Electron microscopic and biochemical study of lipoprotein synthesis in the isolated perfused rat liver

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ABSTRACT The isolated perfused rat liver was used to study the 300–800 A electron-opaque bodies which had previously been described in the liver cell Golgi apparatus, smooth endoplasmic reticulum, and space of Disse.

When the perfusion medium was enriched with linoleate, the number and electron opacity of these particles increased markedly. Sequential biopsies showed that they appeared first in the smooth surfaced terminal ends of the rough reticulum, the smooth endoplasmic reticulum proper, and the Golgi apparatus and later in the space of Disse. After 60 min of perfusion, particles of the same size and shape as those in the liver cells could be isolated in large numbers from the $d < 1.006$ fraction of the perfusate. Control livers perfused with an identical medium but without linoleate did not show these changes.

Puromycin markedly depressed the production of 300–800 A particles by livers perfused with an oleate-rich medium; however, it did not interfere with the formation of large cytoplasmic droplets of neutral fat. In keeping with these findings, puromycin blocked the incorporation of oleate-^{14}C into lipoprotein triglyceride isolated from the perfusate, but did not interfere with the appearance of the labeled fatty acid in tissue triglyceride. Puromycin also blocked the incorporation of leucine-^{3}H into both tissue protein and perfusate lipoprotein. We concluded that the 300–800 A particles observed are, in all likelihood, very low density lipoproteins and that their formation is blocked by puromycin, presumably through interference with the synthesis of their apoprotein.

KEY WORDS rat · perfused liver · fatty acid–albumin · electron microscopy · osmiophilic particles · very low density lipoproteins · endoplasmic reticulum · Golgi apparatus · puromycin

It is now well known that the liver produces most of the circulating lipoproteins (1–12). The theoretical dimensions of the different lipoprotein classes as determined by Oncley (13) from their physicochemical characteristics indicate that such complexes should have dimensions within the resolving power of the electron microscope. Osmiophilic particles of these dimensions, which were thought to be lipoproteins, have been found in the appropriate fraction of human plasma by Hayes and Hewitt (14) and in rat chyle by Casley-Smith (15). Similar electron-opaque particles which had the dimensions of very low density lipoproteins were first noted in liver by Fawcett (16), who found them in the Golgi vesicles and vacuoles. Since then the occurrence of these structures within the Golgi cisternae, and to a lesser extent in the smooth endoplasmic reticulum and space of Disse, has been confirmed by investigators studying the rat (17), bat (18), mouse (19–22), hamster (23, 24), guinea pig (25), and human (26) liver. The number of these particles has been found to increase markedly following partial hepatectomy (21, 22), corn oil ingestion (19, 27–29), and acute ethanol administration (23, 28, 30). Increased numbers of somewhat larger bodies have also been seen in the endoplasmic reticulum and Golgi after the administration of ethionine (31) or orotic acid (32) to rats and carbon tetrachloride (25) to guinea pigs. Despite the resemblance of these osmiophilic bodies to the "lipoprotein" particles found in plasma and chyle, there is still considerable disagreement as to the chemical composition and site of formation of these intracellular particles. They have been interpreted by various authors as albumin (17), chylomicrons absorbed from the circulation (19, 27–29, 33), lipid transported to the liver from fat depots (21, 22), and lipoproteins of hepatic origin (23, 32, 34–36). Most of these observations have been made in whole animals, and hence the liver was subject to a multiplicity of hormonal factors and metabolic control mechanisms that prevail in the internal environment. In a preliminary communica-

tion (37) we recommended the isolated perfused liver as a means of eliminating many of these uncontrollable variables. When this system was used, evidence was obtained which suggested that the 300–800 A electron-opaque bodies seen within the liver cell and space of Disse were very low density lipoproteins synthesized by the liver. A similar conclusion was concurrently reached by Hamilton, Regen, Gray, and LeQuire (36), also on the basis of experiments with the perfused rat liver. The present study confirms and extends our earlier observations and in addition reports the effects of an inhibitor of protein synthesis, puromycin, upon the synthesis of these particles and upon hepatic triglyceride and protein metabolism.

MATERIALS AND METHODS

Sprague-Dawley male rats weighing 200–300 g were anesthetized with Nembutal (3 mg/100 g body weight) after an overnight fast. The bile duct and portal vein were cannulated and a perfusion with oxygenated Krebs-Ringer bicarbonate buffer (KRB) in situ was immediately started. The liver was then removed from the animal and placed in a modified Miller perfusion apparatus (38) in which it was perfused with 90–100 ml of a medium composed of KRB containing, per 100 ml, 4 g of fat-free bovine serum albumin, Cohn fraction V (39) (Mann Biochemical Co., New York), 5 mg of streptomycin, 5 mg of penicillin, and 180 mg of glucose. Heparin (20 mg/100 ml) was used in some perfusions, but was not during the puromycin study. In all cases, the medium had previously been passed through a Millipore filter with interstices of 0.45 μ. During the perfusion the medium was constantly equilibrated with a gas phase of 95% O_2–5% CO_2. In the first series of experiments, 2 μmoles/ml of sodium linoleate were present in the medium and 120 μmoles/hr were added from a constant infusion pump. Ten livers were studied in this fashion. Fatty acid was omitted from the perfusate of six control livers. In two instances, crystalline albumin was substituted for the fat-free bovine albumin in livers perfused with linoleate.

Liver viability was judged by perfusate flow rate, bile production, oxygen extraction, and gross appearance. Biopsies were taken from livers immediately prior to their being placed in the apparatus (0 time), and then after 1, 2, 5, 30, and 60 min of perfusion. The most centrally located section of the biopsy specimen was cut into 1 × 1 mm cubes, fixed in 1% osmium tetroxide buffered with 0.05 M phosphate, and embedded in Epon 812 (40). Thin sections were stained with lead and examined with an RCA EMU 3G or Phillips 200 electron microscope.

Aliquots of initial and final perfusion medium were fixed in equal volumes of 2% OsO_4 as were samples of the d < 1.006 fraction of final medium. The latter fraction consisted of the turbid surface layer seen after ultracentrifugation at 105,000 *g* for 16 hr at 10°C in a Spinco model L ultracentrifuge with a number 40 rotor. The d < 1.006 fraction of human and rat plasma was obtained in a similar manner after a preliminary centrifugation to remove the chylomicron fraction (41). Following a 2 hr "fixation," the mixture was centrifuged at 20,000 *g* for 60 min. In those cases where a pellet was obtained, it was processed and embedded in the same manner as the liver tissue.

In a second series of experiments, live animals received 15 mg of puromycin dihydrochloride (Nutritional Biochemicals Corporation, Cleveland, Ohio) intraperitoneally 45 min prior to surgery. In addition, 15 mg of puromycin was added to the perfusion medium. The six control animals received no puromycin and none was added to the perfusate. The medium differed from that of the previous experiments in that it contained per 100 ml, approximately 6 μc of sodium oleate-U-^{14}C (108 mc/mmole, chromatographically pure; Nuclear-Chicago Corporation, Chicago, Ill.), 0.8 mc of leucine 4,5-^{3}H (5 c/mmole, chromatographically pure; New England Nuclear Corp., Boston, Mass.), and 50 μmoles of carrier leucine. All of the initial perfusates contained 2.5 μmoles/ml of chromatographically pure sodium oleate. Fatty acid was not infused. Tissue biopsies taken after 60 min of perfusion and the post-perfusion media were processed for electron microscopic studies as described above.

Total lipids were isolated from a weighed portion of liver (about 1 g) taken after 60 min of perfusion by the method of Folch, Lees, and Sloane Stanley (42). The lipid extract was evaporated to dryness under a stream of nitrogen and then dissolved in benzene to a lipid concentration of 20 mg/ml; 100 μl of this solution was then applied as a streak or a series of spots on a Silica Gel F plate (Brinkmann Instruments Inc., Westbury, N.Y.) and developed in hexane–diethyl ether–acetic acid 80, 20, 1.5. This system gave a clear separation of cholesteryl esters, triglycerides, fatty acids, cholesterol, and phospholipids. After development, the plate was sprayed with Rhodamine 6G and the triglyceride spot scraped off and suspended in a dioxane and Cab-O-Sil (Cabot Corp., Boston, Mass.) mixture containing naphthalene, 2,5-diphenyloxazole, and *p*-bis[2-(5-phenyloxazolyl)]benzene. Counting was done in a liquid scintillation counter at least 4–6 days after the plates were sprayed to eliminate color quenching due to the Rhodamine 6G. A second sample of liver tissue was homogenized in 10% trichloroacetic acid. The precipitate was washed repeatedly with trichloroacetic acid and ethanol, and then dissolved in 2N NaOH (43). An aliquot was suspended in the scintillation mixture and counted in a liquid scintillation counter.

10 ml of final medium was ultracentrifuged at 105,000 *g* for 16 hr (41). 2–3 ml of normal rat serum was added to provide carrier lipoprotein. The turbid top layer was re-

moved and layered over normal saline, and the centrifugation was repeated. The cloudy lipid layer from the second centrifugation was then separated, extracted, and chromatographed and the triglyceride fraction was isolated and counted as described above.

The β- and pre-β-lipoproteins from a second aliquot of final medium were precipitated with dextran sulfate by a modification of the methods described by Cornwell and Kruger (44) and Oncley, Walton, and Cornwell (45). After preliminary centrifugation to remove debris, 0.4 ml of 10% dextran sulfate (mol wt about 2,000,000) and 2 ml of 1M $CaCl_2$ were added to 20 ml of final medium. The precipitate was washed 5–6 times with 10 ml of a solution containing 4% fat-free albumin, 0.5 mM leucine, 2.4 mM sodium oleate, 0.08% dextran sulfate, and 0.04M $CaCl_2$. It was then redissolved in 2 ml of 2M NaCl, which contained 1% EDTA. From this solution protein was precipitated with trichloroacetic acid, purified, and counted as described above.

