two classes of coated vesicles in epithelial cells of the rat vas deferens. The vesicles were of approximately the same diameters as those in the differentiating 3T3-

L1 cells. The larger ones were “concentrated in the apical cytoplasm” and the smaller ones were “found primarily in the Golgi region.” Also see Anderson et al. (2).

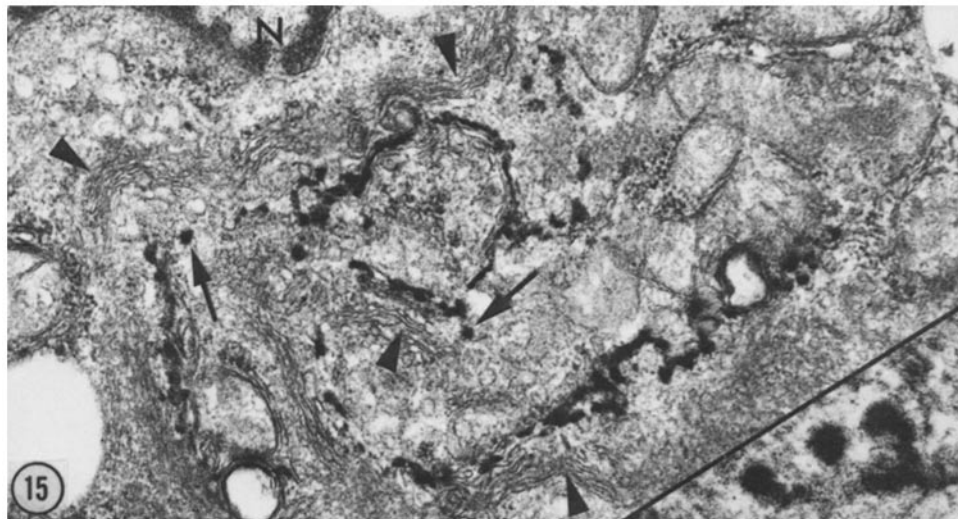
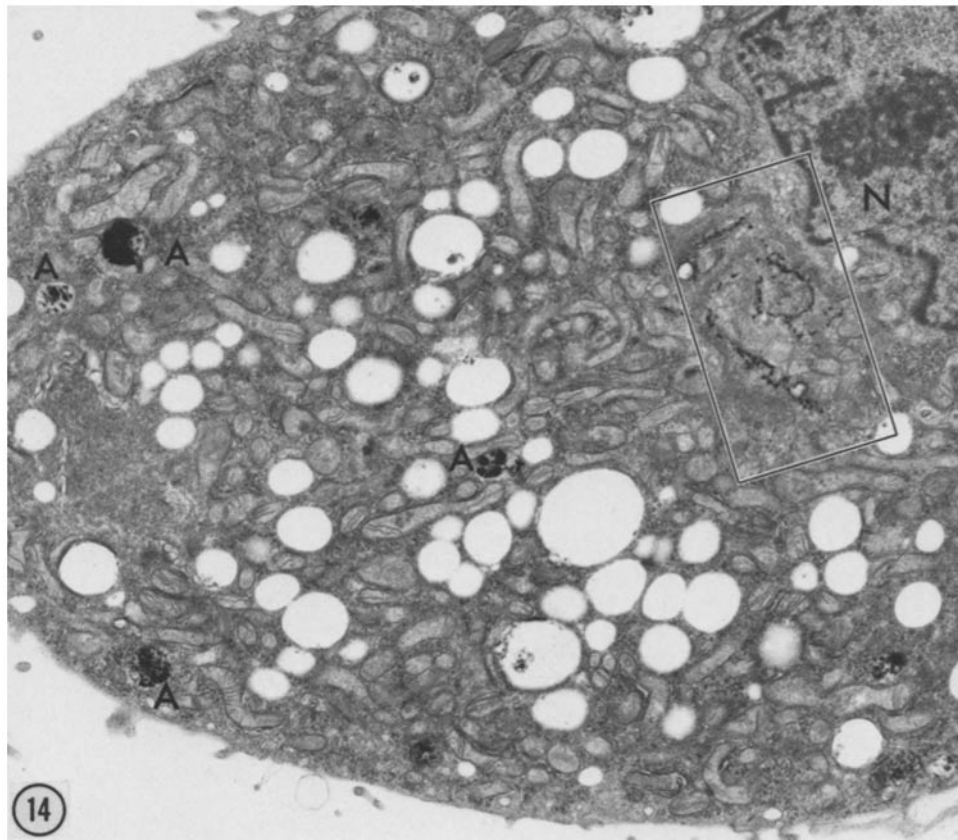


FIGURE 14 After incubation in AcPase medium for 20 min. Reaction product makes the autophagic vacuoles and GERL visible (box; see Fig. 15). $\times 10,000$.

