HEPATIC INTRACELLULAR OSMIOPHILIC DROPLETS

Effect of Lipid Solvents during Tissue Preparation

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ABSTRACT

Lipid solvent extraction of aldehyde-fixed hepatic tissue of rats caused disappearance of all intravascular and hepatocellular osmiophilic droplets normally present, thus indicating their lipid content. Intramitochondrial dense granules and osmiophilic droplets in lysosomes also disappeared after this treatment. Lipid solvents extracted 43.8 to 92.6% of the radioactivity from aldehyde-fixed rat liver with C^{14}-labeled lipids. Only 0.7 to 5.8% of the radioactivity was extracted when the hepatic proteins were labeled. When tissue was fixed with OsO_4, the lipid solvents extracted only 0.7 to 7.2% of the radioactivity from lipid-labeled liver and only 0 to 0.7% when proteins were labeled. Thin layer chromatography of the lipid solvents used in extraction of formaldehyde-fixed tissue revealed that triglyceride, phospholipid, and cholesterol and other lipid classes had been removed. However, acetone extracted less phospholipids than did ethanol or methanol-chloroform. During fat absorption the number and size of osmiophilic droplets increased in the nongranular endoplasmic reticulum. In animals fasted up to 5 days, 250-A osmiophilic particles were still present in the Golgi vesicles, other cytoplasmic vesicles, and in the space of Disse. These were considered possibly to represent lipoprotein being synthesized in the liver cell and secreted into the blood.

INTRODUCTION

Homogeneous osmiophilic droplets are a characteristic component of the cytoplasm of hepatic cells (1–4). Larger droplets 1 to 2 μ in diameter occur randomly in the cytoplasm, while smaller droplets up to 2000 A in diameter may frequently be seen in Golgi vesicles and in cisternae of the smooth endoplasmic reticulum. Very small osmiophilic droplets are also rarely present in cisternae of the rough endoplasmic reticulum. While it usually has been assumed that these osmiophilic droplets are of lipid nature, their composition has not been thoroughly established. Recently, Bruni and Porter (5) suggested that some of these smaller intravesicular osmiophilic particles are of protein composition and might represent synthesized albumen.

In the course of absorption, transport, and utilization of lipids, hepatic parenchymal cells take up lipid from the blood during fat absorption from the intestinal tract (6, 7) or following mobilization of fatty acid from adipose tissue triglyceride (8). Concurrently, the liver cells assemble low density lipoproteins within their cytoplasm, which then are secreted into the blood (9–11). It can be surmised that stages of these processes might be visualized as particulate lipid in the liver cells by electron microscopy.

The present study is an investigation of two factors pertaining to the possible relationship of these osmiophilic droplets in the hepatic cell to the liver's role in lipid metabolism. First, the variations in size and distribution of osmiophilic droplets within

liver cells, in sinusoids, and in the space of Disse were examined by subjecting animals to dietary alteration of the rate of hepatocellular lipid uptake and lipoprotein synthesis and secretion. Second, the extractability of these droplets by lipid solvents employed prior to OsO_4 fixation in the preparation of liver tissue for electron microscopy was studied morphologically and by radiochemical methods.

MATERIALS AND METHODS

The following experiments were undertaken to produce various stages of lipid uptake and secretion by liver cells. Adult male and female Holtzman rats weighing 200 to 300 g were used. (*a*) Six male rats were fed ad libitum by providing a continuous supply of stock diet.[1] (*b*) A state of increased fat absorption was produced in 15 male rats by administering 2 ml of a corn oil emulsion by stomach tube.[2] Three animals at each time interval were sacrificed for study at 30, 45, 90, 180, and 360 min. (*c*) Twelve male rats were fasted but allowed water ad libitum, and 3 animals were studied at each of the time intervals of 24, 48, 65, and 120 hr.

Liver tissue from all animals and tissue from small intestine and kidney of some of the fat-absorbing animals were prepared for electron microscopy by placing 1 mm^3 blocks directly in osmium tetroxide buffered to pH 7.4 with Veronal. Tissues were processed through graded alcohols and propylene glycol and embedded in an epoxy resin (Maraglas, reference 12).

The effect of lipid solvents on osmiophilic droplets was studied by first fixing overnight freehand slices of liver less than 1 mm thick, from animals in the foregoing experiments, in 6.5% glutaraldehyde (13) or 10% neutral-buffered formaldehyde. The thin slices were then placed for 6 hr in either 95% ethanol, absolute acetone, or methanol-chloroform (1:2). Following extraction, small 1 mm^3 blocks were postfixed in osmium tetroxide for 2 hr, dehydrated, and embedded in Maraglas. For a comparison of the effects of lipid solvents upon primarily OsO_4-fixed tissue and aldehyde-fixed tissue, liver which had first been fixed in OsO_4 was subjected to an additional treatment with absolute ethanol, acetone, or methanol-chloroform before being embedded in Maraglas. Thin sections were studied with an RCA EMU 3-F.

Staining with lead or heavy metal other than osmium was omitted in this study, because we have found previously (14, 15) that osmiophilic particles and droplets within hepatic cells appear denser by contrast when heavy metal stains are not employed. We also wanted to avoid the complicating influence of heavy metal stains, other than osmium, in comparing the appearances of OsO_4-fixed tissue with and without prior lipid solvent treatment.

The amounts of lipid and of protein extracted by lipid solvents after formaldehyde fixation prior to OsO_4 postfixation and the amounts extracted by lipid solvents after direct OsO_4 fixation were studied and compared as follows: liver lipids of 4 rats were labeled in vivo with C^{14} by administration of 2.5 μc of triolein-C^{14} 4 hr prior to sacrifice. The specific activity (CPM/g) of fresh, unfixed liver homogenate and of methanol-chloroform extract (total hepatic lipid) was determined, enabling a calculation of the percentage of radioactivity in the total hepatic lipid. Formaldehyde-fixed hepatic tissue slices, before and after lipid extraction with ethanol, acetone, or methanol-chloroform, were homogenized and subjected to radioactivity counting, and the lipid solvent extracts were separately counted. From these data the percentages of radioactivity from lipid-labeled livers ("lipid radioactivity") extracted by the different procedures were calculated. Similar procedures were carried out on hepatic tissue (of 2 of these rats) which had been subjected to direct OsO_4 fixation before and after treatment with ethanol, acetone or methanol-chloroform. A liquid scintillation system (Nuclear-Chicago Corporation, Des Plaines, Illinois) was used in all radioactivity counts.

Phosphomolybdic acid–stained (16), thin layer chromatographs (17) of lipid classes from a methanol-chloroform extract of whole fresh liver homogenate were compared with chromatographs of lipid solvents used in preparing formaldehyde-fixed tissue. In addition, the formaldehyde solution used in primary tissue fixation was subjected to thin layer chromatography.

In 4 additional animals, hepatic proteins were labeled in vivo by the intravenous administration of 2 μc of C^{14}-l-leucine 1 hr prior to sacrifice. The specific radioactivity (CPM/g) of unfixed liver homogenate and of trichloracetic acid precipitate (total hepatic protein) (18) was determined, and the percentage of radioactivity in total hepatic protein was calculated. Radioactivity counts were obtained from slices of formaldehyde-fixed liver of each of the 4 animals before and after the use of lipid solvents, as previously described. The level of radioactivity was similarly determined on liver slices of OsO_4-fixed liver of 2 of these animals before and after the application of lipid solvents. From these data the percentage extraction of radioactivity from protein-labeled livers ("protein radioactivity") was determined in each of the experiments.

[1] Purina Dog Chow.

[2] The corn oil emulsion consisted of Oral Lipomul (The Upjohn Co., Kalamazoo, Michigan). Each 15 cc contains 10 g of corn oil.

