# LIPID SYNTHESIS, INTRACELLULAR TRANSPORT, STORAGE, AND SECRETION

## I Electron Microscopic Radioautographic Study of Liver after Injection of Tritiated Palmitate or Glycerol in Fasted and Ethanol-Treated Rats

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ABSTRACT

A time sequence study of intracellular movement of labeled lipid in the liver was carried out on fasted and ethanol-treated rats injected with either palmitate-$^3$H or glycerol-$^3$H by electron microscopic radioautography. The elimination of water-soluble lipid precursors during specimen preparation was checked and found to be complete. The labeled lipid product in the tissue was identified as mostly triglyceride. A dehydration procedure was adapted to minimize the loss of lipid during specimen preparation. At 2 min after injection, the earliest time interval studied, both precursors were found to have penetrated the liver cells, and the label was found over both rough and smooth elements of the endoplasmic reticulum, which is the site of glyceride esterification. From 5 min on, in fasted and especially in ethanol-treated rats, the label was seen also over lipid droplets 0.5–2.0 $\mu$ in diameter, which represent "storage lipid" (slowly turning over compartment). Mitochondria became labeled mostly at later time intervals after injection. From 10 min on, concentration of label was seen over the Golgi apparatus, containing small osmiophilic particles. Association of label with groups of particles in smooth-surfaced vesicles and vacuoles in and near the Golgi apparatus and in the vicinity of the sinusoidal border was seen, both after palmitate-$^3$H and glycerol-$^3$H. It is proposed that these particles represent lipoproteins which are formed in the endoplasmic reticulum, "processed" in the Golgi apparatus, and transported in vacuoles to the sinusoid surface to be discharged into the circulation.

### INTRODUCTION

During the past decade, many of the biochemical events resulting in lipid synthesis in the liver have been elucidated (17, 39, 40), and the liver has been shown to be the major source of serum lipids (41). On the basis of subcellular fractionation, the site of triglyceride and phospholipid formation in the liver was localized to the microsomal fraction. Following injection of labeled palmitic acid, the specific activity of the lipid in the microsomal fraction decreased with time, indicating rapid turnover. Labeled esterified lipid, mainly triglyceride, was recovered also in the fat cake, which floated during ultracentrifugation (41). The specific activity of this lipid increased with time

and reached an equilibrium with that of the microsomal lipid in 2 hr. Another metabolic process occurring concurrently was the appearance of labeled triglycerides in the circulation 20 min after the introduction of the radioactive fatty acid (41). The present study was undertaken to correlate the findings derived from subcellular fractionation with the biochemical events occurring in the intact cell. Preliminary experiments in which localization of labeled lipids was carried out with electron microscopic radioautography indicated the feasibility of this approach, when labeled glycerol (37) and palmitic acid (38) are used as lipid precursors.

## MATERIALS AND METHODS

Female albino rats of the Hebrew University strain, weighing 60–80 g and fed Purina laboratory chow, were used. All rats were fasted for 16 hr. One group was given a single dose of ethanol by stomach tube (0.6 g/100 g body weight in a 50% solution w/v) and the other was given water. 4 hr after the administration of ethanol or water the animals were injected into the tail vein either with 0.8–1.2 mc of 9,10-palmitic acid-$^3$H (specific activity 350 $\mu$c/$\mu$mole) complexed to rat serum proteins as a sodium salt, or with 5 mc of 2-glycerol-$^3$H (specific activity 0.76 mc/mg) in 0.9% sodium chloride. Samples of blood were taken for the determination of the disappearance of the label and the analysis of the labeled lipid. Liver samples were obtained under ether anesthesia 2 min–4 hr after injection of the labeled precursor. In some experiments, the 2-, 5-, and 10-min biopsies were obtained from the same animal by removing part or a whole lobe of the liver; in others, only one sample was taken.

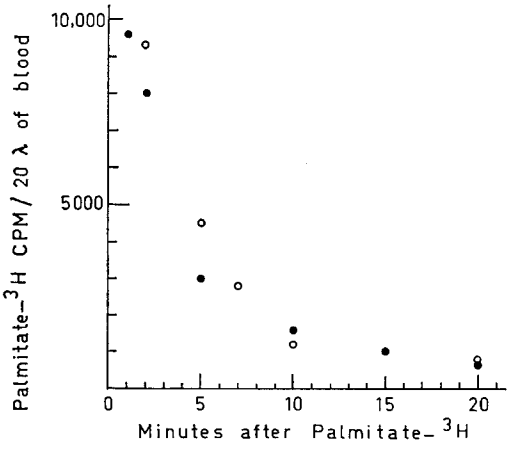



FIGURE 1 Disappearance of palmitic acid-$^3$H from the circulation following intravenous injection into rats. ●—experiment 1; ○—experiment 2.

TABLE I

*Elimination of Water-Soluble Precursors During Fixation and Washing*

| Precursor injected | Phase | Distribution of radioactivity in aqueous and chloroform phases | | |
|---|---|---|---|---|
| | | I | II | |
| | | Unfixed liver | Fixative + washes | Fixed and washed liver |
| | | % | % | % |
| Glycerol-$^3$H | Aqueous | 81 | 81 | 1 |
| | Chloroform | 19 | — | 19 |
| Choline-$^3$H | Aqueous | 42 | 43.5 | 1.5 |
| | Chloroform | 58 | — | 55 |
| Palmitic Acid-$^3$H | Aqueous | 3 | 2.5 | 0.5 |
| | Chloroform | 97 | — | 97 |

The radioactivity in the protein cake from the interphase was less than 1% of total radioactivity.

### *Extraction and Fractionation of Lipids*

A part of the liver samples was processed for electron microscopy (vide infra); the other part was weighed, homogenized in chloroform:methanol (2:1), and washed according to Folch et al. (12). The distribution of radioactivity was determined in the chloroform and aqueous phases as well as in the interphase. The protein cake in the interphase was washed three times with fresh chloroform, prior to analysis. The distribution of radioactivity in lipids was determined on fractions obtained from thin layer chromatography. Separation of triglycerides, free fatty acids, diglycerides, and phospholipids was carried out in petroleum ether (30–60°): diethyl ether:glacial acetic acid (72:28:1). Phospholipids were separated in chloroform:methanol:water (70:25:4).

### *Elimination of Water-Soluble Lipid Precursor*

Prior to the visualization of radioactivity within the specimen and its interpretation as being a lipid product, the following tests were performed. After injection with labeled glycerol, palmitic acid, or choline, aliquots of liver were processed for electron microscopy and the efficacy of elimination of water-soluble lipid precursor and extent of loss of lipid from the specimen were checked. To that end, a weighed aliquot of liver, obtained after the injection of the label, was minced, fixed in glutaraldehyde at 0°, and washed 5 times in sucrose, the last wash being

overnight. Radioactivity was determined in the fixative and sucrose washes. The fixed and washed tissue was homogenized in chloroform:methanol (2:1), and radioactivity determination and lipid analysis were performed as above.

### *Loss of Lipid from Specimen*

For the purpose of checking the loss of lipid label during fixation and dehydration, samples of liver were fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated either in graded ethanols at room temperature and at 0° or with Epon 812, and infiltrated with a complete Epon mixture. All the steps of the procedure were monitored for the appearance of radioactivity, and at the end of the procedures the radioactivity remaining in the sample was determined.

### *Preparation of Tissue for Electron Microscopy and Radioautography*

Fixation was carried out in sodium cacodylate–buffered glutaraldehyde (31) at 0° for 1 hr. The tissue was washed in five changes of cacodylate–buffered 0.25 M sucrose, the last wash being overnight, and postfixed in 2.5% osmium tetroxide (8) for 1 hr at 0°. In some experiments, the tissue was fixed directly in osmium tetroxide. According to a procedure adapted from Idelman (15), dehydration was carried out at 0° with two changes of 70% ethanol (5 min each), one or two changes of 95% ethanol (5 min each), and three changes of pure Epon for 1 hr each. The tissue was transferred to the complete Epon mixture (20) and left overnight at 4°; it was then transferred to a fresh Epon mixture, warmed to 37°C for 1–2 hr, and embedded. Sections showing silver-to-gold interference colors were cut on an LKB or Porter-Blum microtome with glass knives, and picked up on thin meshed grids. Electron microscopic radioautography was carried out according to the method of Caro (6). The specimens were exposed for 8–12 wk, developed in Microdol X for 5 min at 20°, and fixed in Kodak acid fixer for 5 min. The grids were washed very briefly, stained with lead citrate (27), and examined with an RCA EM 3G microscope at 100 kv. For grain counts, both unstained and stained grids were used and micrographs were taken at an instrument magnification of 3000 and enlarged subsequently.

Light microscopic radioautography was carried out on 0.5–1.0 $\mu$ sections obtained from the same Epon blocks. The sections were coated by immersion in the Ilford K5 emulsion diluted 1:1, exposed for 2 wk, and developed in D-19 developer for 5 min at 18°. After being washed briefly, the sections were stained with methylene blue-azure II (29).