FIGURE 15 Boxed area of Fig. 14 enlarged. AcPase reaction product is seen in GERL (unlabeled), the anastomosing nature of which is evident. The Golgi apparatus is without AcPase activity. Arrowheads indicate where the plane of section passes revealing Golgi stacks. When GERL and adjacent Golgi stacks are seen, it is evident that GERL is always trans to the stack. Two arrows indicate vesicles that have probably separated from GERL. The inset is from another cell. Three AcPase-positive, apparently coated vesicles appear to be separating from GERL. $\times 37,000$; Inset $\times 72,500$.

than can be demonstrated in electron micrographs because the process may be extremely rapid. With compaction, the permeability of the ER membrane apparently changes so that AcPase (our marker) and presumably other hydrolases and other materials are transported into the developing autophagic vacuole. This leads to the degradation of the sequestered organelles to forms no longer visible by electron microscopy. Residual bod-

ies, which form from many autophagic vacuoles in other cell types (47), are not seen in the differentiating 3T3-L1 cells.

The rapid autophagy observed in the differentiating cells may be related to the marked structural remodeling and metabolic reorientation that occur during adipocyte conversion. The cells become rounded, the content of cellular actin decreases 80%, and synthesis and/or turnover of ~ 100 cellular

proteins is altered (75). For a striking effect on autophagy, see Dean (9).

Filamentous Structures

Undifferentiated 3T3-L1 cells possess "cables" ("stress fibers") beneath the plasmalemma. From their diameters and arrangement, they resemble organized arrays of actin or actin-like proteins (Fig. 16, arrowhead). Deeper in the cytoplasm are filaments resembling the "100 Å filaments" or "intermediate filaments" (Fig. 16, arrow) described in many cultured cells types (14), including 3T3 cells (18). One of the proteins of the intermediate filaments, with an apparent molecular weight ~57,000, has been named vimentin (13).

In contrast to the undifferentiated cells, the differentiating 3T3-L1 cells exhibit neither the microfilamentlike or the thicker filaments beneath the plasmalemma. However, microfilament-like structures are seen around the cytosolic lipid spheres. These filaments measure 75 Å when cut transversely (Fig. 19) and enclose the lipid spheres in a complex manner, best appreciated in glancing sections (Figs. 20 and 21). The distance between filaments is ~150–350 Å. Such associations of lipid spheres and filaments were first described in 1967 by Wood (84) in developing adipocytes from chick bone marrow; she suggested that the filaments may support the lipid inclusions. Similar filaments were described at the same time in avian subsynovial adipocytes (38).