OBSERVATIONS

Controls

After approximately 5 min of perfusion in situ and before the liver had been placed in the perfusion apparatus (0 time), the hepatic parenchyma looked essentially normal (Fig. 1). The rough-surfaced endoplasmic reticulum was usually in the form of long, parallel, cisternal profiles of ribosome-studded membranes. The smooth-surfaced reticulum, conspicuous when glycogen was lacking (fasted animals), was concentrated in small patches which were distributed randomly throughout the cytoplasm. The Golgi apparatus was either next to the nucleus or near the bile canaliculi. In either case, there was a definite tendency for it to be polarized toward the bile canaliculus. Particles 300–800 A in diameter were occasionally noted within the Golgi vesicles and vacuoles. Sometimes, particles of similar size and density could be found within the smooth endoplasmic reticulum (Fig. 1) and in the space of Disse (Fig. 3). Droplets of neutral fat occurring free within the cytoplasm were easily distinguishable from the 300–800 A bodies by virtue of their larger size and lack of an obvious surrounding membrane. Mitochondria remained unaltered and contained distinct intramitochondrial granules. Lysosomes and microbodies with characteristic crystalline nucleoids were frequently seen.

2 Minutes of Linoleate Perfusion

There were no morphologic changes in the hepatic parenchymal cells after 1 min of fatty acid perfusion; however, after 2 min, a marked increase in the number and electron opacity of the 300–800 A osmiophilic particles was noted. The particles were most prominent in the smooth-surfaced terminal ends of the cisternae of rough endoplasmic reticulum and within the smooth-surfaced reticulum proper, even in areas at some distance from the rough-surfaced form (Fig. 2). There was also a marked accumulation of electron-opaque particles within the Golgi apparatus, where they appeared either as a single particle within a vesicle or, more often, as a cluster of particles within a small vacuole (Fig. 2). No increase in the number of these bodies was seen in the space of Disse at this time (Fig. 4). Livers perfused with a medium devoid of linoleate appeared essentially indistinguishable from livers examined at 0 time.

5 Minutes of Linoleate Perfusion

After 5 min of perfusion with linoleate, there was an even greater accumulation of 300–800 A electron-opaque particles within the smooth-surfaced tubules of the endoplasmic reticulum and in Golgi vacuoles. In addition, a large number of particles were noted within vacuoles and vesicles in the cytoplasm near the space of Disse, in inpocketings of the surface membrane, and free within the space of Disse (Fig. 5).

30 and 60 Minutes of Linoleate Perfusion

After 30 min of perfusion with medium enriched with fatty acid, the number of the osmiophilic particles increased spectacularly in the space of Disse and in the shallow clefts between adjacent parenchymal cells (Figs. 6 and 9). This was found in all livers perfused with fatty acid and was not related to the part of the lobule studied.

As in the livers observed after shorter periods of fatty acid perfusion, numerous highly electron-opaque 300–800 A particles were noted within the tubules of the smooth endoplasmic reticulum (Figs. 7–9), deep in the cytoplasm (Figs. 8 and 9), and near the cell periphery (Figs. 7 and 9). Also, as in the earlier stages studied, increased numbers of particles were observed within vacuoles located near the Golgi and in those opening onto the space of Disse (Fig. 6). Particles located in the smooth-surfaced terminal ends of the cisternae of granular reticulum continued to be a frequent finding (Figs. 10–12). Despite the fact that the 300–800 A dense particles within the Golgi complexes often accumulated near the bile canaliculi, they were never noted within the bile capillaries or ductules. The number of osmiophilic particles in control livers perfused for 30–60 min with a linoleate-free medium was essentially the same or somewhat less than at 0 time (Fig. 9a). Sizable lipid droplets free in the cytoplasm were frequently found in all livers and they appeared to increase moderately both in number and size as a result of perfusion with a fatty acid-enriched medium. Particularly striking was the increase in their electron opacity after perfusion with linoleate (Figs. 7, 8, 12).

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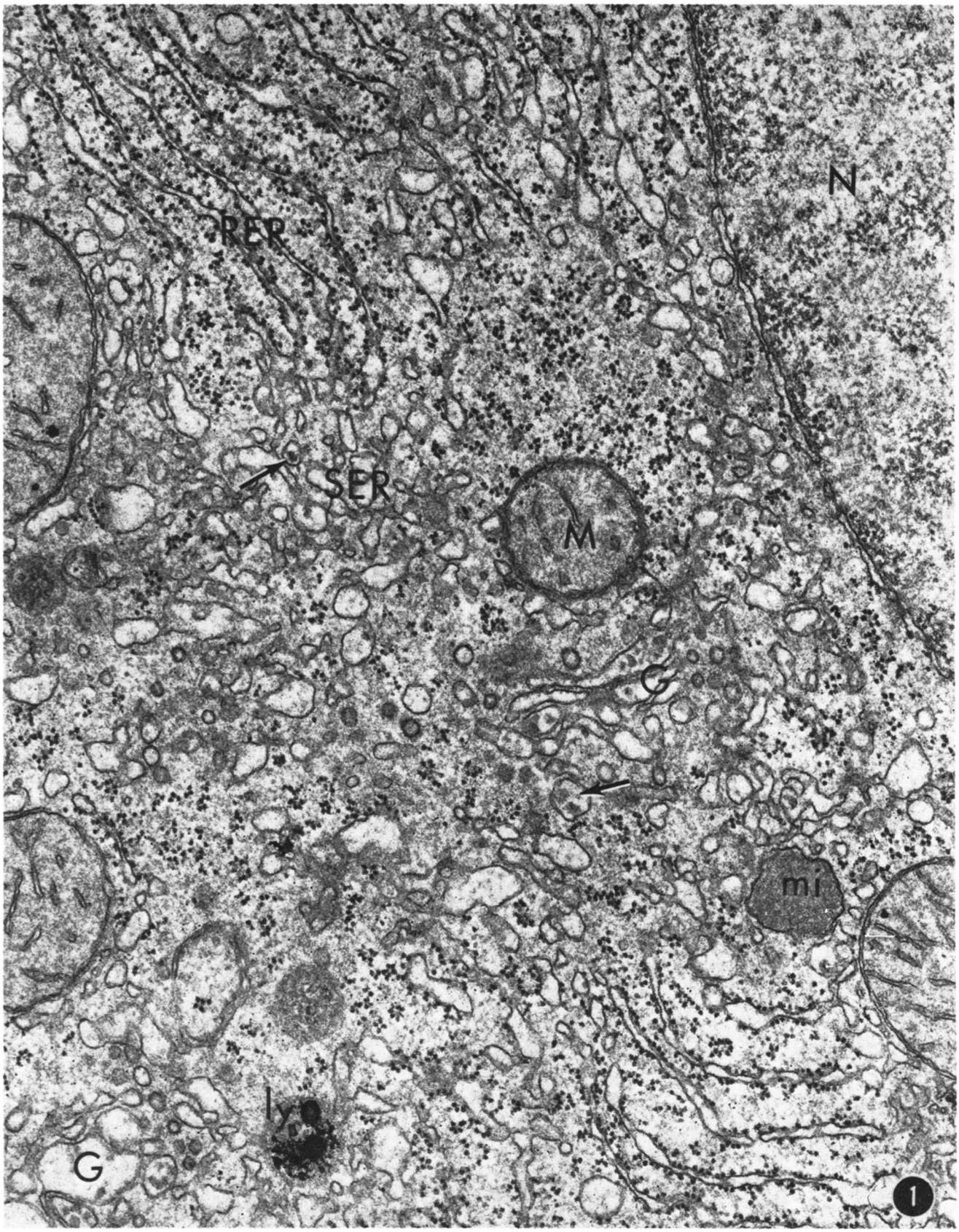


FIG. 1. An electron micrograph of a rat liver cell just prior to fatty acid perfusion (0 time). Rough (*RER*) and smooth (*SER*) endoplasmic reticulum and a Golgi apparatus (*G*) are seen. A few moderately electron-opaque particles are visible (arrows) within the Golgi cisternae and occasionally in the smooth reticulum. A portion of the nucleus (*N*), a lysosome (*ly*), a microbody (*mi*), and several mitochondria (*M*) are also shown. × 38,000.