### *Determination of Radioactivity*

Radioactivity was determined with the Tri Carb liquid scintillation Spectrometer Model 314X. Simultaneous double isotope counting ($^{3}H$ and $^{14}C$) was performed as described before (35). The chloroform extracts were counted in toluene containing 0.4% diphenyl-oxazole and 0.01% p-bis,2-(5-phenyl oxazolyl)-benzene; the aqueous extracts were counted in Bray's solution, and the fractions scraped off the silica gel thin layer plates were counted in the

TABLE II

*The Fate of Labeled Lipid during Processing of Rat Liver for Electron Microscopy*

| | Room t° | 0° | 0° | Room t° | 0° | 0° |
|---|---|---|---|---|---|---|
| | Lipid-$^{14}C$ | | | Phosphatidyl choline-$^{3}H$ | | |
| | % | % | % | % | % | % |
| Ethanol 70% + 95% | 7.5 | 1.6 | 2.1 | 5.0 | 0.8 | 1.2 |
| Ethanol 100% | 6.2 | 6.8 | — | 3.5 | 1.4 | — |
| Epon | — | — | 6.8 | — | — | 3.5 |
| Propylene oxide (Po) | 2.2 | 4.5 | — | 0.4 | 0.2 | — |
| Po + Epon mixture | 1.0 | 3.5 | — | 1.0 | 1.5 | — |
| Epon mixture (37°) | 1.1 | 2.7 | 2.1 | 0.8 | 2.4 | 0.5 |
| Liver tissue | 70.0 | 70.0 | 78.0 | 79.0 | 82.0 | 85.0 |
| Total recovery | 88.0 | 88.1 | 89.0 | 88.7 | 88.3 | 90.2 |

Aliquot of liver was extracted directly with chloroform:methanol (2:1), and the lipid radioactivity recovered was taken as 100%. Other aliquots were fixed in glutaraldehyde, washed in sucrose, postfixed in osmium tetroxide, and dehydrated as described in Table. Only negligible loss of radioactivity into osmium tetroxide was found from samples prefixed in glutaraldehyde.

presence of Cab-O-Sil (Godfrey L. Cabot, Inc. Boston, Mass). The protein cake obtained at the interphase after extraction according to Folch et al. (12) was dissolved in Hyamine, the aliquots of which were counted. For the determination of radioactivity in the tissue after osmium tetroxide fixation, the tissue was homogenized in chloroform:methanol (2:1) and the homogenate was treated with a few drops of 30% $H_2O_2$ and left overnight to bleach; the resulting extract had a pale yellow appearance. Internal standards were used for the estimation of quenching and relative counting efficiency of the respective scintillating system. All labeled materials were obtained from the Radiochemical Centre, Amersham, England.

## RESULTS

### *Disappearance of Labeled Precursors from the Bloodstream*

The disappearance of injected fatty acids from the bloodstream has been investigated and determinations have shown the half-life to be extremely brief (13). Those determinations had been done after the injection of tracer amounts of fatty acid; since in the present study a much higher dose had to be utilized, it seemed pertinent to repeat them. To that end, tracer amounts of palmitic acid diluted with unlabeled palmitate to a final amount of 3 μmoles were injected into several rats. The results of two representative experiments are given in Fig. 1; the half-life of the fatty acid in the bloodstream was estimated to be shorter than 3 min. The disappearance of labeled glycerol from the bloodstream followed a different pattern. Glycerol distributed itself very rapidly in the total body water, but a high level of radioactivity remained in the bloodstream during the next 20 min (34, 46).

### *Fate of Label during Specimen Preparation*

The distribution of radioactivity between the aqueous and chloroform phases in tissue extracted directly or after fixation in glutaraldehyde and subsequent washing in sucrose is shown in Table I. It is evident that the water-soluble label, following both glycerol injection and choline injection, is eliminated completely and that the per cent of radioactivity recovered in the chloroform phase of the fixed and washed tissue is the same as in the untreated tissue. This procedure does not cause any loss of labeled palmitate from the tissue. The protein cake taken from the interphase and dissolved in Hyamine and counted was shown to contain less than 1% of the total radioactivity recovered in the sample. Only traces of radioactive glycogen could be found in *fasted* ethanol-treated rats injected with labeled glycerol.

The loss of labeled lipid during subsequent preparation of the samples for electron microscopy is summarized in Table II. In these experiments, rats were injected simultaneously with palmitic acid-$^{14}C$ and choline-$^{3}H$ in order to follow the loss of $^{14}C$-labeled total lipid and of specifically $^{3}H$-labeled phospholipid. When the time of dehydration in each ethanol solution was reduced to 5 min, and in propylene oxide to 2 min, the total loss of $^{14}C$ label amounted to less than 20%. This loss could be further reduced by eliminating the procedural steps of absolute ethanol and propylene oxide. The loss of $^{3}H$ label represents the

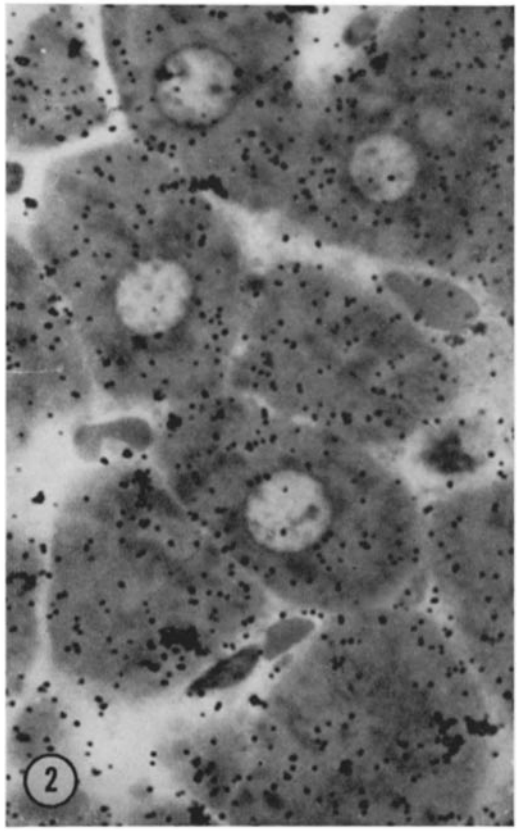
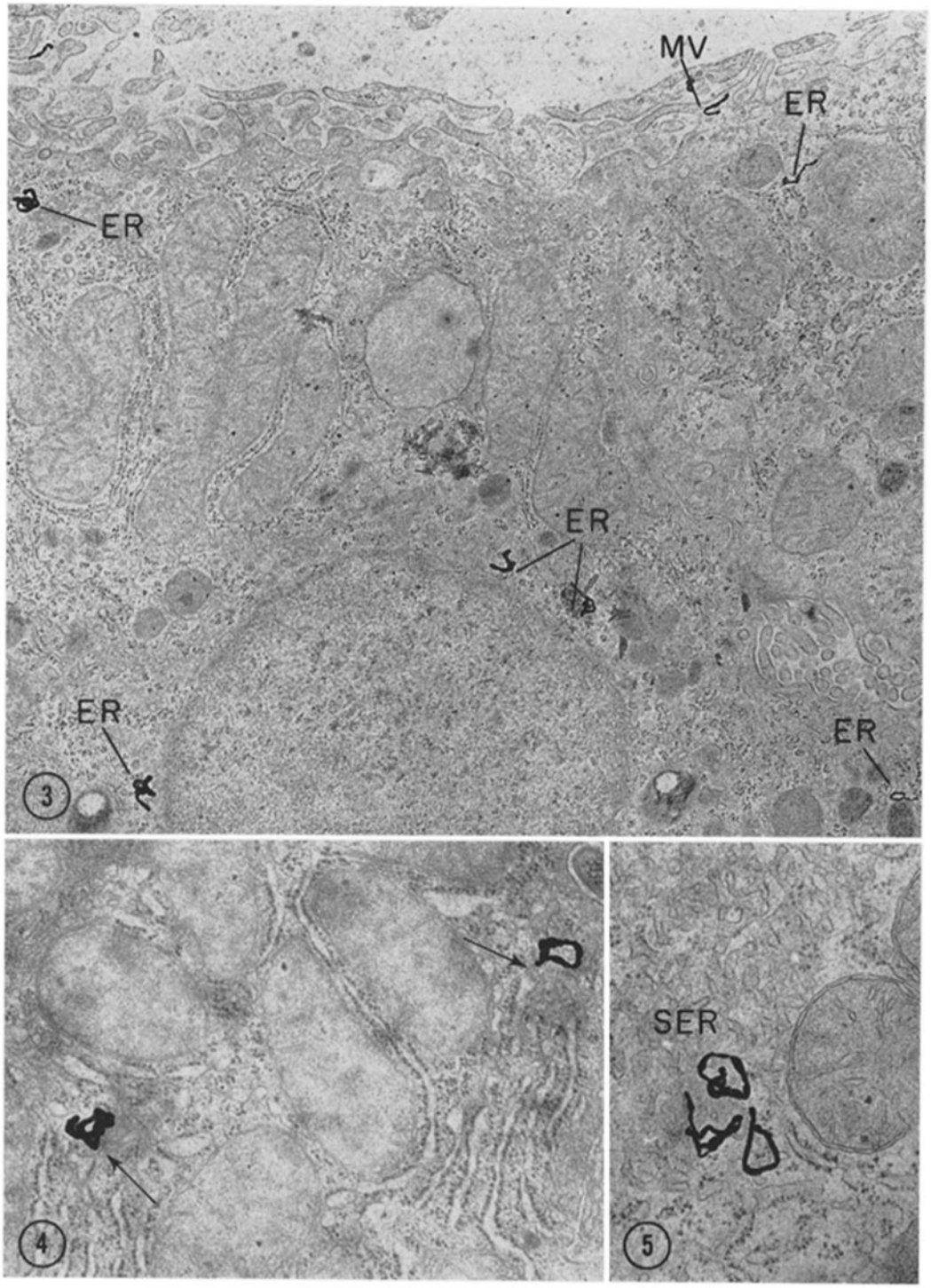



FIGURE 2 Light microscopic radioautograph of rat liver 2 min after injection of palmitate-$^{3}H$. The radioautographic reaction is seen diffusely over the cytoplasm of parenchymal cells. The nuclei and vascular spaces are free of reaction. × 1,500.




FIGURES 3–5 Liver of fasted rats 2 min after injection of palmitate-$^{3}$H.

FIGURE 3 The radioautographic grains are seen over the endoplasmic reticulum (*ER*) and microvilli (*MV*). × 16,000.

FIGURE 4 Two radioautographic grains are shown in the regions of transition between the rough and the smooth endoplasmic reticulum (arrows). × 26,000.