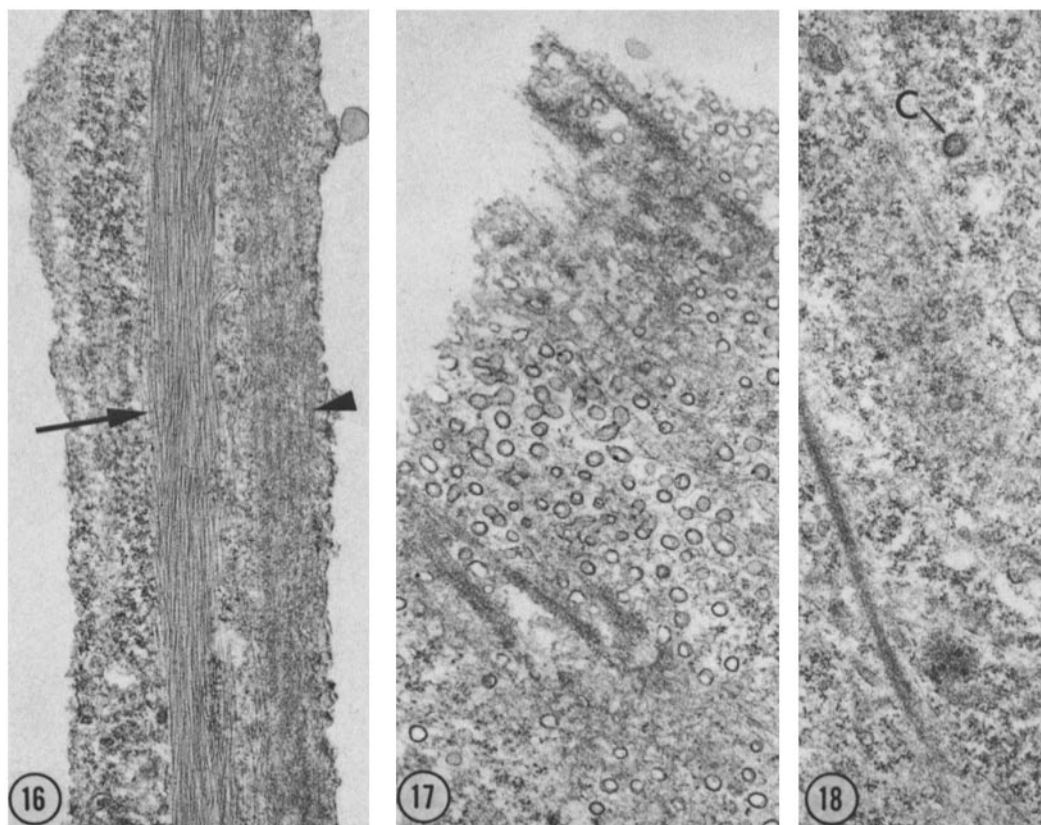
α -Naphthyl-acetate-esterase Activity

Most undifferentiated 3T3-L1 cells show little or no esterase activity. A dramatic increase in staining occurs in differentiating cells (Fig. 1e), with most cells showing intense cytoplasmic staining. In a few cells (e.g., the one at the upper left in Fig. 1e) the staining is much weaker, perhaps because they have not yet sufficiently differentiated.

Because the increased staining does not appear to be particulate or to derive from artifactual diffusion from cell particles, the esterase is probably cytosolic. Although the physiological role of this esterase activity is unknown, it is of considerable interest that such activity appears during differentiation of the 3T3-L1 preadipocytes because it also occurs in developing fat cells isolated from 2–3-d old rats (44, 45). This observation supports utilization of the 3T3-L1 cells as a model system to study adipocyte development.

Glycogen

Light microscopy shows that both undifferentiated (Fig. 1c) and differentiating cells (Fig. 1d) are PAS positive. When treated with diastase before staining they are negative, indicating the presence of glycogen (37). Electron microscopy reveals the glycogen to be relatively uniformly dispersed in most differentiating cells (Figs. 7 and 8) but localized in large areas in some of these cells (Fig. 9; cf. Fig. 1g).



FIGURES 16–18 Unincubated. Three sections of undifferentiated cells close to the plasma membrane, near the carbon film on which the cells were grown. In Fig. 16 the bottom of the cell is at the right. Immediately above the plasma membrane (i.e., to the left in the figure) is a "cable" or "stress fiber" of actinlike filaments (arrowhead). Deeper in the cell is a collection of thicker fibers, probably intermediate filaments (arrow). Fig. 17 shows numerous small vesicles probably continuous with the plasma membrane out of the section plane. In Fig. 18, at C, a coated vesicle measuring ~1,100 Å (or a coated pit sectioned so as to appear like a vesicle) is seen. Fig. 16, $\times 25,500$; Fig. 17, $\times 28,000$; Fig. 18, $\times 26,000$.