RESULTS

Distribution of Osmiophilic Particles in Liver of Fasted and Fed Rats

In rats which had been fed stock diet ad libitum, a few large osmiophilic droplets measuring up to 2 μ were seen in many of the liver cells (Fig. 1). These larger droplets usually appeared to lie free in the cytoplasm, but there was a suggestion of a membrane surrounding some of them. Intravesicular droplets measuring 200 to 400 A in diameter were present but sparse in the smooth endoplasmic reticulum and the Golgi apparatus. Osmiophilic particles 200 to 600 A in diameter were also occasionally found in the space of Disse.

As early as 30 to 90 min after corn oil ingestion, the sinusoids, in which lumen contents were intact, and the spaces of Disse contained an increased number of small osmiophilic droplets 300 to 500 A in diameter, compared with fasting controls. At 180 and 360 min, the droplets had become more numerous, and they were more abundant in the space of Disse than in sinusoids (Fig. 2). In the sinusoids the droplets ranged from 250 A to 1 μ in diameter, while in the space of Disse they measured 250 to 4500 A in diameter.

Many osmiophilic droplets were located inside membrane-bounded vesicles in the peripheral portion (near the space of Disse) of the cytoplasm of liver cells, and some of these vesicles communicated with the cell surface between the microvilli. Most of the cytoplasmic vesicles containing osmiophilic droplets were bounded by smooth membranes; only rarely were small osmiophilic particles seen in cisternae of the granular endoplasmic reticulum. The droplets became smaller and fewer in deeper portions (near the nucleus) of the hepatocytes. While most cytoplasmic vesicles contained a single osmiophilic droplet, a few contained clusters of six or more droplets about 500 A in diameter. Occasionally, vesicles at the periphery of hepatocytes were open at the cell surface and communicated with the space of Disse. At the 180 and 360 min periods, the droplets were comparable in range of size to chylomicrons in the intestinal mucosal lymphatics at the same time intervals (Fig. 3).

The osmiophilic droplets within vesicles of the Golgi apparatus were larger and more numerous during fat absorption than in fasting or in ad libitum–fed animals. The longer the time interval after administration of the fat meal, for as long as 3 to 6 hr, the larger (up to 1000 A) and more numerous (Fig. 2) were the osmiophilic droplets in the Golgi vesicles.

In fasted rats the hepatic cells contained fewer osmiophilic droplets of all types than in animals fed ad libitum or those receiving test doses of fat. The large, homogeneous, densely osmiophilic droplets were reduced in number and size in fasted animals, and they were absent from the great majority of liver cells after 5 days' fasting. Small osmiophilic droplets measuring about 250 A continued to be present in Golgi vesicles, even in 5-day–fasted animals. However, they were fewer and smaller than in fed animals (Fig. 4). In addition to osmiophilic droplets in Golgi vesicles of fasting animals, droplets about 250 A in diameter were also sparsely present in nongranular membrane-bounded vesicles. These small intravesicular particles were randomly located in the cytoplasm. A few similar small osmiophilic particles were found in the space of Disse in fasting rats.

In fat-absorbing animals the lysosomes frequently contained large, homogeneous osmiophilic droplets up to 5000 A in diameter (Fig. 5). In fasting rats the lysosomes occasionally contained osmiophilic droplets, but after 5 days' fasting these droplets were sparse.

Intramitochondrial dense granules were equal in size and number in fasting and fed animals. These bodies ranged from 250 to 450 A in diameter, were homogeneous, highly electron opaque, and the margins were somewhat irregular and finely spiculated (Fig. 6). The degree of their electron opacity was comparable to that of other homogeneously electron-opaque particles and droplets in the cytoplasm.

Effect of Lipid Solvents on Hepatic Cell Ultrastructure

Glutaraldehyde or formaldehyde-fixed tissue which was not subjected to the effects of lipid solvents prior to OsO_4 postfixation revealed the same osmiophilic dense particles and droplets as seen in tissue primarily fixed in OsO_4 (Fig. 7). Treatment of aldehyde-fixed tissue with ethanol, acetone, or methanol-chloroform, prior to OsO_4 fixation, gave essentially similar results. One of the most striking of these effects was disappearance of all cytomembranes (Fig. 8). The dense lines of sectioned membranes of the nuclei, granular and nongranular endoplasmic reticulum, Golgi elements, and mitochondria, as well as the plasma membrane of

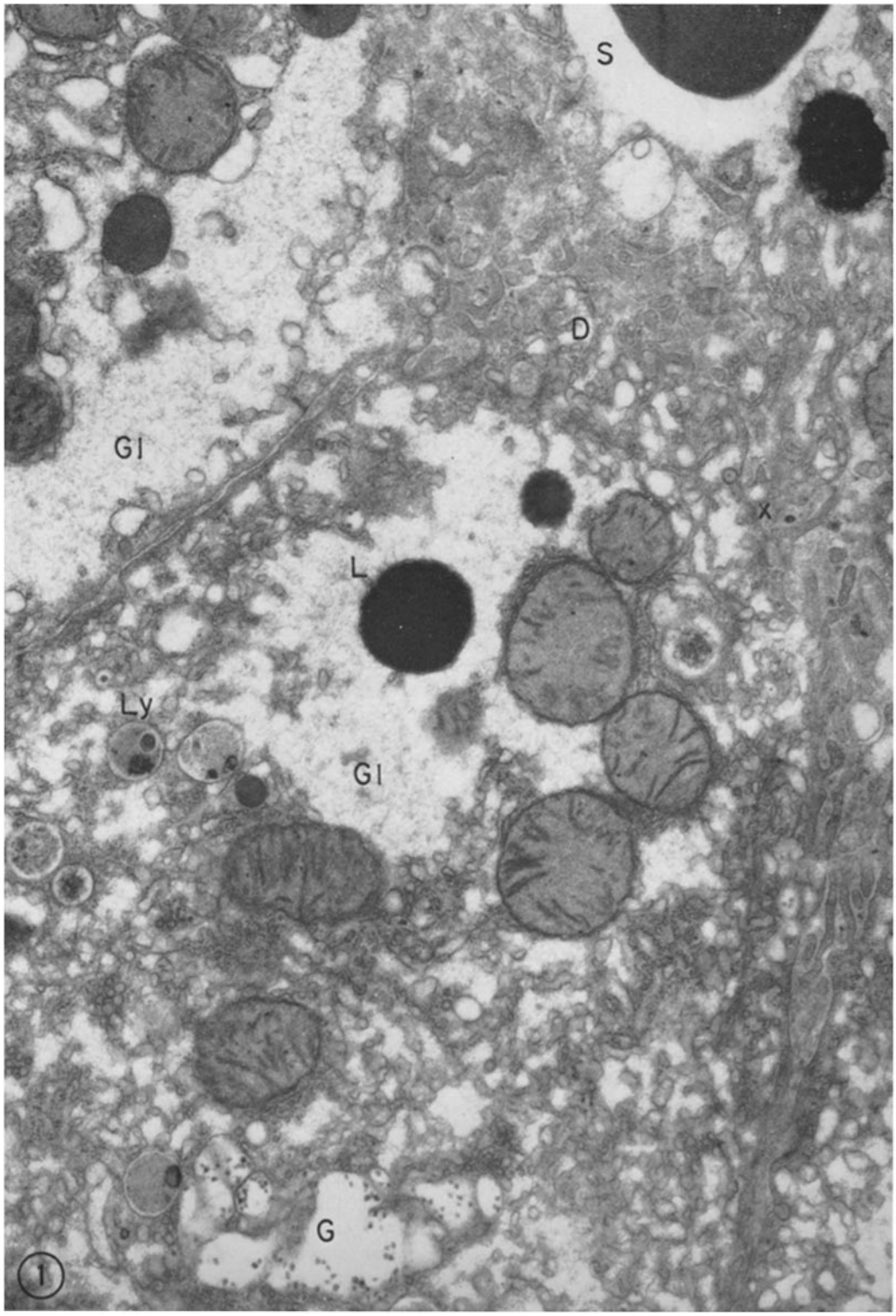


FIGURE 1 Portion of a liver cell from an ad libitum–fed animal. Part of a sinusoid (*S*) is shown. Rare osmiophilic particles (*x*) about 500 A in diameter are present in the space of Disse (*D*). Large osmiophilic droplets up to 1 μ in diameter are present in the cytoplasm. A faintly visible membrane partially surrounds one of these (*L*), which may represent nongranular endoplasmic reticulum displaced by the droplet. Golgi vesicles (*G*) contain numerous small osmiophilic particles about 500 A in size. Osmiophilic droplets are present in some of the lysosomes (*Ly*). Glycogen (*Gl*) in the cytoplasm is of low electron opacity due to absence of lead staining. × 18,000.