FIGURE 5 A concentration of grains in area of smooth endoplasmic reticulum (*SER*). × 30,000.

loss of phospholipids since practically the sole labeled lipid in the liver after injection of choline-$^3$H was lecithin. It is evident that the loss of lecithin was about half of that of combined neutral lipid and phospholipid. The loss of lipid in ethanol solutions determined chemically correlated well with the loss determined by radioactivity measurement.

### *Radioautography*

2 MIN AFTER PALMITATE-$^3$H: In 0.8–1.0 $\mu$ sections prepared from the Epon-embedded material, a diffuse radioautographic reaction was seen over the cytoplasm of liver cells (Fig. 2). Very little or no label was seen over the nuclei and sinusoids. On electron microscopic radioautography, it became evident that the radioactivity in the cytoplasm was associated mainly with elements of the endoplasmic reticulum (Figs. 3–5). The nucleus and the area of the sinusoid were free of grains, but some grains could be seen in association with the microvilli in the space of Disse (Fig. 3). In some areas, the radioautographic grains were localized in the areas of transition between rough and smooth endoplasmic reticulum (Fig. 4), and concentrations of radioactivity were seen over elements of smooth endoplasmic reticulum (Fig. 5). Very few grains were seen over mitochondria.

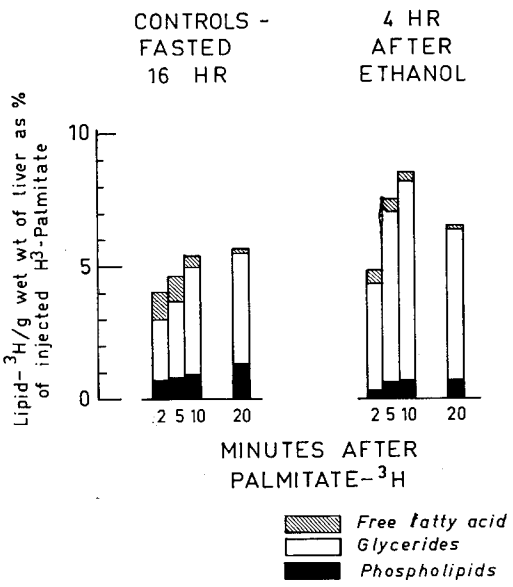
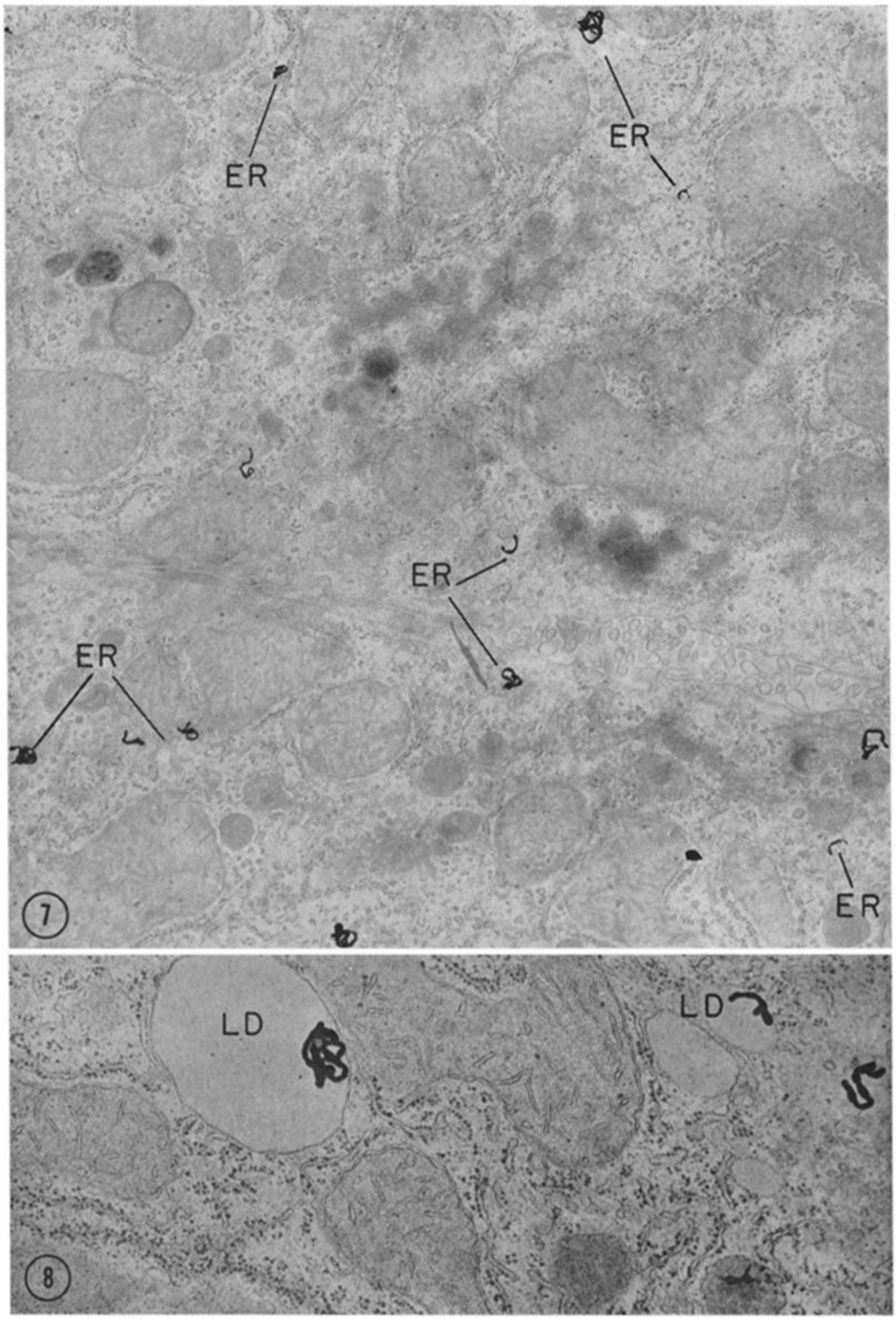



FIGURE 6 Four representative examples of uptake of palmitate-$^3$H by liver of fasted and ethanol-treated rats. Samples of liver 2–10 min after injection were derived from the same rat.

In order to enhance fatty acid uptake and esterification, the experiments were repeated in rats pretreated with ethanol. As seen in Fig. 6, at 4 hr after ethanol administration there was an increased uptake of injected palmitic acid. Analysis of the labeled lipids in the liver showed that in the fasted rats about 20% of the radioactivity was still in the form of free fatty acids, whereas in the ethanol-treated rats the extent of esterification was greater and much less free fatty acid was found even 2 min after injection. The intracellular distribution of radioactivity was essentially the same as in the livers of fasted rats, the grains being associated mainly with elements of the endoplasmic reticulum (Fig. 7). In the livers of ethanol-treated rats there was an accumulation of lipid in the form of droplets of varying size. These droplets were usually surrounded by elements of the endoplasmic reticulum, and some became labeled (Fig. 8) 2 min after injection of palmitic acid. The results obtained so far indicated that the penetration of palmitic acid into liver cells is quite rapid and that the primary site of esterification is the endoplasmic reticulum.

2 MIN AFTER GLYCEROL-$^3$H: It seemed of interest to verify these findings by using a different glyceride precursor, namely glycerol-$^3$H. This label was found especially useful when it became necessary to ascertain that the labeled lipid in the liver was formed in situ. Incorporation of glycerol into liver lipids was shown to be enhanced very significantly by pretreatment with ethanol (Fig. 9). It can be seen that the liver is the main organ of glycerol incorporation into lipids and that the lipid formed is predominantly triglyceride. On light microscopic radioautography, the reaction was seen diffusely over the cytoplasm of the liver cell, and, as with after palmitate, no reaction was observed over cell nuclei or the sinusoidal lumen (Fig. 10). In the cytoplasm the grains were associated with the endoplasmic reticulum and some mitochondria (Figs. 11 and 12).

5 MIN AFTER EITHER PALMITATE-$^3$H OR GLYCEROL-$^3$H: The label was found over lipid droplets 0.5–2.0 $\mu$ in diameter, as well as over the endoplasmic reticulum. These droplets were most prominent in livers of ethanol-treated rats (Figs. 13 and 14), and the steps leading to their formation are suggested in Figs. 15–17.




FIGURES 7 and 8 Liver of ethanol-treated rats 2 min after injection of palmitate-$^{3}$H.

FIGURE 7 The radioautographic grains are seen (arrows) over the endoplasmic reticulum (*ER*). Note that the mitochondria are not labeled. × 16,000.

FIGURE 8 Label is seen over two lipid droplets (*LD*). × 30,000.