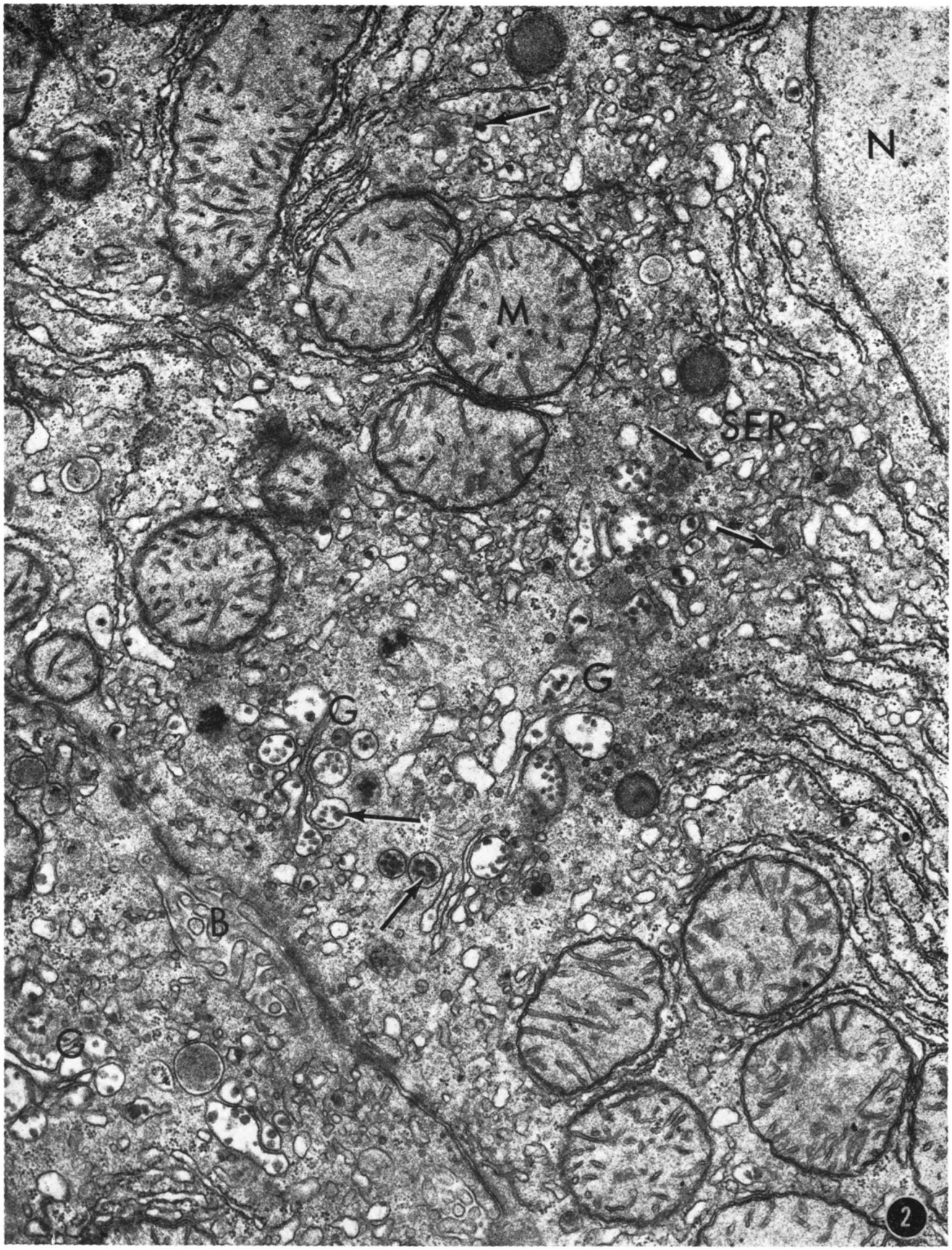
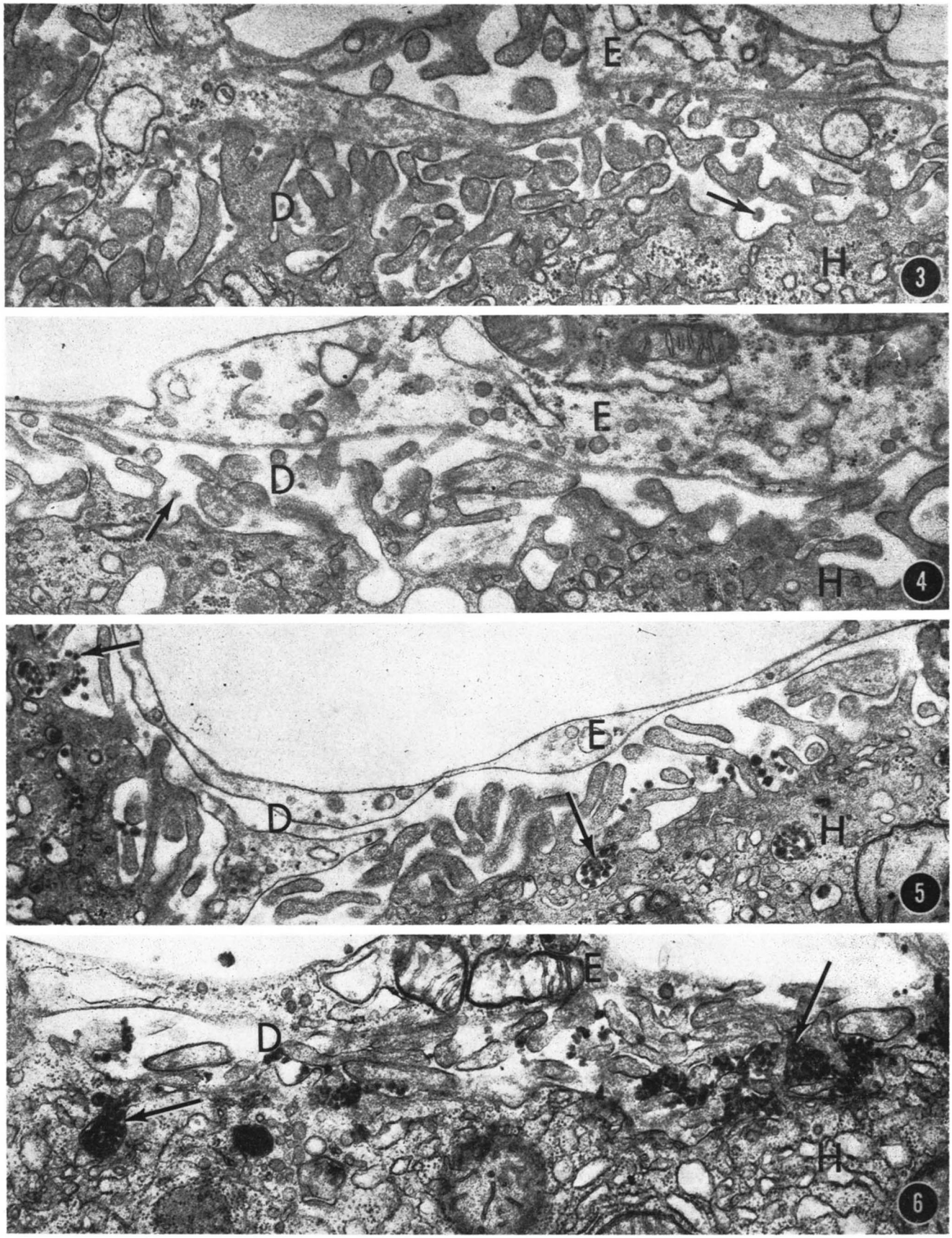
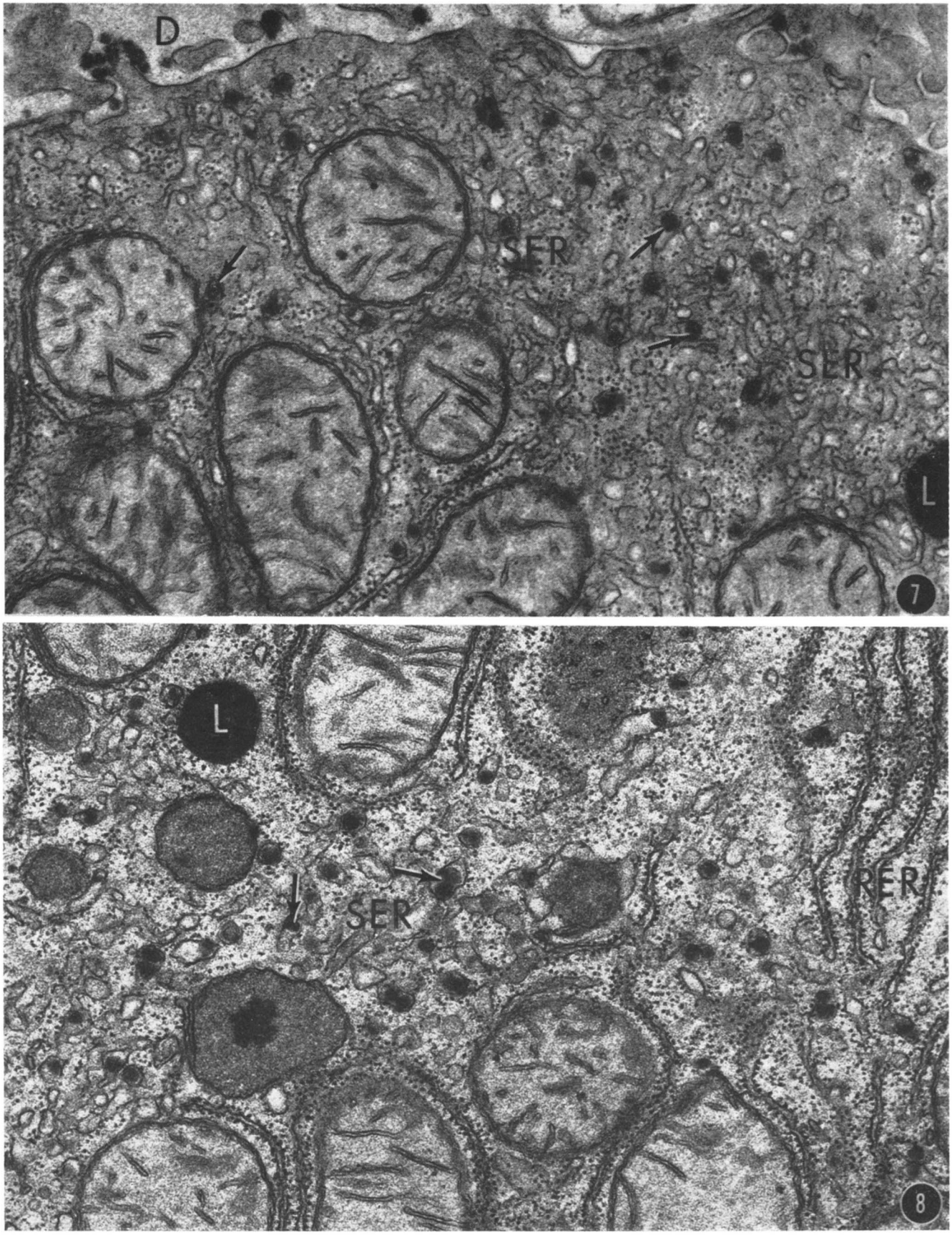