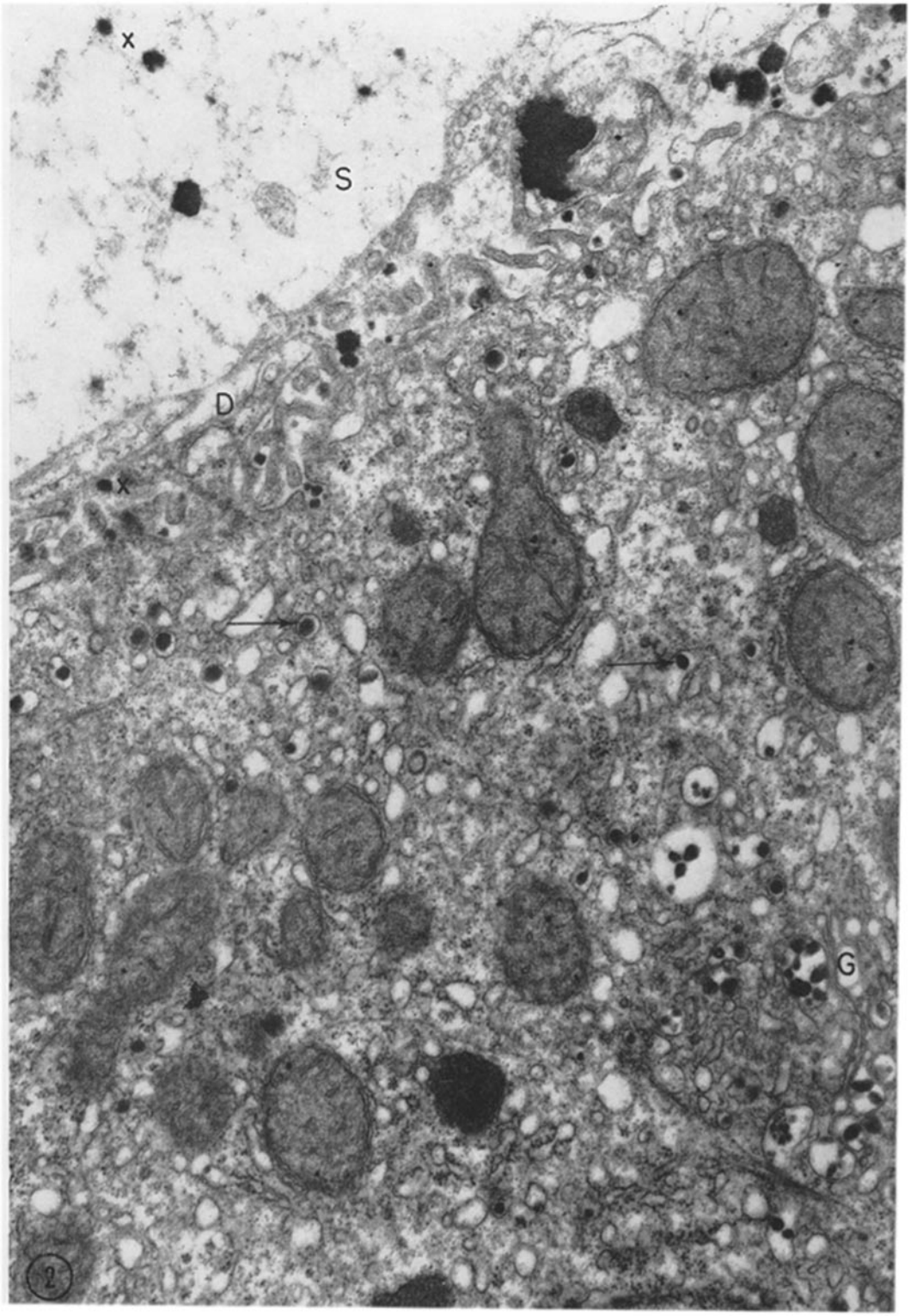


FIGURE 2 Portions of sinusoid (*S*) and hepatocyte in rat which had received corn oil by stomach tube 3 hr previously. Osmiophilic droplets 500 to 2000 A in diameter (*x*) are present in the sinusoidal blood, and similar droplets are present in the space of Disse (*D*). Droplets 500 to 1000 A in size are observed in membrane infoldings at the surface of the hepatic cell and in vesicles bounded by nongranular membranes (arrows). The Golgi vesicles (*G*) contain osmiophilic droplets up to 1000 A in size which are larger than the osmiophilic droplets in the Golgi vesicles of ad libitum–fed animals. × 27,000.