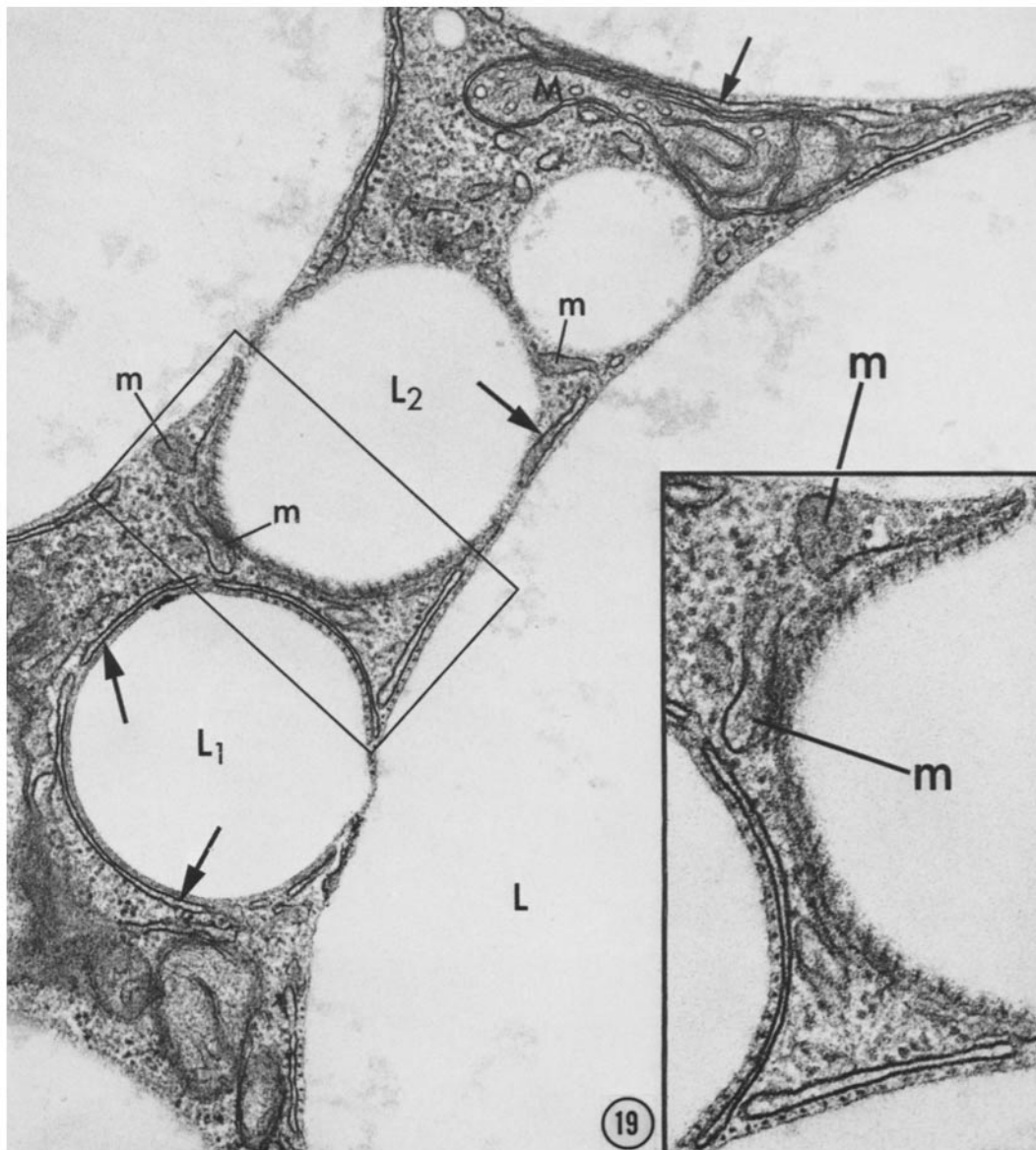


FIGURE 19 Unincubated. Microfilamentlike structures are seen between the limits of the lipid spheres and the surrounding ER. In the boxed area, these structures are sectioned transversely adjacent to L_1 ; they are sectioned slightly tangentially adjacent to L_2 . The structures show more clearly in the enlargement. The thin, electron-opaque limit of the lipid sphere, L_1 , may also be seen in the enlargement. $\times 45,000$; enlargement, $\times 80,000$.