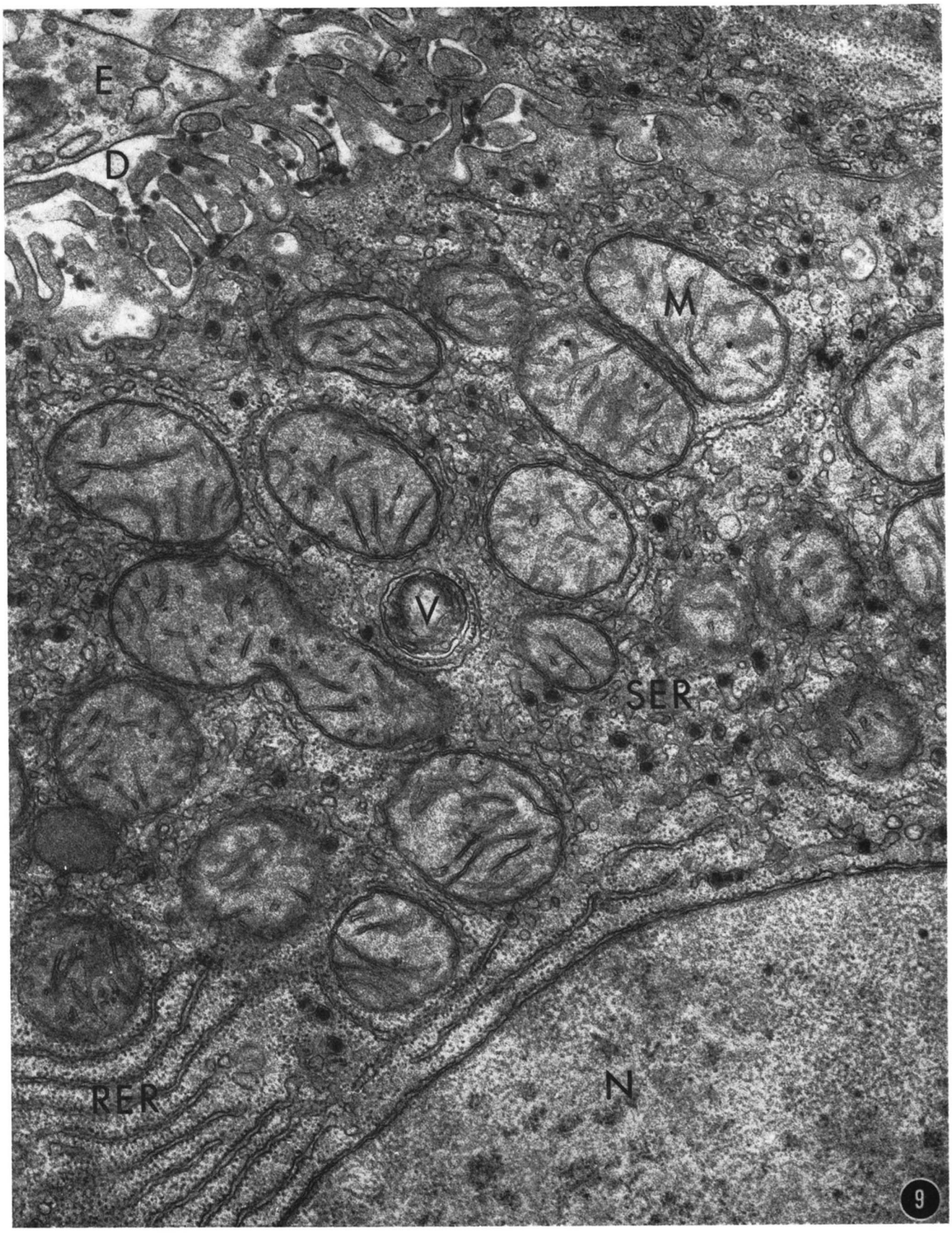
FIG. 2. Portions of two liver cells after 2 min of perfusion with linoleate. Numerous 300–800 A electron-opaque particles (arrows) have accumulated within the smooth reticulum (*SER*) and Golgi apparatus (*G*). Note the polarization of the Golgi towards the bile canaliculus (*B*). *N*, nucleus; *M*, mitochondrion. × 25,000.



FIGS. 3–6. Electron micrographs of hepatic subendothelial spaces (*D*, space of Disse) after various periods of perfusion with a linoleate-rich medium. At 0 time (Fig. 3) and after 2 min of perfusion (Fig. 4), an occasional moderately electron-opaque particle can be seen in the space of Disse (arrows). *E*, endothelial cell. After 5 min of perfusion (Fig. 5) the particles are more numerous and electron opaque. They appear either singly or in clusters packed within infoldings of the parenchymal cell (*H*) surface. Except for the lack of a limiting membrane, they appear identical with the particles found within the liver cell cytoplasm. After 60 min of perfusion (Fig. 6) the accumulation of particles in the space of Disse is even more striking. Figs. 3 and 4, × 30,000. Figs. 5 and 6, × 25,000.



FIGS. 7 and 8. Portions of liver cells after 60 min of fatty acid perfusion showing the osmiophilic particles (arrows) within the tubules of the smooth reticulum (*SER*). In Fig. 7, they are located near the vascular surface of the cell, whereas in Fig. 8 they are in a more central portion of the hepatocyte. The electron opacity of the 300–800 A membrane-bounded particles and the cytoplasmic lipid droplets (*L*) has markedly increased. *RER*, rough endoplasmic reticulum; *D*, space of Disse. × 42,000.