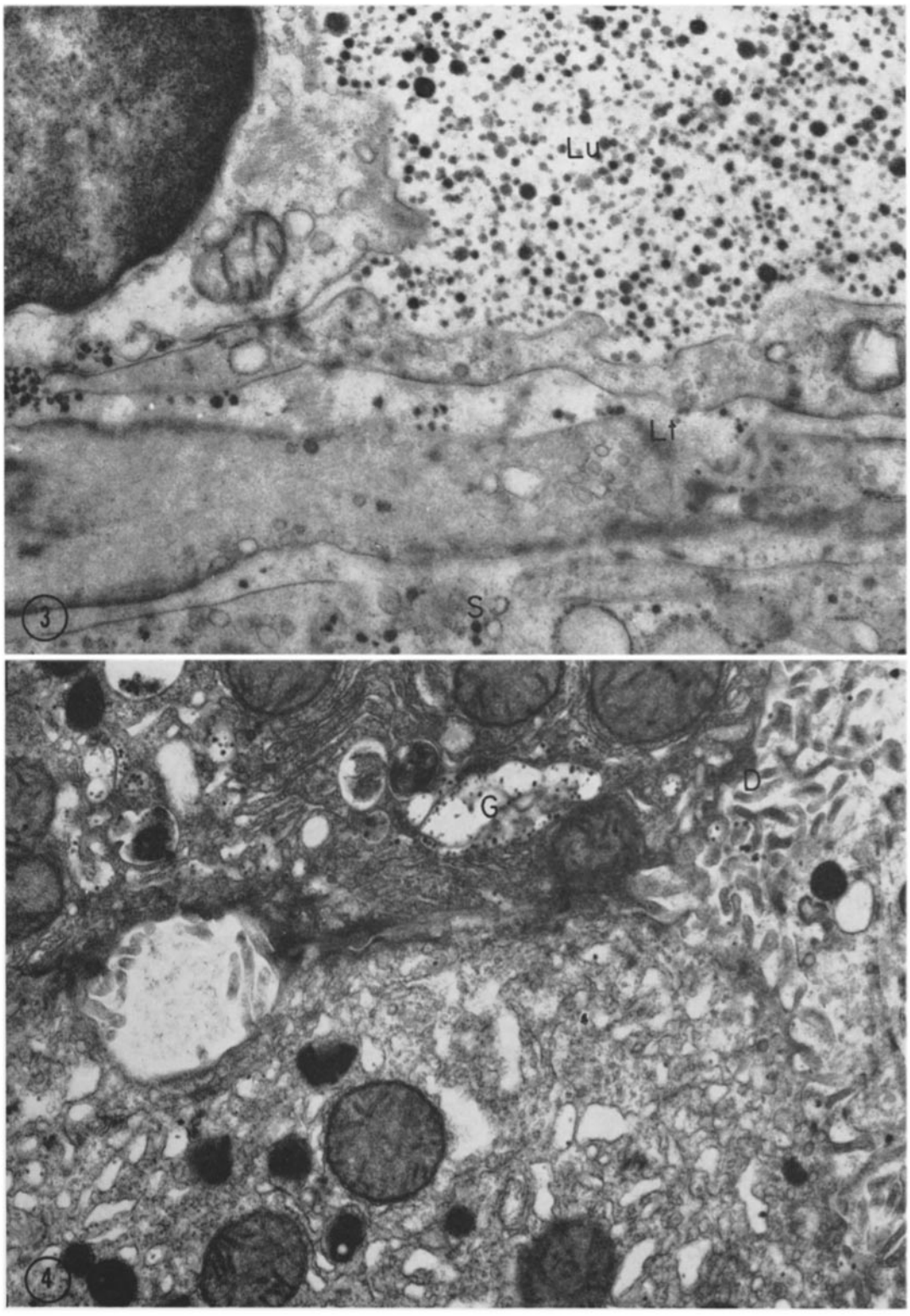


FIGURE 3 Portions of lumen and wall of intestinal mucosal lymphatic of rat 3 hr after corn oil administration. Chylomicrons in the lumen (*Lu*) range from 300 to 1500 A in diameter. Similar osmiophilic droplets are present in the wall of the lymphatic (*Lt*) and in the interstitial spaces (*S*) of the lamina propria. × 28,000.

FIGURE 4 Liver cell of rat which had been fasted for 120 hr. The total amount of osmiophilic droplet material demonstrable under the electron microscope is much reduced over that seen in ad libitum–fed animals. Osmiophilic droplets about 300 A in diameter are present, however, in the Golgi vesicles (*G*) and in the space of Disse (*D*). × 22, 000.

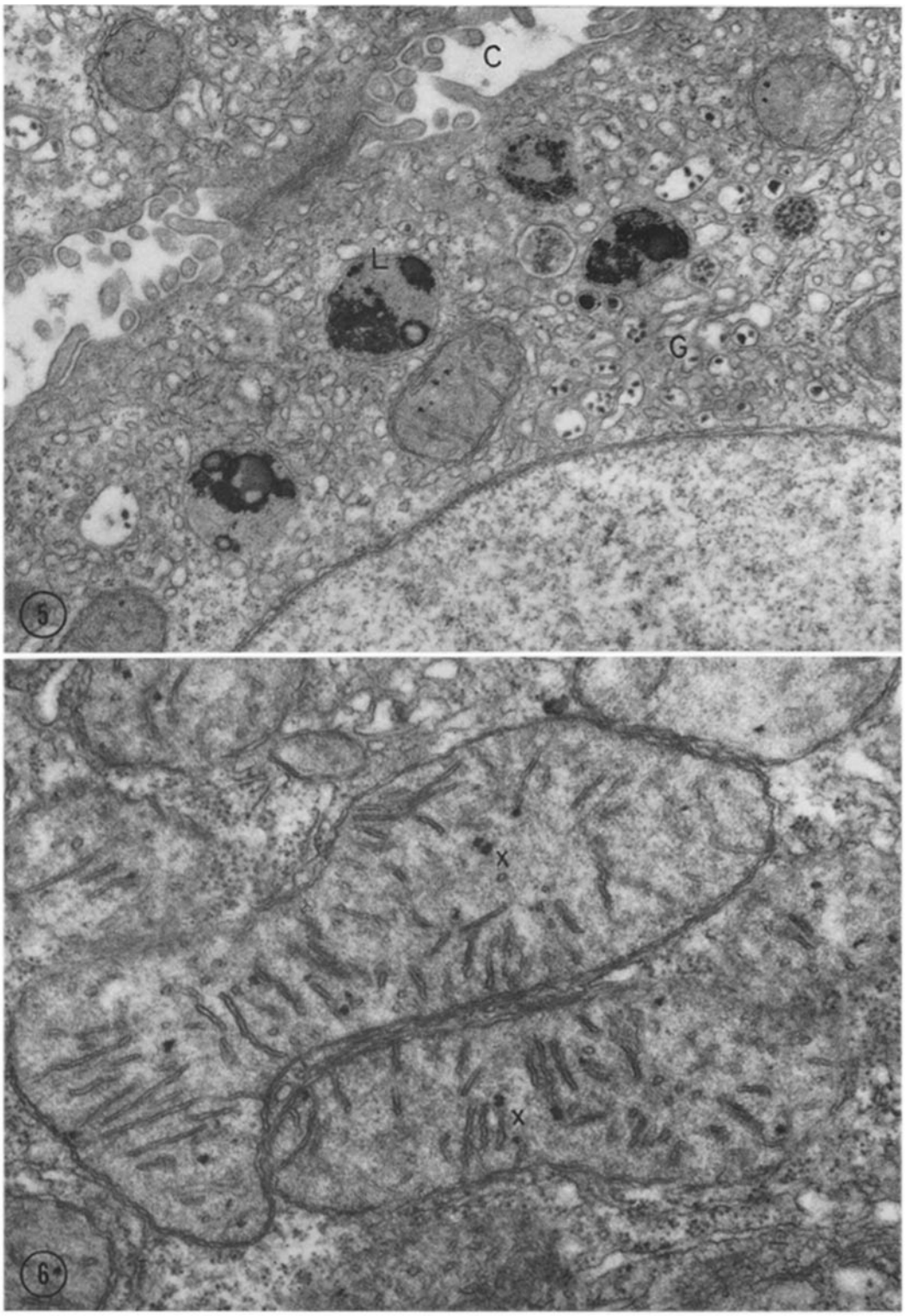


FIGURE 5 Portion of liver cell of rat fed fat 1½ hr previously. The tissue was fixed immediately in OsO_4. Numerous osmiophilic droplets are present in Golgi vesicles (*G*). The lysosomes contain numerous large, dense osmiophilic droplets (*L*). Part of a bile canaliculus (*C*) is shown. × 33,000.

FIGURE 6 Mitochondria of liver cell of rat which was fed ad libitum. Visible in the matrix of the mitochondria are discrete, generally rounded osmiophilic droplets, the "dense intramitochondrial granules" (*x*). These have slightly irregular, spiculated margins and average about 350 A in diameter. × 60,000.