CONCLUSIONS

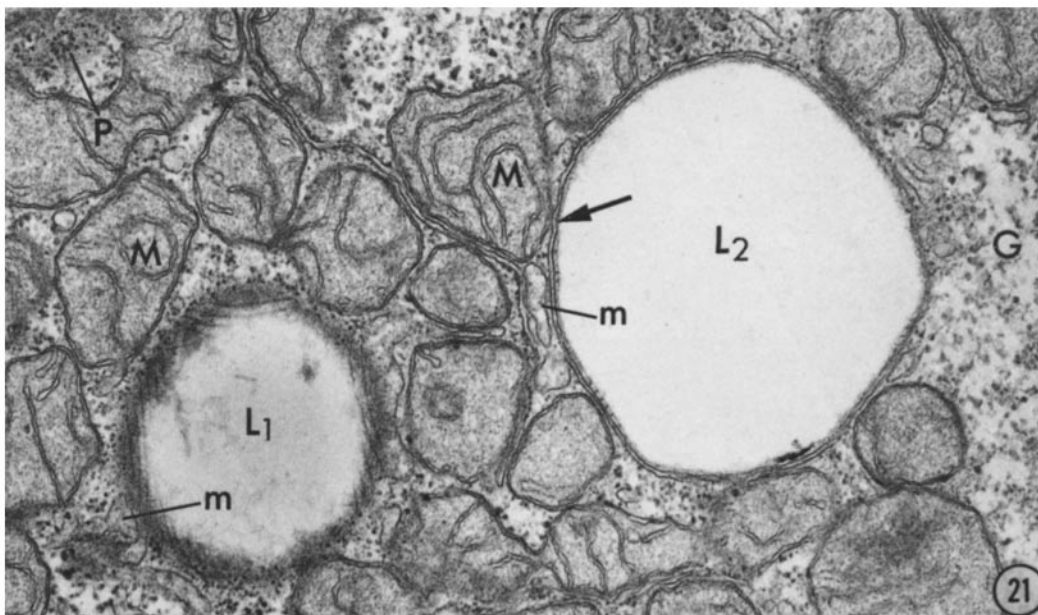
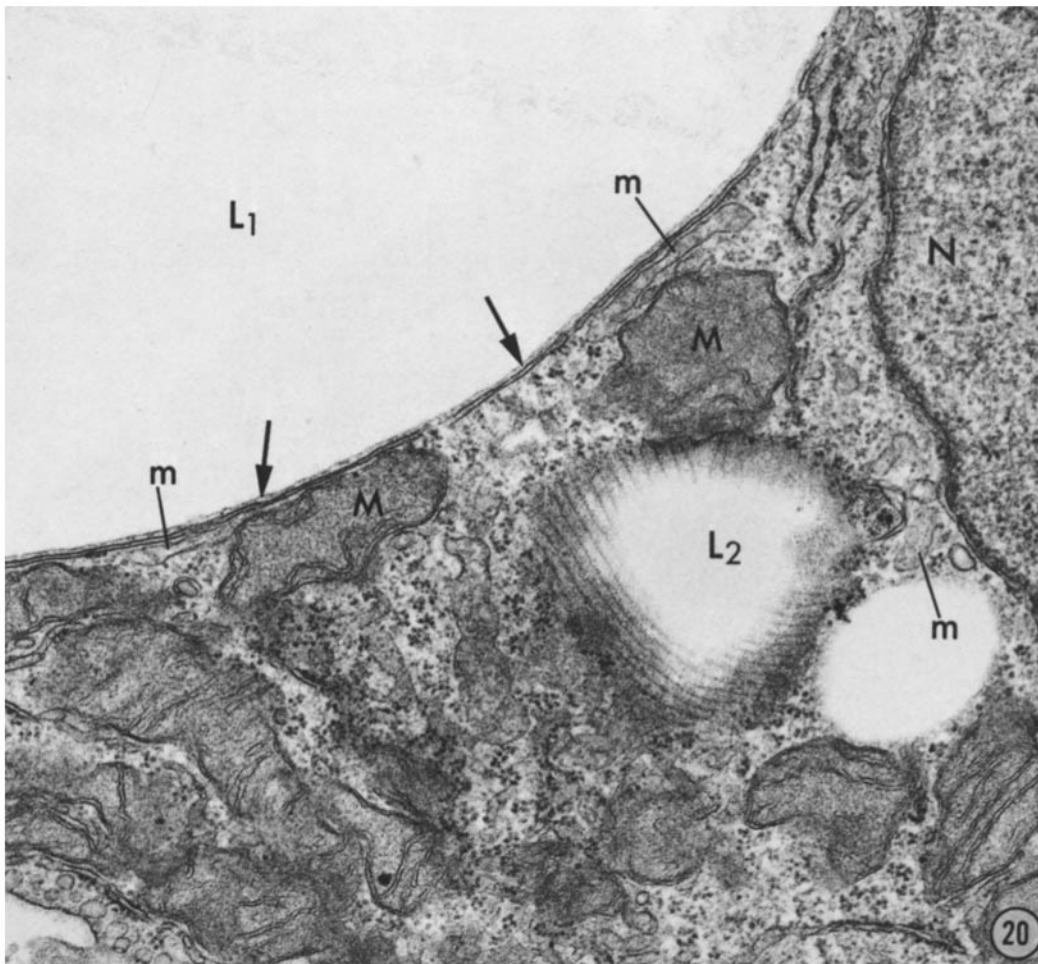
(a) Undifferentiated and differentiating 3T3-L1 preadipocytes have been used as a model system to initiate and analyze the ultrastructural features of cells before and after adipocyte conversion. The cells provide a unique system for studying cellular organelles in adipocyte development and for ultimately correlating the biochemical changes that accompany adipocyte development with organelle function. Although the 3T3-L1 preadipocyte cell line is being used increasingly to investigate the biochemistry of developing adipocytes, the observations presented here provide the first documentation that the ultrastructure of the differentiating 3T3-L1 cell is consistent with the principal metabolic functions of the cell and with what is known about the ultrastructure of mature adipocytes.