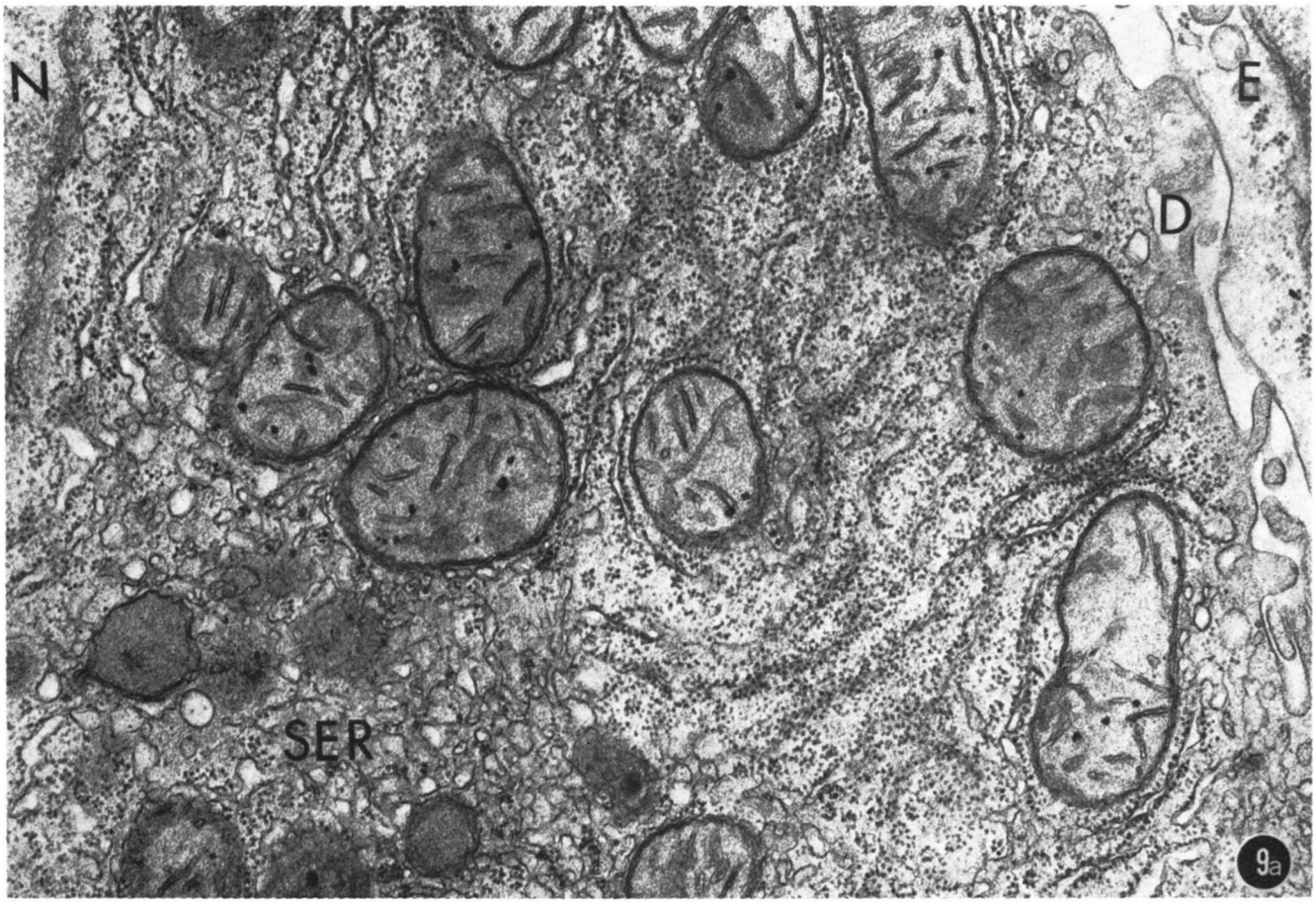
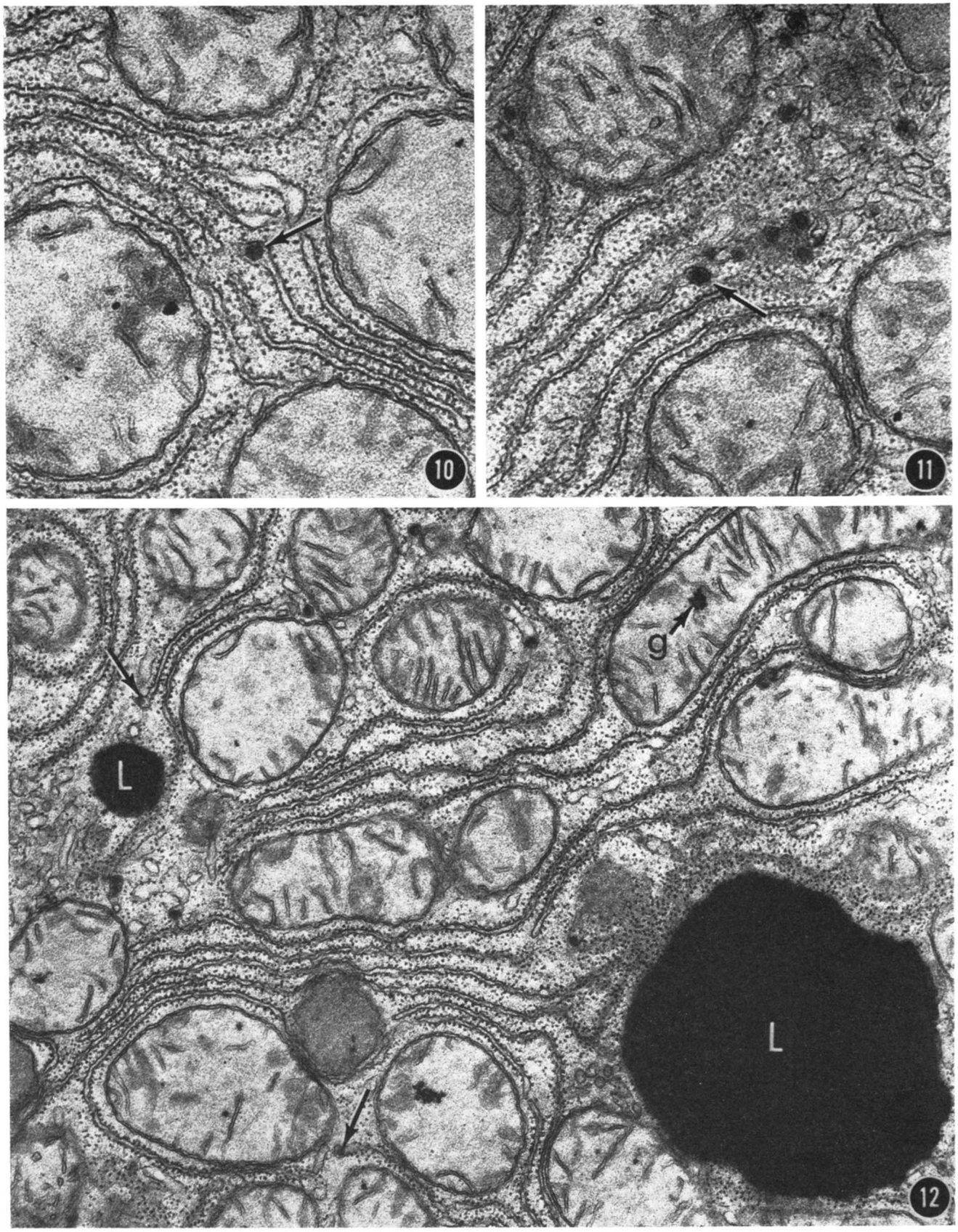


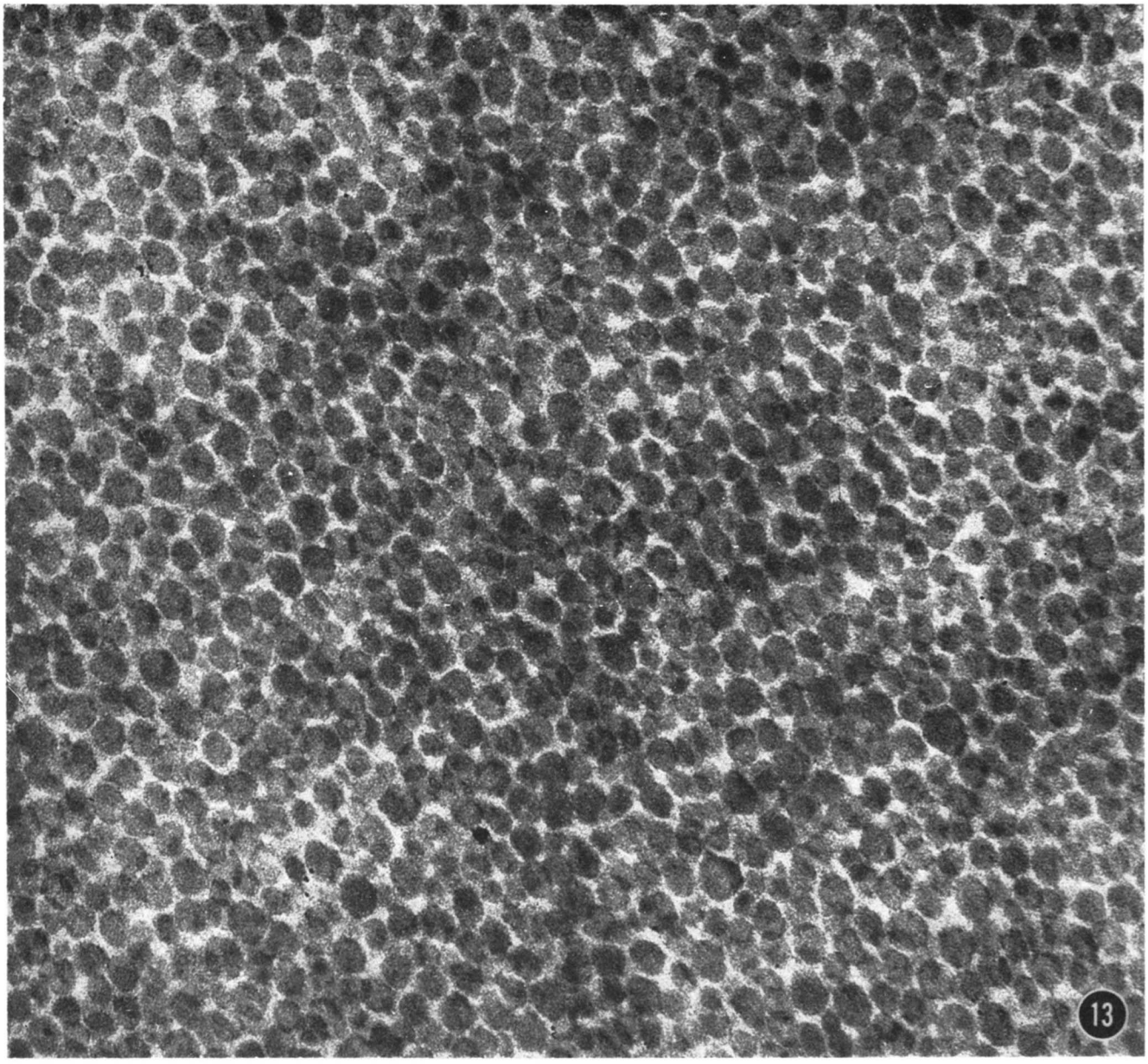
FIG. 9a. Control liver perfused for 30 min with medium devoid of fatty acid. 300–800 A particles within either the smooth endoplasmic reticulum (*SER*) or space of Disse (*D*) are exceedingly rare. *E*, endothelial cell; *N*, nucleus. × 30,000.

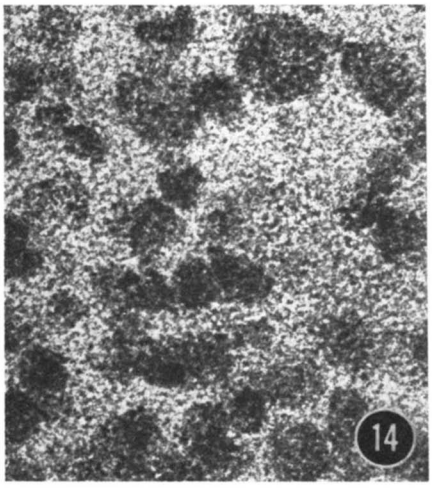
FIG. 9. After 5 min of perfusion with a fatty acid-rich medium and until the termination of the perfusion (60 min shown here), the electron-opaque particles are widely distributed throughout the cell and are also seen in the space of Disse (*D*). An autophagic vacuole (*V*) is observed midway between the nucleus and cell surface. *E*, endothelial cell; *M*, mitochondrion; *N*, nucleus; *RER*, rough endoplasmic reticulum; *SER*, smooth endoplasmic reticulum. × 32,000.