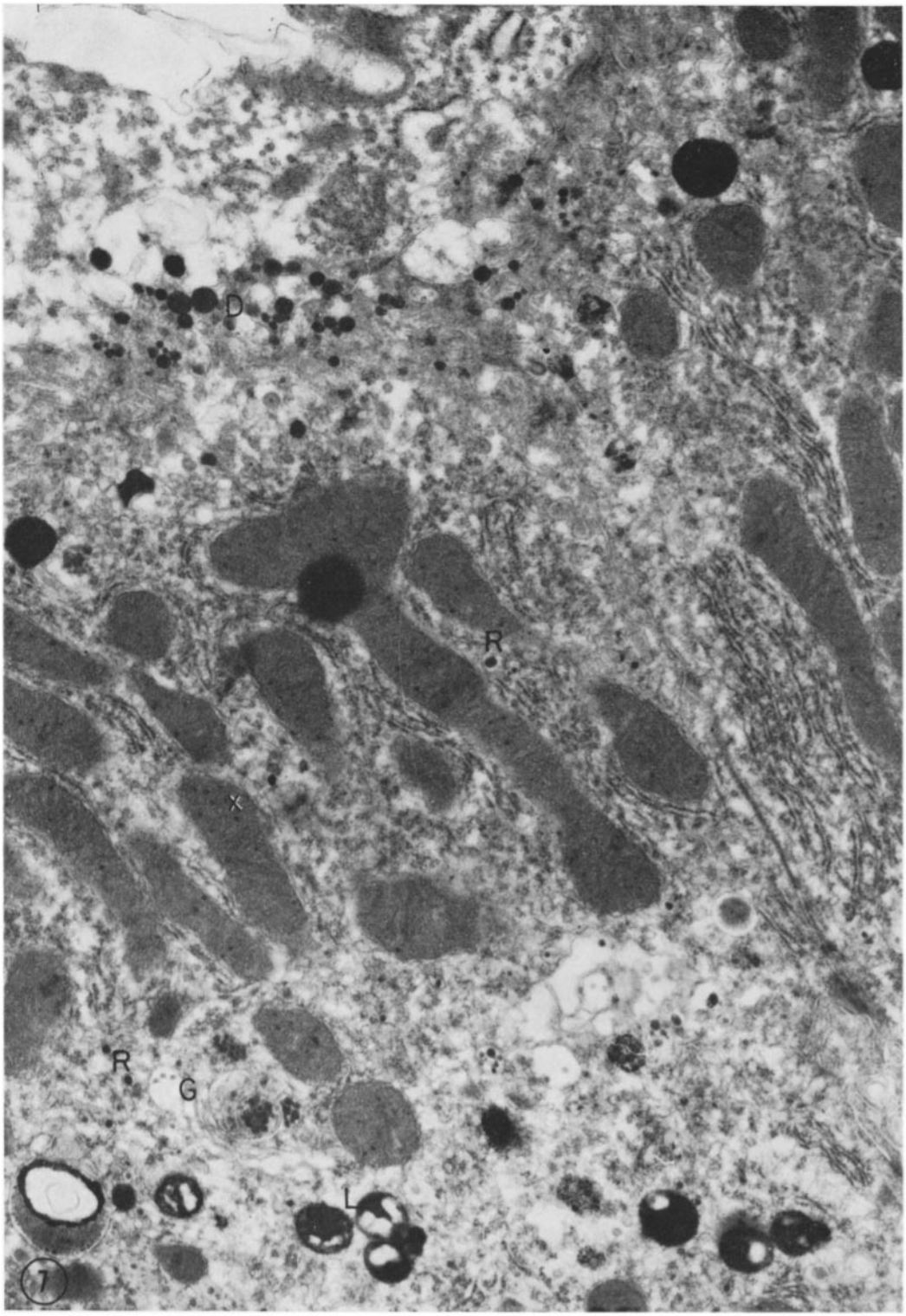


FIGURE 7 Electron micrograph of liver tissue of the same animal shown in Fig. 8. The tissue was first fixed in formaldehyde, and then treated with OsO_4. Osmiophilic droplets are present in the space of Disse (*D*), in Golgi vesicles (*G*), and in cisternae of the endoplasmic reticulum (*R*). Intramitochondrial dense granules are visible (*x*) as are the membranes of mitochondria, endoplasmic reticulum, and other cytomembranes. Lysosomes contain numerous large osmiophilic droplets (*L*). × 22,000.

the cells, could no longer be demonstrated. While these cytomembranes became invisible, many other protoplasmic structures, such as nuclear chromatin granules, ribosomes, lysosomes, dense bodies, mitochondrial matrix, and ground cytoplasm, remained essentially undisturbed. In contrast with formaldehyde- or glutaraldehyde-fixed tissue treated with lipid solvents before postfixation with OsO_4, tissue fixed only with OsO_4 and then treated with ethanol, acetone, or methanol-chloroform during the process of dehydration revealed that osmiophilic cytomembranes remained undisturbed and that osmiophilic droplets in the sinusoids and in the cytoplasmic vesicles remained (Fig. 9). Osmiophilic droplets in tissue subjected to this treatment, however, were of less electron opacity than in OsO_4-fixed tissue which had not been subjected to additional and prolonged treatment with lipid solvents.

Effect of Lipid Solvents on Homogeneous Osmiophilic Droplets

Homogeneous osmiophilic droplets were visible in formaldehyde- or glutaraldehyde-fixed hepatic tissue when lipid solvents were not employed prior to OsO_4 postfixation, but they disappeared when lipid solvents were used prior to OsO_4. Specifically, in fasting, ad libitum–fed, and fat-fed animals, there was a loss of visibility of all osmiophilic droplets in the sinusoids and space of Disse, in the nongranular membrane-bounded vesicles in the cytoplasm, and in the Golgi vesicles, and of the cytoplasmic osmiophilic droplets 1 μ in diameter or larger (Figs. 8, 10, and 11). While usually the osmiophilic droplets had completely disappeared when acetone was the lipid solvent used, collapsed, finely membranous, myelin figurelike deposits frequently remained (Fig. 12). The osmiophilic droplets contained within lysosomes and the dense intramitochondrial granules (Figs. 6 and 7) also were completely removed by the lipid solvents (Figs. 8, 10, and 11). The nucleoids of dense bodies were not, however, affected by lipid solvents.

Chemical Studies of Lipid and Protein Extraction by Lipid Solvents

The effects of lipid solvents on the radioactivity of hepatic lipids and proteins in formaldehyde-fixed and OsO_4-fixed tissue are shown in Tables I and II. In animals receiving C^{14}-l-leucine, an average of 76.1% of the radioactive label in liver tissue was in recoverable hepatic proteins. In those receiving triolein-C^{14}, an average of 72.1% of the hepatic tissue radioactivity was in extractable hepatic lipids. During OsO_4 fixation there was a loss into the OsO_4 solution of 8.0 and 8.6% of the radioactivity in protein-labeled livers (protein radioactivity) and of 10.7 and 23.9% of the radioactivity in lipid-labeled liver (lipid radioactivity) in the two animals of each group studied. Formaldehyde fixation was accompanied by a loss into the fixative solution of 10.6 to 11.6% of the protein radioactivity and of only 0.8 to 2.2% of the lipid radioactivity. Ethanol extraction of OsO_4-fixed tissue was followed by loss of only 0.7% of protein radioactivity and of only 0 to 2.4% of lipid radioactivity. When formaldehyde-fixed tissue was treated with ethanol, 2.0 to 3.9% of the protein radioactivity was removed, while 67.3 to 92.6% of lipid radioactivity was extracted. Acetone and methanol-chloroform extraction produced results which were similar to those of ethanol extraction, except that usually the percentage of extraction was slightly greater with ethanol.

Comparison of thin layer chromatographs of methanol-chloroform extracts (total lipid) of fresh, unfixed liver homogenate with chromatographs of the lipid solvent extracts from formaldehyde-fixed liver showed that all lipid classes present in fresh, unfixed liver were also represented in the lipid solvent extracts (Fig. 13). By inspection, the relative amounts of the lipid classes were similar in lipid solvent extracts of unfixed as compared with extracts of formaldehyde-fixed tissue, except in the case of acetone (Fig. 13). In the acetone extracts of 3 of the 4 animals, spots representing extracted phospholipids were considerably smaller than those of ethanol and methanol-chloroform extracts.