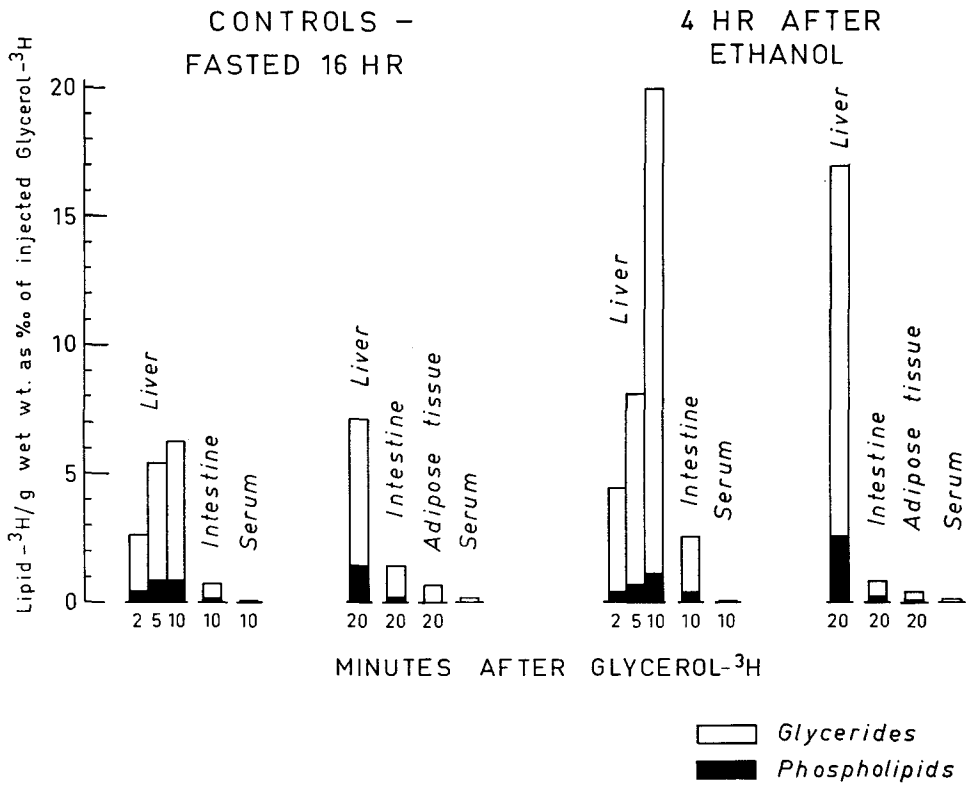



FIGURE 9 Four representative examples of incorporation of glycerol-$^3$H into lipids of liver, intestine, adipose tissue, and serum of fasted and ethanol-treated rats. Samples of liver 2–10 min after injection are derived from the same rat.

10 AND 20 MIN AFTER EITHER PALMITIC-$^3$H OR GLYCEROL-$^3$H: The radioautographic reaction was still present over the endoplasmic reticulum and lipid droplets,[1] and became much more pronounced over the mitochondria (Fig. 18).

At these time intervals, the label became apparent over the Golgi apparatus, and a few representative examples are given in Figs. 19–22. In many instances, the Golgi vesicles and vacuoles were filled with particles[1] 40–100 mμ, and similar particles were seen to be also single and surrounded by a membrane. Silver grains were found over the particles located in the Golgi apparatus and in the smooth-surfaced vesicles (Figs. 23–27). Vacuoles filled with particles, associated with silver grains, were seen in and near the Golgi apparatus, in some areas of the cytoplasm, and in the vicinity of the sinusoid border (Figs. 28–30). Particles devoid of membranes were also seen in the space of Disse, but, though radioactive grains were also present in that region (from 10 min on after injection of label), it was difficult to demonstrate an association between an individual grain and a particle. At 1 and 2 hr after the injection of labeled palmitate, a concentration of silver grains was seen in the space of Disse; label was still apparent over mitochondria and the endoplasmic reticulum (Fig. 31).

In order to gain more insight as to the relative distribution of intracellular radioactivity, grain counts were performed on sections of liver of fasted rats 2–20 min after injection of palmitate-

[1] The visible lipid is referred to as either (1) lipid droplets, which are more than 0.2 μ in diameter and usually not surrounded by an easily demonstrable membrane (except Fig. 15), or (2) particles, of varying electron opacity, 40–200 mμ in diameter, surrounded by well demonstrable membranes. Structures similar to the latter have been called granules (9), droplets (36), bodies (43), masses (22), or liposomes (10). The term "particle" has been chosen in order to differentiate these bodies from the lipid droplets, defined above.

$^3$H. The results of a representative experiment are summarized in Table III, which shows a rise in grain concentration over the mitochondria and the Golgi apparatus at later times after injection.

## DISCUSSION

In studies concerned with localization and intracellular movement of labeled products, it is important to ascertain the duration of the availability of the precursor. The results of the present experiments, which are in agreement with those of previous investigations (13), indicate that palmitic acid, even at a high load, disappears from the bloodstream quite rapidly. One can consider, therefore, the conditions of labeling as pulse labeling. In view of the more prolonged half-life of glycerol, we are dealing here with a continuous type of labeling and hence the comparison between the two sets of experiments can be qualitative only. One of the major methodological problems encountered in the study of intracellular localization of lipids is the loss of lipids during preparation of the specimen for electron microscopy (18); however, by using the modified dehydration procedure this loss is reduced considerably. It is also pertinent to stress that extractability of lipids by dehydrating agents varies considerably among different tissues[2]. Although labeled lipid present in the specimens is lost during preparation, no dislocation of label was seen on examination of the Epon adjacent to the sections by both light and electron microscopic radioautography. The background was invariably negligible, as shown by the absence of label from areas of the nucleus and of the sinusoid.

The penetration of the labeled substrates used was extremely rapid, and at 2 min after injection the label was distributed throughout the cell. The presence of occasional grains over the sinusoidal microvilli might indicate that these microvilli are a site of fatty acid entry into the liver cell, but no concentration of radioactivity was seen in that area at the earliest time studied. It is hoped that under in vitro conditions it will be possible to shorten the time of labeling to a few seconds and thus gain more information as to the primary site of fatty acid uptake. At 2 min after injection of palmitic acid, the major part of the radioactivity was recovered in triglycerides. Thus, the radioautographic reaction present over the endoplasmic reticulum at that time indicates that this organelle is the primary site of fatty acid esterification. The relative participation of the rough and the smooth endoplasmic reticulum in the process of fatty acid esterification was studied by Brindley and Hübscher in cat intestinal mucosal homogenates (4), and the activity of the glyceride-synthesizing enzymes was found to be highest in a rough-surfaced vesicle fraction. Schneider (32), who studied lecithin synthesis in rat liver homogenates, concluded that both the rough and the smooth elements of the endoplasmic reticulum were equally active. Since, in the present study, the label appeared over both the rough and the smooth elements of the endoplasmic reticulum at the earliest time examined (2 min), it seems that the enzymes operative in the process of glyceride formation in

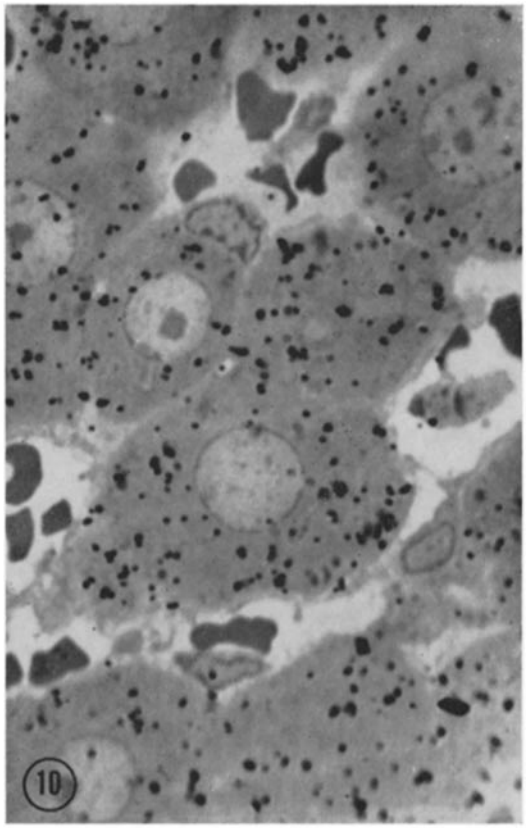
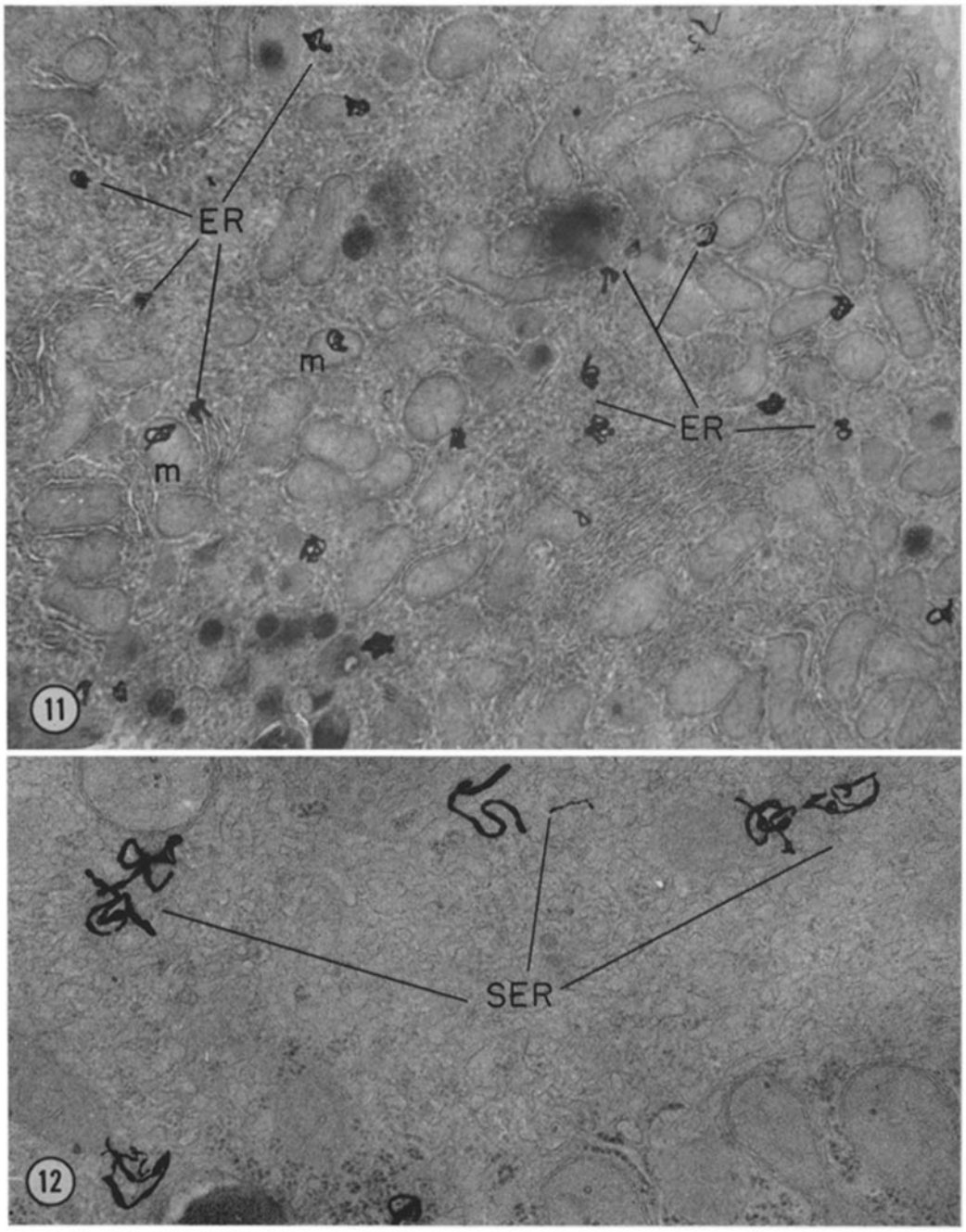



FIGURE 10 Light microscopic radioautograph of rat liver 2 min after injection of glycerol-$^3$H. The radioautographic reaction is seen diffusely over the cytoplasm of parenchymal cells. The nuclei and vascular spaces are free of reaction. × 1,660.