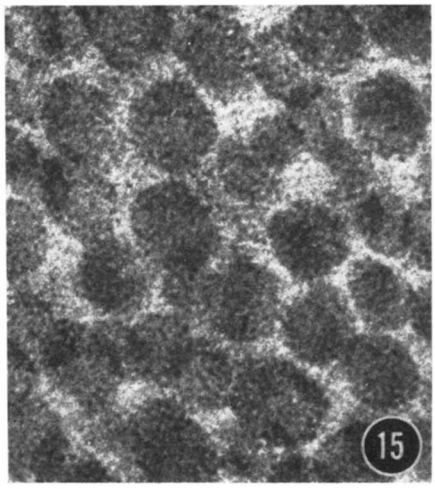


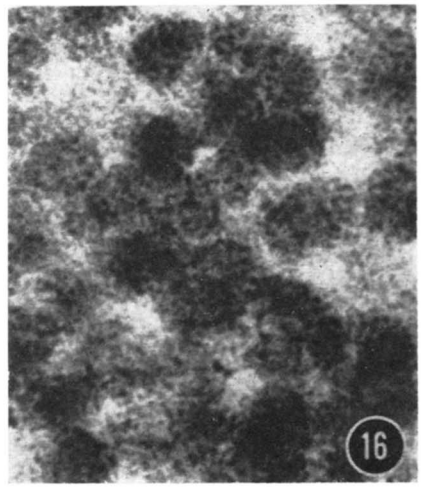
FIGS. 10 and 11. Osmiophilic particles (arrows) within the smooth-surfaced terminal ends of the rough reticulum after 60 min of fatty acid perfusion. × 53,000.

FIG. 12. 300–800 A particles (arrows) within the smooth reticulum in close relationship with the rough reticulum. A highly electron-opaque cytoplasmic lipid droplet (*L*) is shown, as is an example of clumping of intramitochondrial granules (*g*). 60 min of fatty acid perfusion. × 26,000.



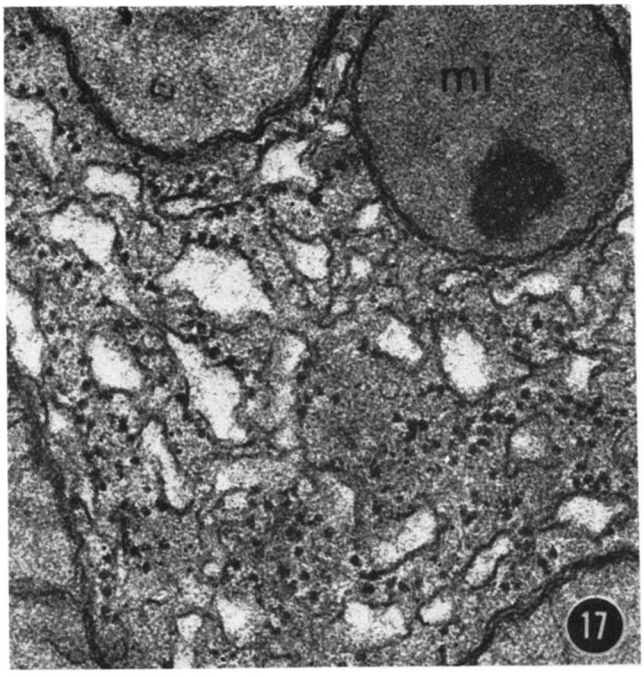


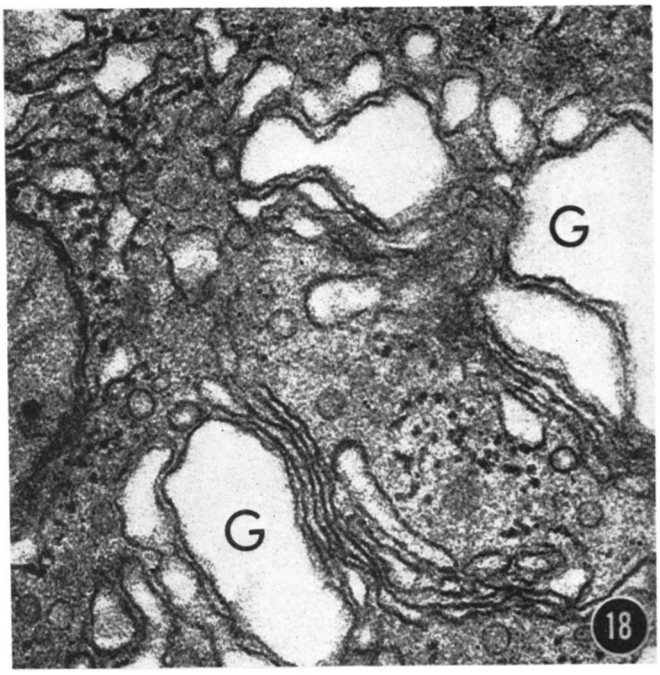


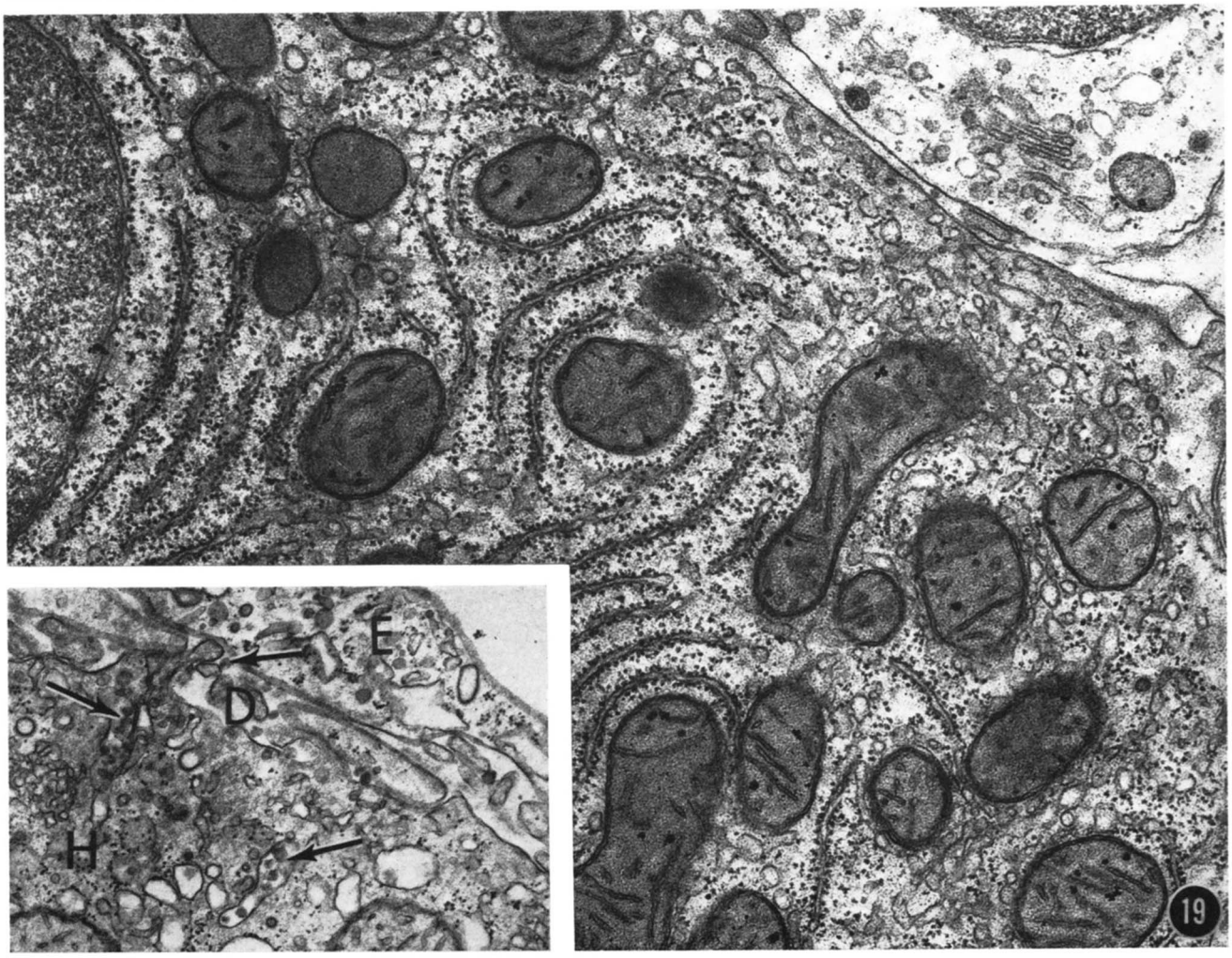


FIGS. 13 and 15. Osmium fixation of the d $<$ 1.006 fraction of fatty acid-rich medium after 60 min of perfusion yielded a small dense pellet consisting of 300–800 A particles. These are seen at higher magnification in Fig. 15. Fig. 13, $\times$ 87,000; Fig. 15, $\times$ 150,000.

FIGS. 14 and 16. Electron micrographs of osmium fixed d $<$ 1.006 fraction of human and rat plasma after removal of the chylomicrons. The particles found in this fraction are approximately the same size as those found in the postperfusion medium. $\times$ 150,000.