DISCUSSION

Pertinent to the interpretation of our findings on lipid solvent effect upon liver cell ultrastructure is the nature of OsO_4 reaction with tissue elements. Recent studies (19, 20) have added confirmation to the earlier view (21) that osmium tetroxide staining depends upon the presence of unsaturated bond sites in fatty acid residues. Osmium tetroxide staining results from this reaction although the osmium is deposited at other sites on the lipid molecule. These studies have shown also that protein is stained only slightly by osmium tetroxide. Accordingly, disappearance of densely osmiophilic droplets and particles after treatment with lipid solvents prior to OsO_4 fixation indicates lipid content. Structures of the cell which remain

after treatment with lipid solvents and which are inherently electron opaque or stain lightly with osmium tetroxide are believed to represent the protein components of the cell devoid of their attached lipids.

In his discussion on tissue fixation for electron microscopy, Pease (22) commented on the effects of lipid solvents on formaldehyde-fixed tissue. He noted that cytomembranes disappeared from view and he attributed this to the lipid solvent effect. In his illustration of tissue prepared with formaldehyde fixation, it is apparent that particulate lipids have also been removed from the tissue, as we observed.

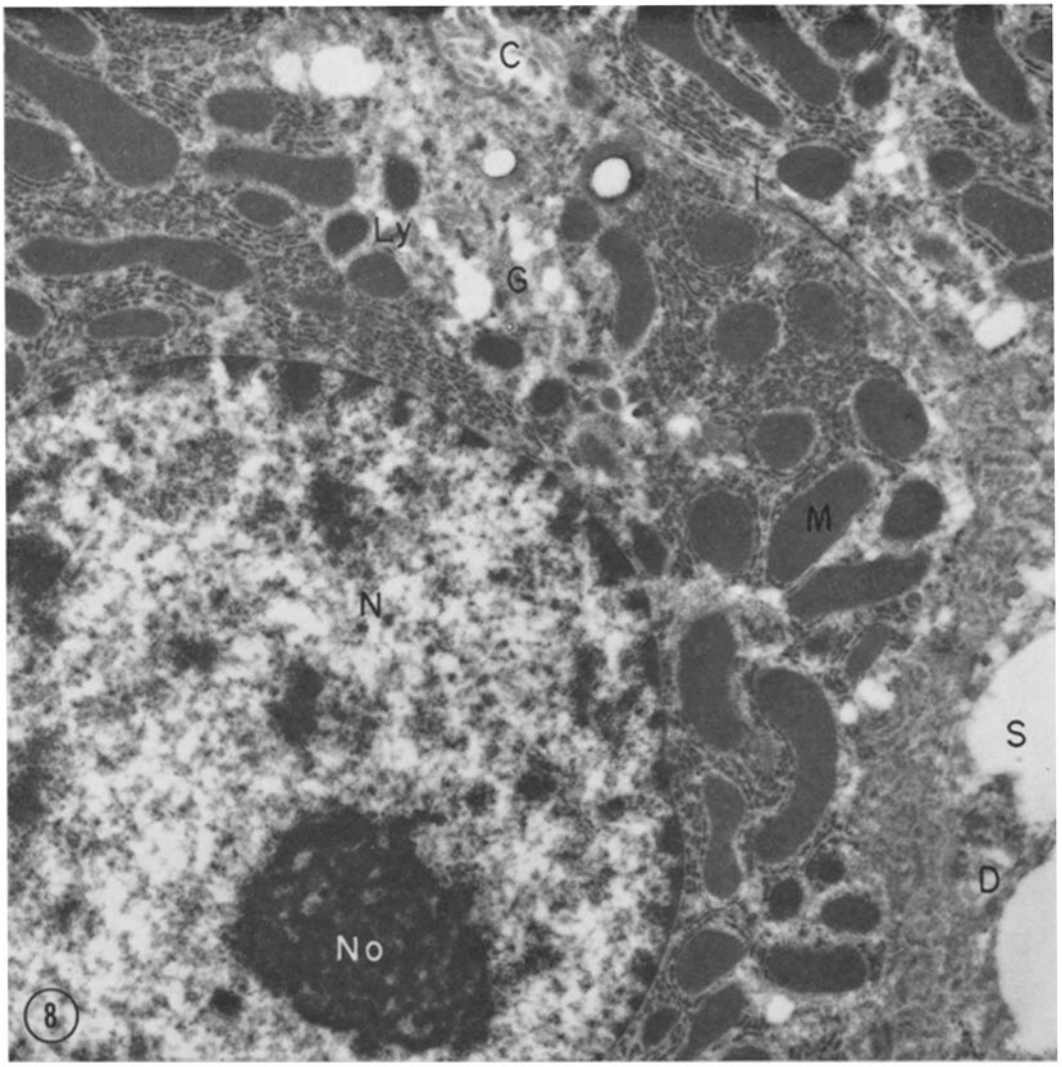
Although lipid solvents have been used for many years in the preparation of tissues for histological (23) and electron microscope studies (22), the degree of lipid extraction and the effects upon the various lipid classes have not been extensively studied (24). Relative preservation of lipid droplets in tissue fixed and maintained in formaldehyde is the basis for frozen section demonstration of lipid by sudanophilia, while formaldehyde-fixed tissue processed through lipid solvents to paraffin blocks contains no sudanophilic droplets (25). Thus, it appears that lipids may, to some degree, be extracted from formaldehyde-fixed tissue by lipid solvents. On the other hand, OsO_4-fixed tissue, even though it is passed through lipid solvents prior to embedment, continues to exhibit osmiophilic lipid droplets. This suggests that lipid solvents do not readily extract lipids following OsO_4 fixation. Our chemical and radiochemical studies substantiate these observations.

In vivo labeling of hepatic lipids and proteins by the procedures used in our experiments might not be expected to result in complete distribution of the label in total hepatic lipids and total hepatic proteins, respectively. Possibly some of the C^{14} in the course of the experiments might be metabolically diverted to compounds other than protein or lipid within the liver. Nor is it certain that the procedures of separation of total lipids (methanol-chloroform extraction) or of total proteins (trichloracetic acid precipitation) result in complete collection of these fractions. Possibly for these and other reasons it was found that the percentage of the C^{14} radioactivity in the "total protein" of the liver of animals having received C^{14}-l-leucine was 76% of that in the whole liver homogenate and that, in those animals receiving triolein-C^{14}, 72% of the label was in the "total lipid" fraction of the liver. Since not all the radioactivity appeared in total protein or lipid fractions of fresh, unfixed liver homogenates, the radioactivity extracted from formaldehyde- or OsO_4-fixed liver tissue by lipid solvents is referred to as "protein radioactivity" and "lipid radioactivity," respectively. Interpretations of the significance of the degrees of extraction of radioactivity by lipid solvents should take into account the fact that the figures obtained are not representative of absolute values of lipid and protein extractions; the observations, by indicating a trend, are believed, however, to be valid.

From 43.8 to 92.6% of lipid radioactivity in lipid-labeled rat livers was extracted from formaldehyde-fixed tissue by lipid solvents, while only 0 to 7.2% of the lipid radioactivity was extracted when the tissue had been primarily fixed in osmium tetroxide. Much smaller amounts of protein radioactivity were extracted by lipid solvents from

FIGURE 8 Electron micrograph of liver cell of the same animal shown in Fig. 7. The tissue was fixed with formaldehyde, followed by treatment with lipid solvent (ethanol), and subsequently with OsO_4. A portion of sinusoid (*S*) and space of Disse (*D*) are visible. In the cell can be seen the nucleus (*N*), nucleolus (*No*), mitochondria (*M*), lysosomes (*Ly*), Golgi vesicles (*G*), intercellular zones (*I*), and bile canaliculi (*C*). Notable in this electron micrograph is the absence of lipid droplets from the sinusoid, space of Disse, and from all cytoplasmic vesicles and cisternae. Dense osmiophilic droplets are also absent from lysosomes. Cytoplasmic membranes of all types are absent, although demarcation of the margins of cell components is clear-cut due to variations in electron opacity. There is also a complete absence of dense intramitochondrial granules. × 14,000.