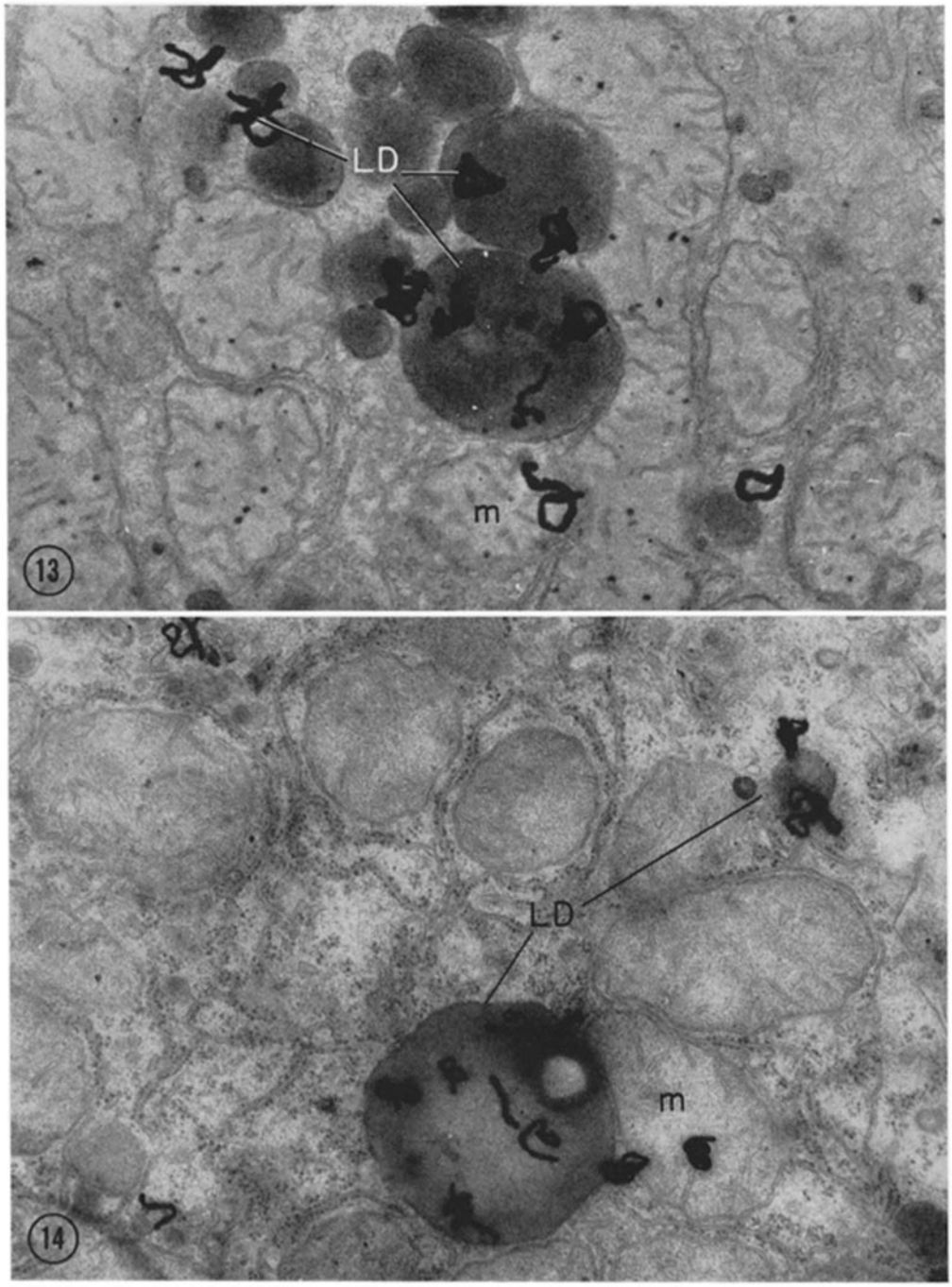
[2] O. Stein. Unpublished results.




FIGURES 11 and 12 Liver of ethanol-treated rats 2 min after injection of glycerol-$^3$H.

FIGURE 11 The label is seen over endoplasmic reticulum (*ER*) and some mitochondria (*m*). × 12,000.

FIGURE 12 A portion of a liver cell with silver grains over the smooth endoplasmic reticulum (*SER*). × 30,000.




FIGURES 13 and 14 Liver of ethanol-treated rats 5 min after injection of palmitate-$^3$H (Fig. 13) or glycerol-$^3$H (Fig. 14). Note concentration of label over lipid droplets (*LD*) and the presence of grains over some mitochondria (*m*) and the endoplasmic reticulum. Fig. 13, × 23,000; Fig. 14, × 26,000.

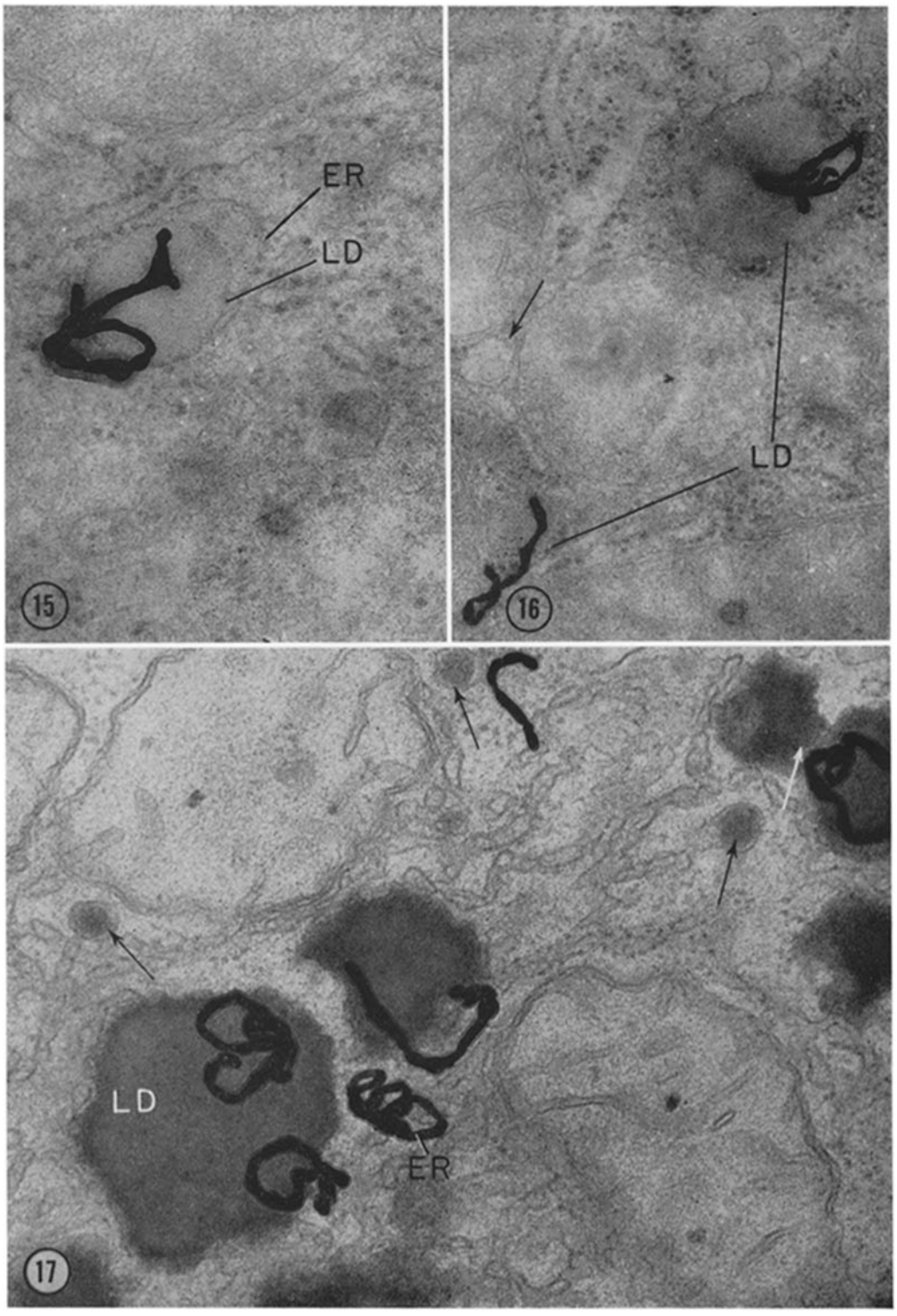
the liver are present throughout the endoplasmic reticulum.