There was no evidence that the 300–800 A particles fused with the larger droplets as has been reported by others (21, 22, 32, 36).

The endoplasmic reticulum, which is considered by some to be a sensitive indicator of cell injury, maintained its normal architecture during the 60 min of perfusion in both control livers and those perfused with fatty acid (Figs. 7–12). Some alteration in the intramitochondrial granules was found in several, but not all, of the livers of this group (Fig. 12). Instead of appearing as discrete individual densities, they appeared as a cluster of smaller, denser granules closely applied to one another. In some instances, the number of the intramitochondrial granules was apparently reduced. There did not appear to be any quantitative change in microbodies, but autophagic vacuoles were observed more frequently after 60 min of perfusion regardless of whether the liver was perfused with the basic medium or one enriched with fatty acid (Fig. 9). Lysosomes, however, appeared to increase in number, but only in those livers perfused with fatty acid. When livers were perfused with linoleate in a medium containing crystalline albumin, rather than the usual fat-free bovine serum albumin (Cohn Fraction V), there was no substantial difference in the number or appearance of the newly synthesized 300–800 A particles.

Electron Microscopy of the Perfusate

Osmium tetroxide fixation of the linoleate-rich media after 60 min of perfusion or of the d < 1.006 fraction from the same media yielded on centrifugation a pellet which contained a relatively homogeneous population of spherical or slightly oval particles (Figs. 13 and 15). These were of the same size, shape, and density as those seen in thin sections of osmium tetroxide-fixed liver tissue (Figs. 7, 8, 10, 11). Bodies of similar size and appearance could also be found in the d < 1.006 fraction of human and rat serum (Figs. 14 and 16), as well as in the dextran-precipitable fraction of the postperfusion medium. However, the particles isolated by the dextran sulfate technique were always less electron opaque than those isolated directly from the perfusate. No pellet was obtained from media devoid of linoleate. As an additional control, a linoleate-containing perfusate was circulated through the perfusion apparatus for 60 min without a liver in the system. Under these conditions, there was no evidence of particle formation.

Puromycin Treatment

Livers perfused with a medium that contained oleate were very similar in appearance to livers perfused with linoleate. The only difference was that the newly formed 300–800 A particles were of lower electron opacity (Fig. 19, inset) and somewhat less in number than those observed after 60 min of linoleate perfusion. Treatment with puromycin inhibited the production of these particles, as indicated by their almost complete absence from the endoplasmic reticulum and Golgi apparatus (Figs. 17–19) and by the markedly diminished size of the pellet obtained upon osmium fixation and centrifugation of the medium after 60 min of perfusion. On the other hand, puromycin did not appear to interfere with the moderate increase in the number and size of the cytoplasmic droplets of neutral fat. The smooth and rough endoplasmic reticulum and Golgi apparatus, however, often appeared dilated after puromycin treatment and frequently the cisternal profiles of rough reticulum were shortened and somewhat degranulated (Figs. 17 and 18). The extent of these latter effects was variable even within the same liver (Fig. 19).

In addition to these morphologic alterations, puromycin markedly affected hepatic lipid and protein metabolism (Table 1). The incorporation of leucine-^{3}H into liver and lipoprotein protein and of oleate-^{14}C into lipoprotein triglyceride were all decreased to less than 10% of the values obtained in the control livers. In contrast to this, incorporation of oleate-^{14}C into hepatic triglyceride was not affected. The perfusate of one of the puromycin-treated livers was exceptional in that upon osmium fixation it yielded a pellet similar to that of the controls. The incorporation of oleate-^{14}C and leucine-^{3}H into the lipoprotein fraction of this perfusate also approached control values.

DISCUSSION

The isolated, perfused rat liver was utilized in the present study in order to eliminate many of the problems that

FIG. 17. Marked dilatation and partial degranulation of the rough endoplasmic reticulum in a puromycin-treated liver. *mi*, microbody. × 55,000.

FIG. 18. Dilatation of the Golgi (*G*) and the almost complete absence of particles within its cisternae in a puromycin-treated hepatocyte. × 55,000.

FIG. 19. A portion of a cell which maintained a relatively normal appearance after exposure to puromycin. Even in these cells, however, there is a conspicuous absence of the 300–800 A membrane-bounded particles which normally accumulate after 60 min of fatty acid perfusion. *H*, parenchymal cell. × 29,000.

Inset, a portion of liver cell perfused with oleate in the absence of puromycin. Moderately electron-opaque particles (arrows) are seen in the smooth reticulum and space of Disse (*D*). × 24,000.

TABLE 1 EFFECT OF PUROMYCIN ON INCORPORATION OF OLEIC ACID-^{14}C AND LEUCINE-^{3}H INTO TISSUE AND LIPOPROTEIN TRIGLYCERIDE AND PROTEIN

	Radioactivity Incorporated		
	Control	Puromycin	*P*
	*10^{-3} × dpm/g liver dry wt ± sem**		
Liver protein	32,370 ± 4194 (n = 6)	451 ± 100 (n = 5)	<0.001
Liver triglyceride	937 ± 250 (n = 6)	956 ± 480 (n = 5)	NS
Lipoprotein protein	410 ± 58 (n = 6)	74.9 ± 50.0 (n = 4)	<0.005
		24.5 ± 5.9† (n = 3)	<0.001
Lipoprotein triglyceride	18.0 ± 3.3 (n = 6)	2.84 ± 1.9 (n = 5)	<0.005
		1.0 ± 0.5† (n = 4)	<0.005

* Corrected for differences in total radioactivity of initial medium.

† For undetermined reasons, one puromycin-treated liver had a 10 times greater incorporation of label into lipoprotein triglyceride and protein than did the others. It also had a much larger pellet after osmium fixation and centrifugation of the postperfusion medium. When it was excluded from the calculations these results were obtained.

result from the use of an in vivo system. The perfusion system used in this investigation had previously been shown to allow adequate liver function as judged by bile production, oxygen extraction (46, 47), biochemical behavior (46–49), and even mitochondrial respiratory control (F. L. Hoch and N. B. Ruderman, unpublished data). The present study also demonstrates that these livers are intact morphologically after 1 hr of perfusion. This is evidenced by our failure to find the alterations in liver cell nuclei, endoplasmic reticulum, and (or) lysosomal membranes that have been noted by morphologists studying the effect of hypoxia on the liver (50–54). Changes in the appearance of the mitochondrial granules were noted; however, their significance is not clear.

Identification of the Particles

Previous investigations have shown conclusively that the isolated perfused liver is able to synthesize lipoprotein (3, 5, 8, 55, 56). The present study strongly suggests that the membrane-bounded electron-opaque particles within the liver cell, as well as those found free in the vascular space, are very low density lipoproteins. First, they are of the same size, shape, and relative electron opacity as the particles in the very low density lipoprotein fraction isolated from human and rat serum in this and other studies (14), and are themselves recovered in the d < 1.006 and dextran-precipitable fractions of the postperfusion medium. Second, they increase markedly in number and electron opacity in both the liver cell and the perfusate when a highly unsaturated (and therefore osmiophilic) (57–59) fatty acid is added to the perfusing medium. It has previously been shown by Nestel and Steinberg (60) and Heimberg, Weinstein, Dishmon, and Fried (61) that isolated rat livers produce and release considerably more triglyceride when perfused with a fatty acid-rich medium than when perfused with a fatty acid-poor medium. Finally, fatty acid stimulation of the 300–800 A particle formation was markedly reduced when the livers were treated with puromycin, an agent which depressed the incorporation of oleate and leucine into the lipoprotein fraction of the medium. Puromycin also depressed the incorporation of leucine into liver protein but had no effect on the incorporation of oleate into hepatic triglyceride. This finding is important, for it indicates that protein synthesis rather than lipid synthesis was rate limiting with respect to the appearance of the "lipoprotein" particles. It is also consistent with the view that the small spherical bodies seen inside the liver represent lipid already complexed to protein rather than simple lipid droplets.

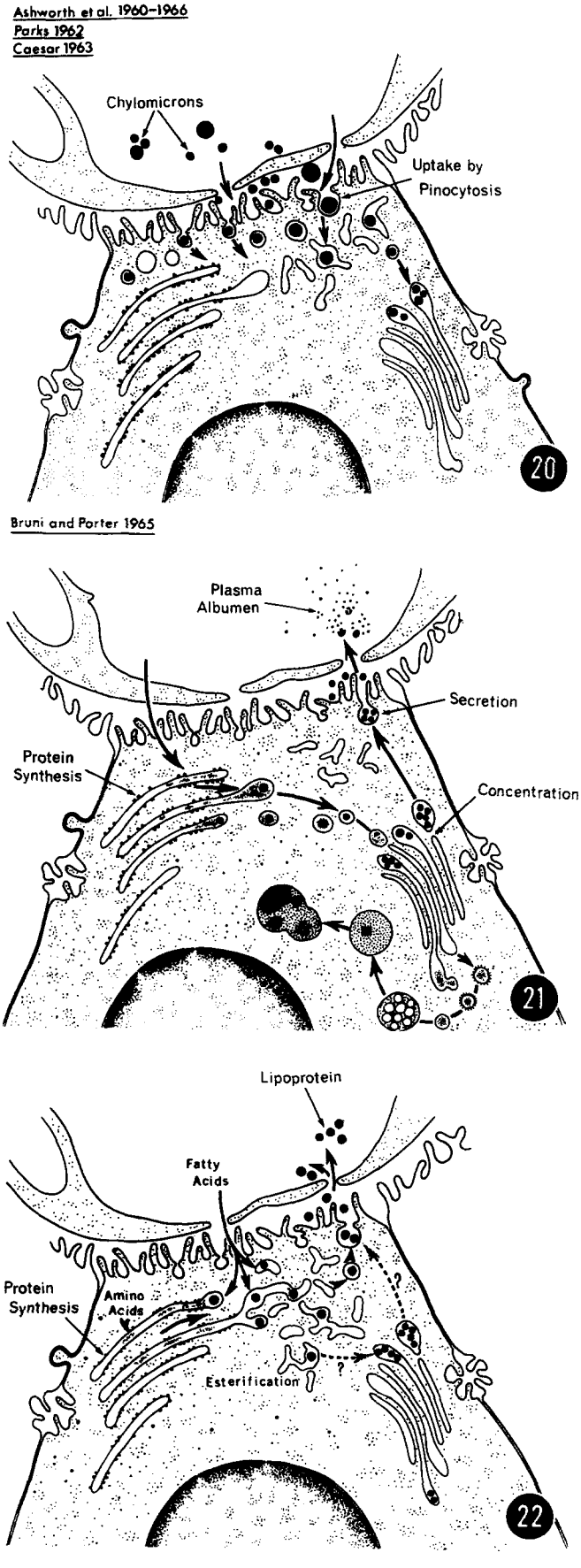
Many of the above findings make it appear most unlikely that the membrane-bounded particles are serum albumin produced by the liver cell, as has been suggested by Bruni and Porter (17) (Fig. 21). Moreover, the particles described here appeared in all of the cells examined whereas serum albumin can be detected by a sensitive immunohistochemical method in only about 10% of liver cells (62).