(b) A prominent ultrastructural feature of differentiating 3T3-L1 cells is the widespread occurrence of constellations

composed of large triacylglycerol droplets bordered by the ER and closely associated with microperoxisomes and mitochondria. Knowledge of enzymic activities of these organelles in other cells (16, 25, 36, 41, 42) suggests that such constellations may serve as foci for the biosynthesis, accumulation, and metabolism of adipocyte lipid.

(c) Very high densities of specialized plasmalemmal structures with unique three-dimensional configurations have also been observed in association with the ER in differentiating 3T3-L1 cells. Such structures have earlier been designated rosettes, and it has been speculated that these structures, in adipocytes, may play important roles in lipid uptake (81) and in insulin action (31).

(d) Undifferentiated 3T3-L1 cells contain typical actinlike stress fibers associated with the internal surface of the plasma membrane and bundles of intermediate filaments. In contrast, the differentiating cells are depleted of both stress fibers and



FIGURES 20 and 21 Unincubated. Transverse sections of microfilamentlike structures do not show as well as in Fig. 19. However, glancing sections of these structures may be seen at L_2 of Fig. 20 and L_1 of Fig. 21. These suggest a complex encasement of the lipid spheres by the filaments. In Fig. 21, a polysome is indicated by *P*. Fig. 19, $\times 30,000$; Fig. 20, $\times 33,000$.

intermediate fibers, and their cytosolic lipid spheres are encased in a complex network of actinlike microfilaments. The loss of stress fibers parallels the conversion of the highly spread and flattened 3T3-L1 cells to spherical differentiating cells and

correlates with an 80% reduction in cellular actin content (75).

(e) Small AcPase-positive coated vesicles appear to arise from GERL at the trans aspect of the Golgi apparatus (52) in differentiating 3T3-L1 cells. These vesicles are far more nu-

merous than the vesicles apparently derived from coated pits at the cell surface, and they are much smaller than those derived from the surface.

(f) Differentiating 3T3-L1 cells contain large populations of autophagic vacuoles (49), which are seen to contain cytoplasmic organelles in various states of autolysis. These vacuoles may reflect the dramatic remodeling that accompanies differentiation.

(g) α -Naphthyl-acetate-esterase activity rises from cytochemically undetectable levels in undifferentiated cells to strikingly elevated levels in the cytoplasm of differentiating cells. It has been suggested that esterolytic activity is a property of α -glycerolphosphate dehydrogenase (1, 79). A marked increase in the activity of this dehydrogenase during the differentiation of preadipocytes has been demonstrated by Kuri-Harcuch et al. (35) and Wise and Green (83).

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