The origin of mitochondrial esterified lipid has not been clarified completely. Wilgram and Kennedy (45), who studied the intracellular distribution of enzymes active in lipid synthesis in rat liver, found very low activity of diglyceride-acyl transferase and of phosphorylcholine-glyceride transferase in the mitochondrial fraction, and almost all of the activity was recovered in the microsomal fraction. These authors concluded, therefore, that the mitochondrial phospholipids are formed in the endoplasmic reticulum. The results obtained in the experiments with palmitate-$^3$H indicate that the mitochondria became labeled at later time intervals after injection, when very little, or no unesterified precursor was present in the blood plasma or in the liver tissue.

The metabolic nonhomogeneity of the liver lipid has been demonstrated previously (41), and presently the large lipid droplets were interpreted to be the structural counterpart of the larger pool with a slower turnover rate. The increase of lipid in the livers of fasted and ethanol-treated rats permitted the study of the mode of formation of lipid droplets. The enzymes which catalyze fatty acid esterification are bound to the membranes of the endoplasmic reticulum, and hence this process must evidently occur on or near the membrane. The next step, which involves aggregation of triglyceride molecules until a visible droplet is formed, has been localized to the intracisternal portion of the endoplasmic reticulum, as shown in Fig. 15. The subsequent growth of the lipid droplet could proceed both by intracisternal accretion and by fusion of smaller droplets. In the case of accretion, one could expect to find a membrane surrounding a large lipid droplet, which might be difficult to visualize owing to the presence of lipid. In the case of coalescence, the membrane material could become incorporated into the storage droplet, or be returned to the "membrane pool" after discharge of the lipid into the cytoplasmic matrix.

The triglycerides and phospholipids synthesized in the liver either are retained in the cell for cell membrane formation and for storage in the form of droplets, or are destined for secretion in the form of serum liproproteins. The morphology of certain serum and lymph lipoproteins has been investigated by Casley-Smith (7) who described them as osmiophilic electron-opaque particles 100–500 A in diameter. Similar particles have been observed also in various locations in the liver, under normal and pathological conditions. Their chemical composition and their site of origin have been widely debated; some of the more recent opinions are summarized in Table IV. In a previous report (36) dealing with ultrastructural changes in the liver of rats with acute ethanol intoxication, it was suggested, on circumstantial evidence, that these particles originate most probably in the liver and are the structural representation of lipoprotein secretion. It was hoped that the use of radioactive tracers would make it possible to define their chemical nature and fate more closely. The limitations of resolution in radioautography make it difficult to determine and localize the exact position of the source of electrons which are responsible for the developed grain (6, 19). The difficulty is not less when one is dealing with particles the diameter of which is much smaller than that of the silver grain. However, a more precise correlation between the

FIGURES 15–17 Liver of ethanol-treated rats 5 min after injection of glycerol-$^3$H (Figs 15, 16) or palmitate-$^3$H (Fig. 17). The labeled lipid droplet (*LD*) in Fig. 15 is about 0.3 $\mu$ in diameter and is seen inside a dilated end of a cisterna of the endoplasmic reticulum (*ER*) with a few remaining ribosomes. Both lipid droplets (*LD*) in Fig. 16 are surrounded by elements of the endoplasmic reticulum, but a membrane is not seen clearly. A smaller unlabeled lipid particle (arrow) is seen in a dilated end of the rough endoplasmic reticulum partly devoid of ribosomes. In Fig. 17 are illustrated the different steps in lipid accumulation. The grains over the elements of the endoplasmic reticulum (*ER*) indicate the site of esterification. Three unlabeled lipid particles, similar to, but smaller than, the labeled lipid droplet in Fig. 15, are seen surrounded by a membrane (black arrows). Around the larger labeled lipid droplets no membrane is discernible, and two droplets seem to be near fusion with each other (white arrow). Fig. 15, × 67,500; Fig. 16, × 48,000; Fig. 17, × 60,000.


ER
LD
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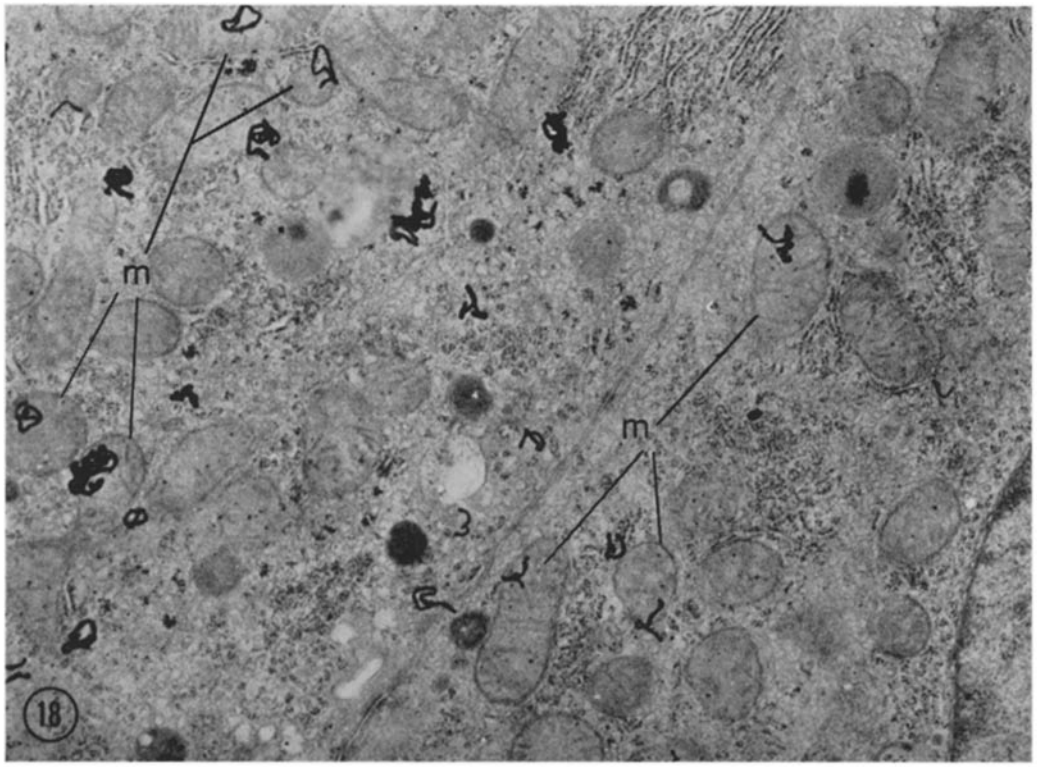
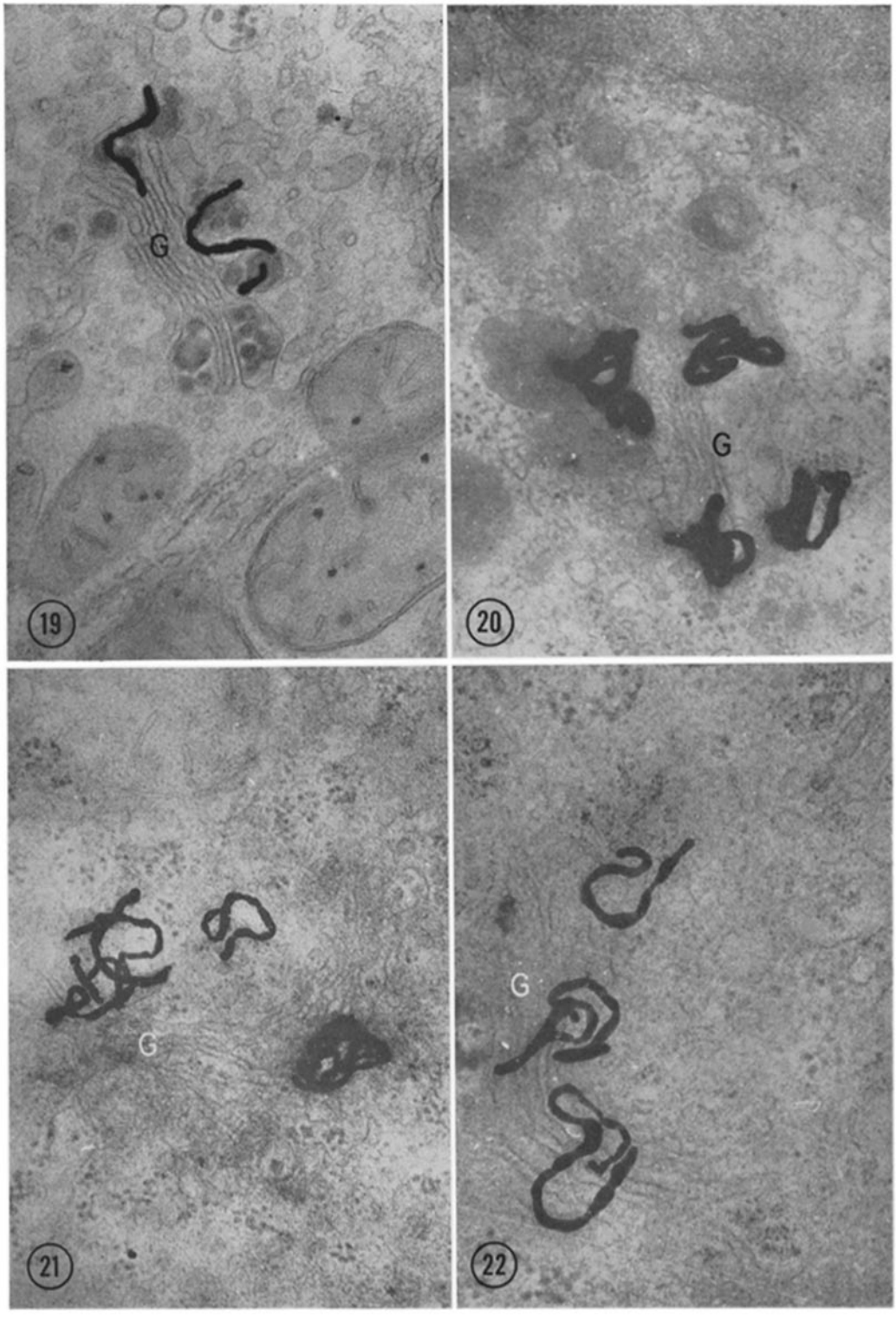



FIGURE 18 Liver of fasted rat 20 min after injection of palmitate-$^3$H. Note many labeled mitochondria (*m*). × 16,000.

position of the developed grain and the electron source became possible in instances in which the particles were not single, but in groups or clusters (Figs. 23, 24, 27, 28, 30). More rarely, single particle-grain association could also be shown, as in Figs. 25 and 26. The use of palmitate-$^3$H as tracer provided information concerning the chemical nature of these particles, and it can be concluded that they contain lipid. With the help of glycerol-$^3$H, the origin of the particles could be localized to the liver, as the liver is the main site of glycerophosphokinase, an enzyme mandatory for glycerol utilization in glyceride synthesis. In addition, no lipid radioactivity could be detected in the circulation at a time when the label appeared in the particles.