The identification of the 300–800 A particles as very low density lipoproteins casts a new light on many of the previous interpretations of morphologic events associated with hepatic lipid metabolism. For this reason, the ultrastructural aspects of hepatic lipid uptake, triglyceride synthesis, and lipoprotein synthesis, transport, and release deserve more detailed discussion.

Lipid Uptake

The method by which various lipids enter cells has been the concern of a number of investigators. Palay and Karlin (63) and Palay and Revel (64) have suggested that the absorptive cell of the gut takes up fat from the intestinal lumen by pinocytosis. Although the existence of such a mechanism has been demonstrated beyond doubt, its quantitative significance has been questioned even by these investigators. More recently, Strauss (65) and Strauss and Ito (66), using radioautography together with electron microscopy, clearly demonstrated that intestinal mucosa incubated at 4°C could take up a considerable amount of fatty acid and (or) fatty acid micelles without any morphologic evidence of pinocytosis. Because of the low temperature at which the fatty acid was taken up by the cell, they suggested that it entered by simple diffusion.

It appears likely that fatty acid enters the liver by a similar mechanism. In the present study, perfusion with a fatty acid-rich medium produced no morphologic evidence of lipid uptake at the cell membrane after 2 min, although at this time a markedly increased number of osmi-



ophilic particles was noted within the endoplasmic reticulum and Golgi vacuoles. Since particles appeared at the cell surface only after 5 min of perfusion, it seems more likely that in this location they represent lipid leaving rather than entering the cell.

The substrate used in this study, i.e., fatty acid bound to albumin, is not visible as a particle by electron microscopy according to Stein and Stein (30). This was confirmed by the present study, in which there was no evidence of particle formation within the perfusate when the liver was omitted from the system.

On the other hand, Ashworth and coworkers (27–29), Parks (19), and Caesar (33) have suggested that liver takes up particulate lipid, i.e., chylomicrons, by means of pinocytosis (Fig. 20). As evidence for this, they noted that increased numbers of osmiophilic particles appeared in the liver cell after a fat meal. However, the diameter of the membrane-limited intracellular particles which they described more closely approximated that of a very low density lipoprotein (300–1000 A) than that of a chylomicron (0.1–1 μ). In view of our findings, we suggest that the particles in question were newly synthesized, very low density lipoproteins that had been derived in part from chylomicron lipid, and that the lipid of the latter had entered the cell by a means other than pinocytosis. The recent studies of Felts (67) and Mayes and Felts (56) revealed that an isolated perfused rat liver would take up very little, if any, chylomicron triglyceride unless heparin was added to the perfusing medium. This finding is explained by the observation (56, 67–71) that heparin promotes the secretion and activation of a hepatic lipoprotein lipase which can hydrolyze chylomicron lipids. Definitive evidence that pinocytosis of particulate lipid is an important mechanism of hepatic lipid uptake is therefore lacking.

Fatty Acid Esterification and Lipoprotein Synthesis

In the liver cell, fatty acids may be oxidized or incorporated into triglycerides, phospholipids, or cholesteryl esters. The enzymes necessary for the latter processes are for the most part in the microsome fraction (72–76), which in the intact cell is composed of smooth and rough endoplasmic reticulum and Golgi apparatus (77–79). Phosphatidic acid phosphatase may be exceptional in that it seems to be located in the lysosomes (80).

The precise site of fatty acid esterification is unknown,

FIG. 20. A schematic diagram of a liver cell in the process of taking up intact chylomicrons by pinocytosis (19, 27–29, 33).

FIG. 21. The Bruni-Porter conception of the origin and fate of the 300–800 A particles (17).

FIG. 22. Schematic view of the synthesis, transport, and release of the 300–800 A particles, as suggested by the present investigation.

but the smooth endoplasmic reticulum, an organelle already implicated in lipid metabolism in both the liver and intestinal mucosa (24, 65, 81, 82), seems the most likely possibility. This view is not accepted by all investigators (83) but is supported by the observation that agents such as carbon tetrachloride (30) and puromycin which disrupt the rough endoplasmic reticulum of the liver cell, do not interfere with fatty acid esterification. Studies utilizing relatively pure fractions of hepatocyte rough and smooth endoplasmic reticulum as isolated either by Dallner (84) or Rothschild (78) may provide the answer to this question.

The ultrastructural localization of hepatic triglyceride synthesis is also made difficult by the fact that liver contains at least two triglyceride pools. One pool, which turns over rapidly, has been localized in the microsome fraction (73). Morphologically it is probably represented by the membrane-bounded 300–800 A particles. A second pool, which has a slower turnover, has been localized in the soluble portion of the cytoplasm (73) and is probably represented morphologically by the free cytoplasmic lipid droplets. In the present study, it appears that fatty acid was incorporated into both pools, since the membrane-bounded particles and the cytoplasmic lipid droplets both become more osmiophilic upon perfusing with linoleate. At present, it is difficult to say how these two morphologic pools interrelate. Baglio and Farber (31), Trotter (21, 22) and Hamilton et al. (36) have suggested that the membrane-bound particles may, under certain circumstances, fuse to produce the large cytoplasmic lipid droplets. Such an occurrence was not noted in the present study. It has also been suggested that the enzymes necessary for neutral lipid synthesis might be located on both sides of the endoplasmic reticulum, thereby allowing fat synthesis to go on simultaneously in both the lumen of the endoplasmic reticulum and the cell sap (81). This explanation, however, is not supported by any firm experimental evidence.

Although the precise interrelation of the two triglyceride pools remains in doubt, the availability of the protein moiety of the lipoprotein (apolipoprotein) appears to be a key determinant of whether newly synthesized triglyceride is incorporated into the lipid droplet or exported from the cell as lipoprotein. In our experiments, puromycin inhibited triglyceride transport out of the liver while it had no effect on the incorporation of fatty acid into tissue triglyceride. In addition, Robinson and Seakins (85) have found that puromycin administered in vivo markedly depresses the ability of rat liver to produce lipoprotein and results in a fatty liver.

The apolipoprotein is in all likelihood synthesized, like other proteins, in the granular reticulum. Even though most of the 300–800 A particles observed in the livers perfused with a high concentration of fatty acid appear in the smooth endoplasmic reticulum, they are frequently present at the sites of confluence of smooth and rough surfaced reticulum. These well-documented continuations between the rough and smooth endoplasmic reticulum conceivably represent a pathway by which the specific acceptor protein could reach the smooth reticulum, there to combine with the lipid moiety of the lipoprotein. It is unlikely that significant stores of preformed apolipoprotein are present in the liver, since puromycin blocked the incorporation not only of amino acid but also of lipid into lipoproteins. If this is the case, our data would imply that perfusion with a medium rich in fatty acid stimulated the synthesis of the apoprotein.

Lipoprotein Transport and Release

It is likely that at least a portion of the newly formed lipoprotein arrives at the cell periphery by moving through the tubules of the agranular reticulum (Fig. 22). In this location, smooth-membraned vesicles containing the particles may bud off the reticulum, travel to the cell surface, and there release the particles. The finding of packets of particles in the Golgi region after 2 min of fatty acid perfusion, and at the cell surface after 5 min, suggests an alternative pathway of lipoprotein transport involving the Golgi apparatus (Fig. 22), as has been emphasized by Hamilton et al. (36). Since lipoproteins are actually glycolipoproteins (86–88), it is also conceivable that the Golgi complex plays a role in the addition of the carbohydrate moiety, a function suggested by the experiments of Neutra and Leblond (89–90).

Although this investigation has left many questions unanswered, we hope that the identification of the 300–800 A intrahepatic particles as lipoproteins will enable the morphologist to play an important role in the understanding of lipoprotein metabolism in health and disease. We also hope that this study has served to establish the value of the isolated perfused liver as a useful experimental system for combined morphologic and biochemical studies of hepatocyte function.

Note Added in Proof. While this article was in press a report appeared concerning the effect of diet on the osmiophilia of 300–1000 A membrane-bounded particles in mouse liver (91).

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