FIGURE 9 Electron micrograph of liver tissue which had first been fixed in OsO_4 and was subjected to additional extraction with methyl alcohol–chloroform during preparation for Maraglas embedding. The cytomembranes and the osmiophilic droplets of the cytoplasm and in the sinusoid (*o*) remain undisturbed. × 18,000.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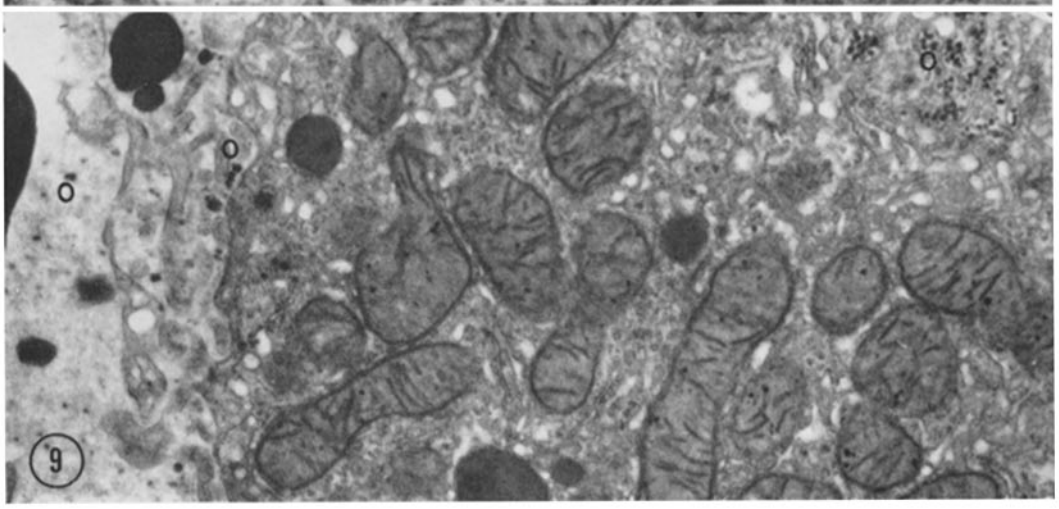
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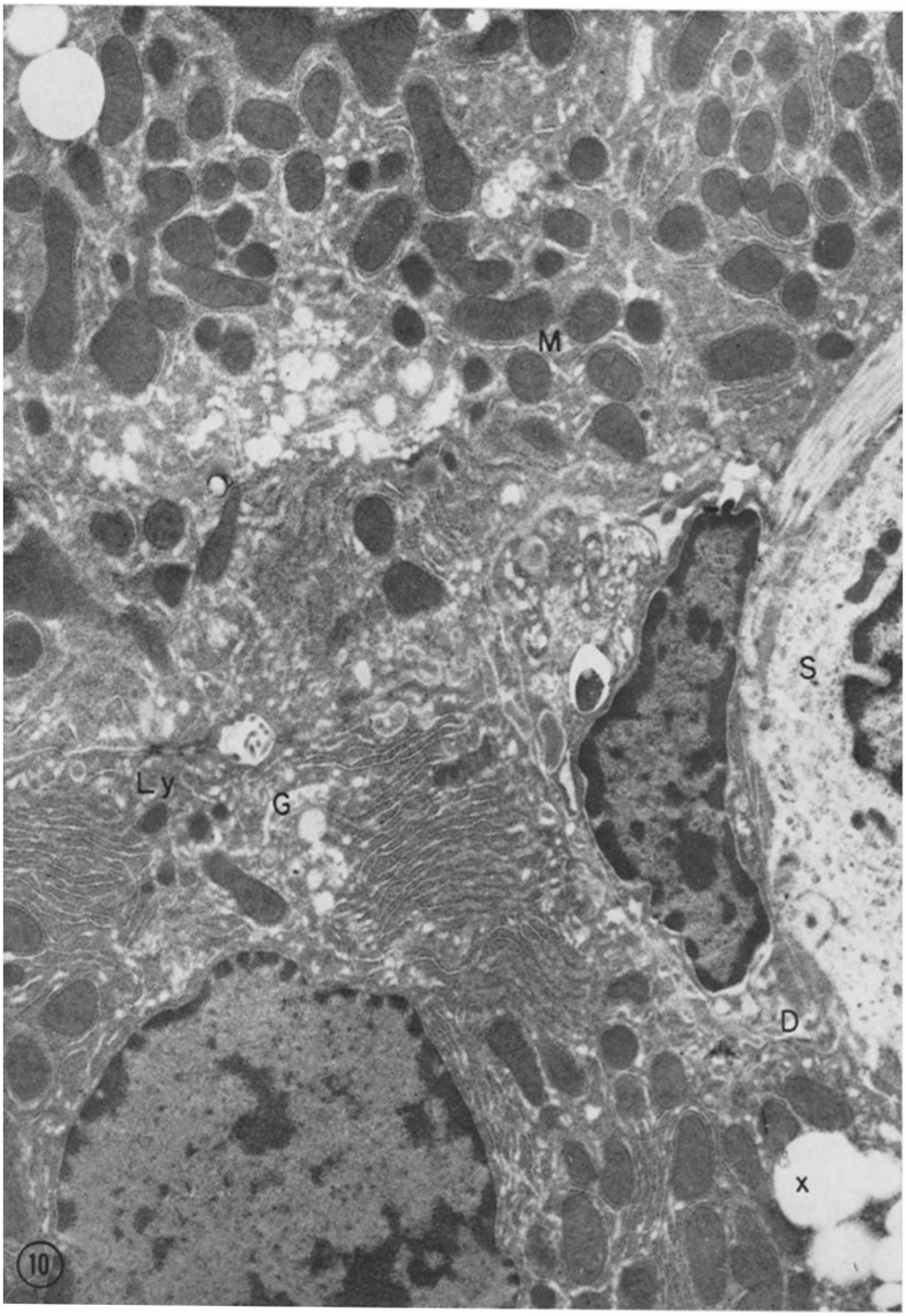

FIGURE 10 Electron micrograph of liver of animal 3 hr after fat administration. The tissue was first fixed in glutaraldehyde, followed by treatment with lipid solvent (ethanol), and then with OsO_4. A portion of a sinusoid (*S*) and space of Disse (*D*) are shown, and no lipid droplets are visible in these locations. Absence of osmiophilic droplets from Golgi vesicles (*G*) and other cytoplasmic vesicles is noted also. Larger, clear, round spaces (*x*) are present in the cytoplasm which represent sites from which large cytoplasmic osmiophilic droplets have been removed by the preparation of tissue with lipid solvents prior to OsO_4 postfixation. Lysosomes (*Ly*) and mitochondria (*M*) are visible. × 14,000.

hepatic tissue fixed either with formaldehyde or with OsO_4 (range of 0.7 to 3.9% from formaldehyde-fixed and of 0.2 to 0.7% from OsO_4-fixed tissue). Thus it appears that formaldehyde fixation does not render tissue lipids insoluble to lipid solvents, whereas OsO_4 does. During the course of our study, Korn and Weisman (26) reported on the effects of ethanol extraction of lipid on amebae fixed by glutaraldehyde or osmium tetroxide alone or in combination. They showed that lipid in aldehyde-fixed amebae is readily extractable, which is in agreement with our findings. Their result indicating a 20 to 25% loss mainly of triglycerides and some phospholipids from amebae following OsO_4 fixation is considerably greater than our finding of a 0 to 7.2% loss for hepatic tissue. The major portion of their label was incorporated into fatty acids and phospholipids which contain no or one double bond. This may account for the difference, since their fatty acid analyses of the extracts revealed that over 90% of the fatty acids extracted following OsO_4 fixation contained no double bonds. Thus, determinations excluded those fatty acids which had reacted with osmium.