The participation of the Golgi apparatus in the process of lipid absorption has been shown by Palay and Karlin (23) who described lipid droplets in the Golgi area of mucosal cells of the small intestine after a fatty meal. In addition, the small particles in the Golgi vesicles of liver cells have been interpreted as containing lipid (3, 9, 22, 42). In the present study, it became possible to identify the product in the Golgi apparatus as containing lipid. The number of silver grains over the Golgi apparatus relative to the number found over the endoplasmic reticulum increased with time, especially between 5 and 20 min after injection of label, when no free fatty acids were found in the liver. This increase would have been even more pronounced if expressed per unit area of organelle, as the Golgi apparatus amounts to about 3.5% of the cell surface area. These results have lent support to the conclusion that the lipid product found in the Golgi vesicles is synthesized in the endoplasmic reticulum and delivered to the Golgi apparatus as a secretory product. The migratory process of secretory proteins, which, after their synthesis in the endoplasmic reticulum, reach the Golgi apparatus and emerge from there in secretory granules or vesicles to be discharged at the cell




FIGURES 19–22 Liver of fasted (Figs. 19, 21) and ethanol-treated rats (Figs. 20, 22), showing localization of radioautographic grains over the Golgi apparatus (*G*).

FIGURE 19 10 min after palmitate-$^3$H. × 40,000.

FIGURE 20 10 min after glycerol-$^3$H. × 50,000.

FIGURES 21 and 22 20 min after palmitate-$^3$H. Fig. 21, × 45,000; Fig. 22, × 70,000.

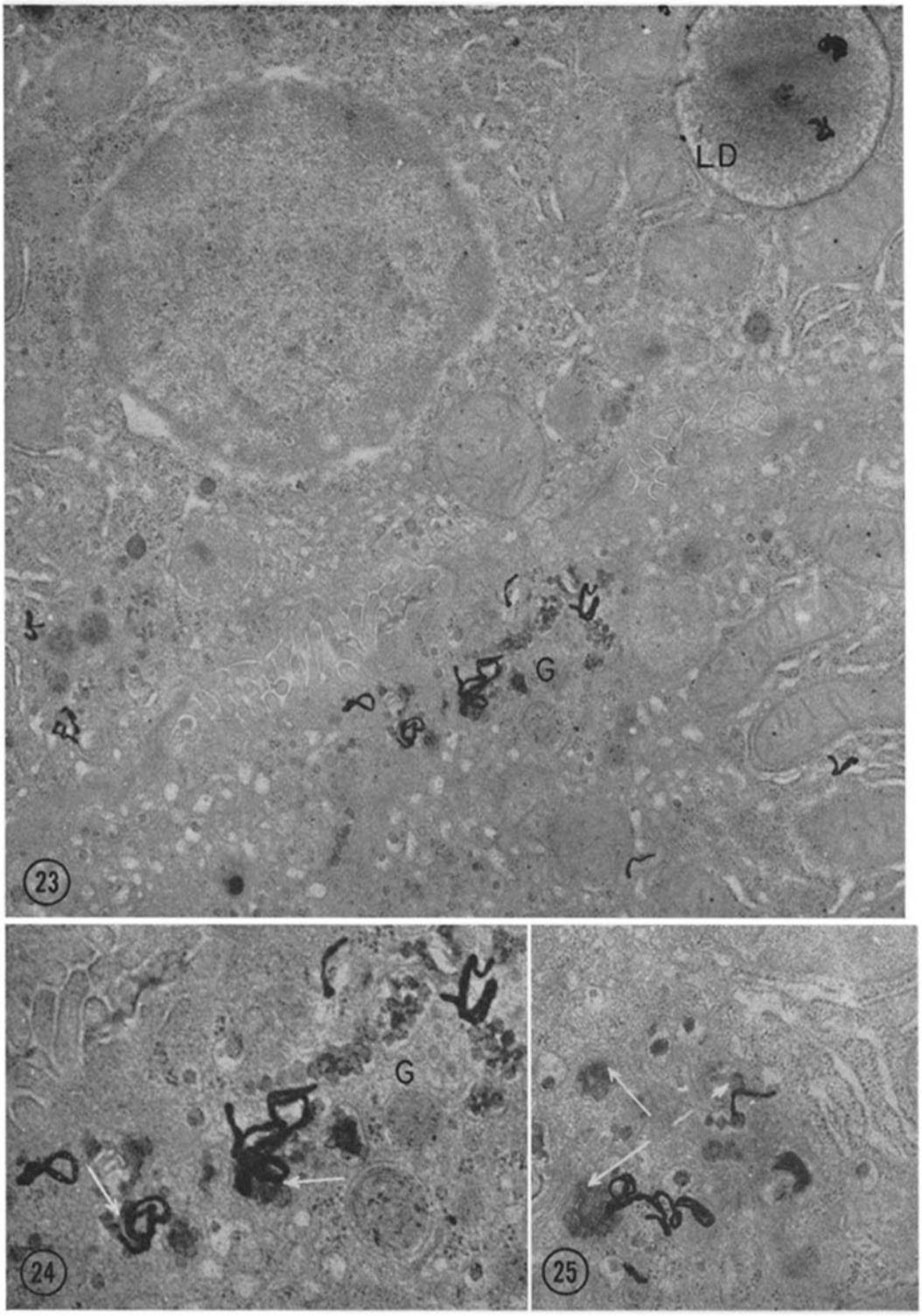
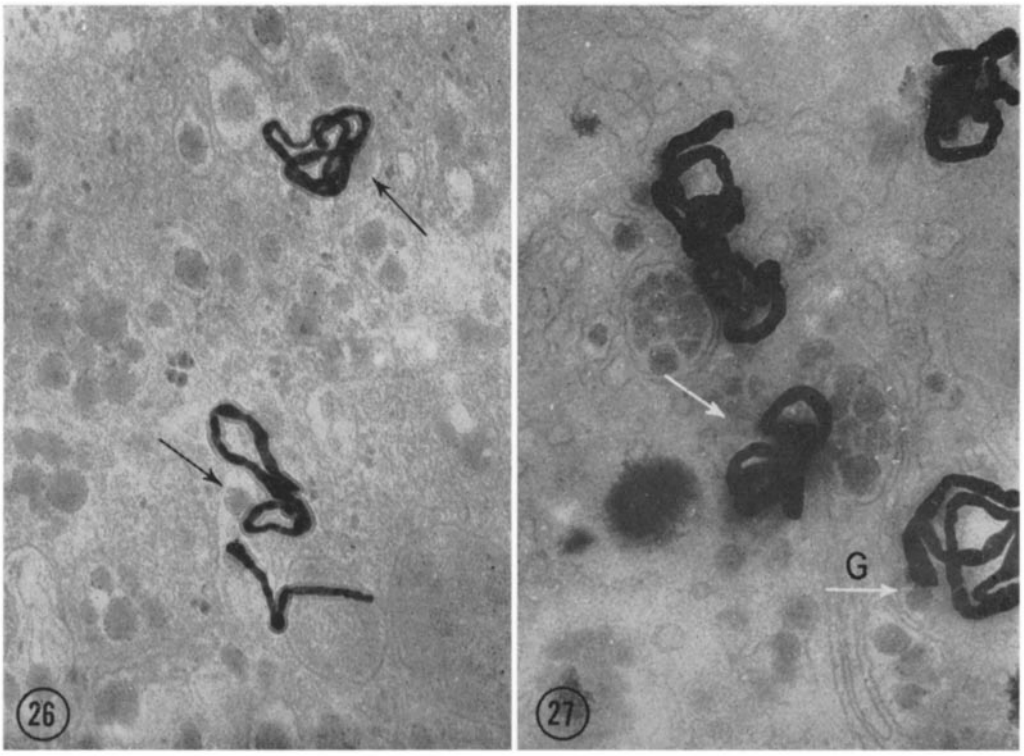



FIGURE 23 Liver of ethanol-treated rat 20 min after injection of palmitate-$^3$H, showing concentration of radioautographic grains over the Golgi apparatus (*G*), which is filled with electron-opaque particles. × 17,500.

FIGURE 24 Detail of Fig. 23, showing the relation of the radioautographic grains to the particles (arrows) in the Golgi vacuoles (*G*). × 35,000.

FIGURE 25 Liver of fasted rat 10 min after injection of palmitate-$^3$H. The radioautographic grains are seen over membrane-bounded particles. The arrows indicate labeled and unlabeled particles. × 30,000.




FIGURES 26 and 27 Liver of ethanol-treated rats 10 min after injection of palmitate-$^3$H (Fig. 26) or glycerol-$^3$H (Fig. 27).

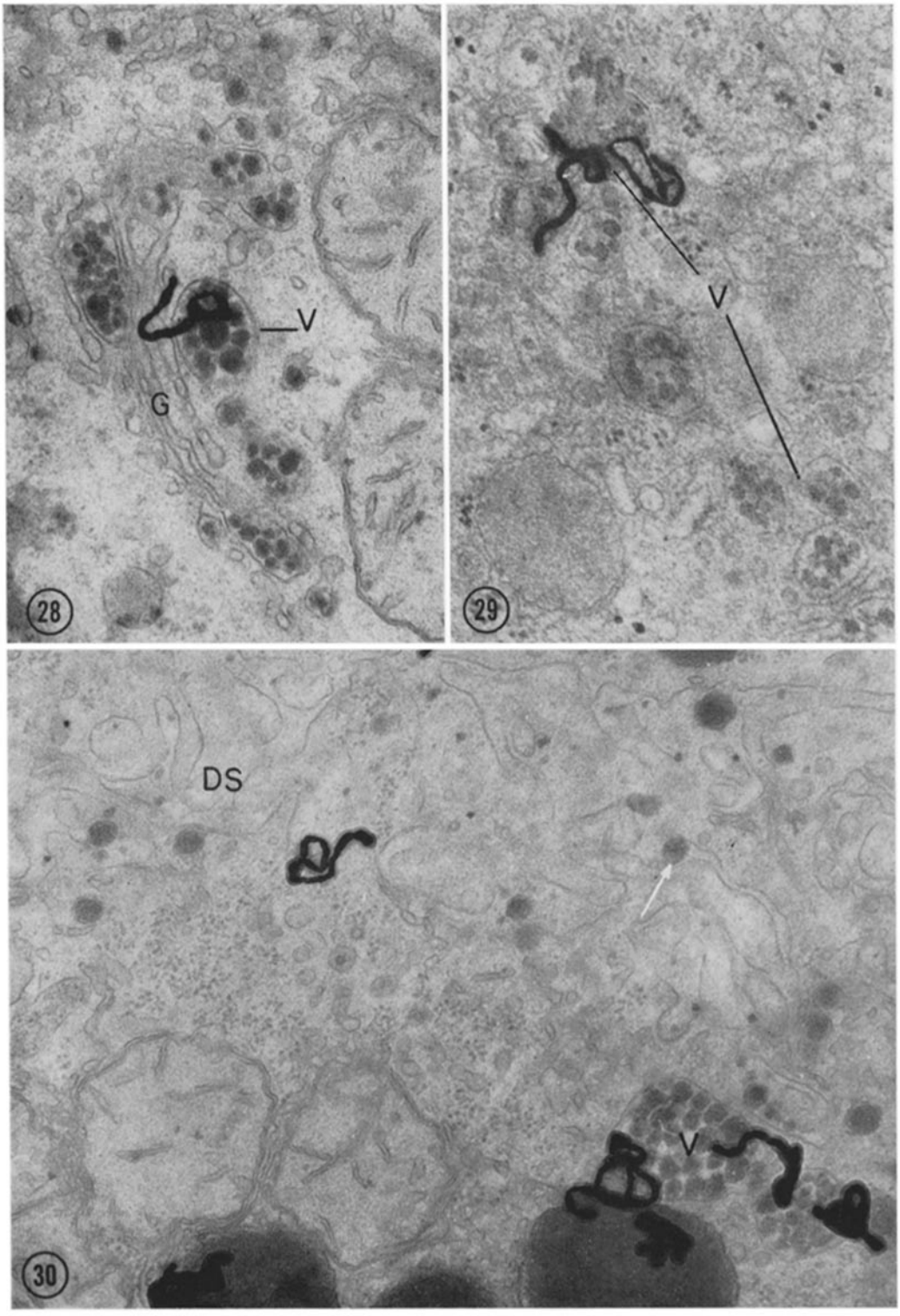
FIGURE 26 Autoradiographic grains over membrane-bounded particles (arrows). × 56,000.

FIGURE 27 Concentration of radioautographic grains in the Golgi area (*G*) filled with electron-opaque particles (arrows). × 70,000.

surface, has been described in numerous cells (5, 11, 21, 26, 30, 44). By analogy, one could envisage that in the liver the Golgi vacuoles perform the function of transport vehicles for the lipid-containing particles, which reach the sinusoidal surface and are discharged into the space of Disse. This sequence of events was first proposed by Chandra (9) who studied the morphology of secretion in livers of young hamsters and deduced that the particles consist of lipoproteins. The formation of compound proteins involves two steps: synthesis and conjugation. As has been shown for glycoproteins, the two steps need not occur in the same location: the protein part is synthesized in the endoplasmic reticulum and the carbohydrate moiety is added in the Golgi region (14, 19, 24, 26). In the case of the lipoprotein molecule, though both lipid and protein are synthesized in the endoplasmic reticulum, it is not known whether the complexing occurs at the site of synthesis or in the Golgi apparatus.

The present investigation has provided information mainly concerning the triglyceride part of the lipoprotein molecule, which contains also phospholipids and cholesterol. Preliminary data obtained after injection of choline-$^3$H into choline-deficient rats have shown that the phospholipids also are formed in the endoplasmic reticulum, and that label appears in the Golgi apparatus (35).

Impairment of protein synthesis has been implicated in the pathogenesis of fatty liver under diverse conditions (10, 22, 25, 28, 33). One could envisage also that the rate of synthesis of the protein moiety relative to the rate of lipid synthesis will determine whether certain triglyceride molecules will be channeled for secretion or will




FIGURES 28–30 Liver of ethanol-treated rats 10 (Fig. 28) and 20 min (Figs. 29, 30) after injection of palmitate-$^3$H. The figures are intended to illustrate a tentative sequence of events in which the particles (lipoproteins), after final segregation in the Golgi vacuole (*v*) (Fig. 28), are transported through the cytoplasm inside the vacuoles (*v*) (Figs 29 and 30), until they reach the sinusoidal surface upon which they are discharged (white arrows) into the space of Disse (*DS*). Fig. 28, × 43,000; Fig. 29, × 45,000; Fig. 30, × 40,000.

be stored in the liver cell. Thus, lipid accumulation in the liver, as after fasting or after acute ethanol intoxication, could be the result of an increased fatty acid esterification, without actual impairment in protein synthesis.

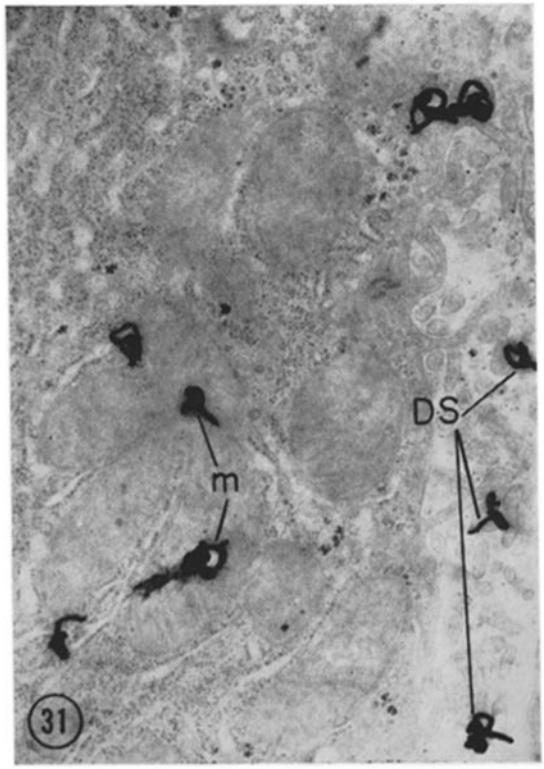



FIGURE 31 Liver of fasted rat 2 hr after injection of palmitate-$^3$H. Most of the grains are in the vicinity of, and within Disse's space (*DS*). Label is also seen over mitochondria (*m*) and endoplasmic reticulum. × 18,500.

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*Note Added in Proof*

Since the manuscript was submitted for publication, two papers (by Jones, A. L. et al. 1966. *Proc. Soc. Exptl. Biol. Med.* **123**:4; and Ashworth, C. T. et al. 1966. *J. Cell Biol.* **31**:301) dealing with the lipoprotein nature of small electron-opaque granules in the liver were published. The conclusions of both groups, derived from data obtained by different techniques, support the conclusions of the present authors.

TABLE III

*Distribution of Grains over Liver Subcellular Structures after Injection of 9,10-Palmitic Acid-$^3$H into Rats Fasted for 16 hr*

| Time after injection | Distribution of grains: Endoplasmic reticulum* | Mitochondria | Golgi region | Disse space | Nucleus | Total grains counted |
|---|---|---|---|---|---|---|
| *min* | % | % | % | % | % | |
| 2‡ | 87 | 6 | 1 | 4 | 2 | 243 |
| 5‡ | 86 | 7 | 2 | 3 | 2 | 242 |
| 10‡ | 67 | 15 | 11 | 5 | 2 | 434 |
| 20 | 65 | 16 | 11 | 6 | 2 | 297 |

* Includes cytoplasmic matrix.
‡ Samples obtained from the same rat.

TABLE IV

*Recent Interpretation of "Lipid Particles" in the Liver Described under Diverse Conditions*

| Lipid accumulation in | Location of particles | Interpretation | Reference |
|---|---|---|---|
| Choline deficiency | ER, Disse space | Uptake | (1) |
| Normal liver | ER, Golgi complex, Disse space | Secretion | (9) |
| Liver regeneration | ER, Disse space | Uptake, secretion? | (42–43) |
| Liver regeneration | ER, Golgi complex | Uptake | (16) |
| Ethanol fatty liver | ER, Golgi complex, Disse space | Uptake, secretion? | (2) |
| Ethanol fatty liver | ER, Golgi complex, Disse space | Secretion | (36) |
| Ethionine fatty liver | ER, Golgi complex, Disse space | Secretion | (3) |

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