Thin layer chromatography revealed essentially similar patterns of distribution and amounts of the various lipid classes in both ethanol and methanol-chloroform extracts from formaldehyde-fixed liver tissue as compared with methanol-chloroform extracts of unfixed whole liver. This suggests that there was no significant selective extraction of specific lipid classes by these two solvents. On the other hand, acetone extracts usually showed a decreased amount of phospholipid in the chromatographs, as compared with ethanol and methanol-chloroform extracts. This is in accord with the known relative insolubility of phospholipid in acetone (27).

These radiochemical studies lend support to our electron microscope findings on the nature of osmiophilic droplets within the hepatocytes. While it has usually been tacitly assumed that many of the dense membranes and densely osmiophilic particles and droplets seen with electron microscopy in hepatic cells contain lipid, conclusive evidence on this point has not previously been adduced. Since lipid solvents cause removal of these particles, but fail to affect presumed nonlipid constituents of the cells, it appears that those particles and droplets under discussion do contain lipid which is responsible for their dense osmiophilia.

It may be significant that, in acetone-extracted, formaldehyde-fixed tissue, electron microscopy reveals the presence of membranous osmiophilic formations resembling myelin figures at sites of dense osmiophilic droplets as seen in tissue prepared as usual for electron microscopy. Since acetone is not a suitable extractant of phospholipid (27), and since thin layer chromatographs indicated less extraction of phospholipid by acetone than by ethanol or methanol-chloroform, it is possible that the membranous osmiophilic remnants represent unextracted phospholipid.

The large osmiophilic droplets which accumulate in the liver cells in hepatic steatosis would seem, a priori, to be of lipid composition. Baglio and Farber (28) discussed the evidence favoring the lipid nature of osmiophilic droplets occurring in hepatic steatosis due to ethionine, and Novikoff et al. (29) concluded that osmiophilic droplets forming in the liver after administration of orotic acid were lipid. In fasting and in normal fat-absorptive states, however, the nature of small osmiophilic particles and droplets in hepatic cells is more problematical than in hepatic steatosis. Bruni and Porter (5), in their studies of the normal, fasting rat liver, described osmiophilic particles similar to those we observed in nongranular membrane-bounded vesicles and in the Golgi system. Reasoning from the known role of the granular endoplasmic reticulum in the synthesis of protein and the hypothesis of release of protein into the Golgi vesicles, these authors concluded that the osmiophilic droplets were protein in nature. The intravesicular particles illustrated in their report possesed less electrons opacity than those we have studied, but lead staining used in their material tends to diminish the density of osmiophilic droplets by decreasing contrast. The fact that in their position and size the particles studied by Bruni and Porter are similar to those we have studied in the Golgi vesicles, endoplasmic reticulum channels, and the space of Disse strongly suggests that they are identical. Our studies obviously do not present any evidence on the processes of protein elaboration and secretion, but our demonstration that lipid solvents remove the small osmiophilic droplets in the Golgi and other cytoplasmic vesicles in fasting and in fat-fed rats indicates that the dense osmiophilia of these droplets is due to their lipid rather than their protein content.

Many recent biochemical studies have dealt with the subject of lipoprotein elaboration by

TABLE I

Degree of Labeling in Trichloracetic Acid Precipitate (Total Protein) in Liver after C^{14}-l-leucine and in Methanol-Chloroform Extract (Total Lipid) after Triolein-C^{14}

Animal No.	C^{14} label used	CPM/g in whole liver homogenate	% of label in lipid	% of label in protein
1	l-Leucine	58,240		74.0
2	l-Leucine	58,280		78.5
3	l-Leucine	63,760		70.6
4	l-Leucine	57,760		81.4
5	Triolein	2,520	67.5	
6	Triolein	10,680	66.8	
7	Triolein	13,150	83.8	
8	Triolein	5,550	71.2	

hepatic cells and the subsequent release of these lipid particulates into the blood (9–11, 30), and electron microscopy has been employed in the effort to study the significance of intracellular lipid droplets (2, 14, 28, 29, 31, 32). The exact sites and morphologic expressions of these processes have so far not been clarified, however. Since our studies have shown that intravesicular droplets in hepatic cells contain lipid which is responsible for their marked osmiophilia, it seems highly probable that at least some of them represent stages of lipid incorporation and lipoprotein synthesis and secretion. This does not, of course, deny that other forms of intracellular lipid formations and droplets may exist. It is difficult to determine which of the lipid droplets represent absorbed chylomicron fat entering the liver cells and which represent lipoprotein droplets being assembled and secreted, but some of our observations bear upon this question. Lipoprotein droplets range from 300 to 700 A in diameter as measured under the electron microscope (33, 34). In the animal absorbing fat, osmiophilic droplets as small as 300 A and as large as 2000 A occur in the space of Disse and in vesicles near the periphery of the liver cell. We found such droplets to be comparable in range of size to those seen concurrently in the intestinal lymphatics of the fat-absorbing animals. Therefore, it seems likely that at least these larger-sized droplets (and probably some of the smaller ones) are chylomicrons entering the liver cell. Other osmiophilic droplets of the smaller sizes probably represent lipoprotein which has been synthesized in the liver cell and is in the process of being secreted. We have been unable to ascertain any difference between populations of the 300 to 700 A class of lipid droplets within the liver cell which would differentiate smaller chylomicrons entering from lipoprotein droplets leaving the cells. Studies are under way in our laboratory to correlate the presence, size, location, and number of intravesicular lipid droplets in liver cells following C^{14}-labeled fat absorption with the time of hepatic uptake of radioactivity and incorporation into hepatic lipids and serum lipoproteins.

Pertinent also to the question of identity of the small intravesicular lipid droplets are observations on fasting animals. In the fasting state it can be assumed that mobilized fatty acids are being taken up by the liver and converted partly to lipoproteins, which in turn are being secreted into the blood (8). Since mobilized fatty acid bound to albumen is not recognizable under the electron microscope (35), any observed lipid droplets in the space of Disse or in the serum would presumably be lipoprotein which has been secreted by liver cells. In such animals, osmiophilic droplets are sparse, but a few, ranging from 250 to 450 A in size, are found in the space of Disse, in Golgi vesicles, and

FIGURE 11 Electron micrograph of portions of liver cells and a sinusoid of animal which had been fasted for 120 hr, for comparison with Fig. 4. The tissue was first fixed in formaldehyde, then treated with lipid solvent (ethanol), and subsequently with OsO_4. The small osmiophilic particles which were present in the Golgi vesicles (*G*) and space of Disse (*D*) (see Fig. 4) of tissue primarily fixed with OsO_4 are no longer visible. A bile canaliculus (*C*) is visible. × 14,000.

FIGURE 12 Electron micrograph of liver of animal fed corn oil 3 hr previously. The tissue was fixed in formaldehyde and treated with acetone prior to OsO_4 postfixation. The osmiophilic particles which are seen in the sinusoid (*S*) and space of Disse (*D*) in tissue fixed primarily in OsO_4 are no longer present. The larger cytoplasmic osmiophilic droplets, however, are represented by collapsed and sometimes concentrically arranged membranous material in otherwise empty spaces (*x*). × 14,000.

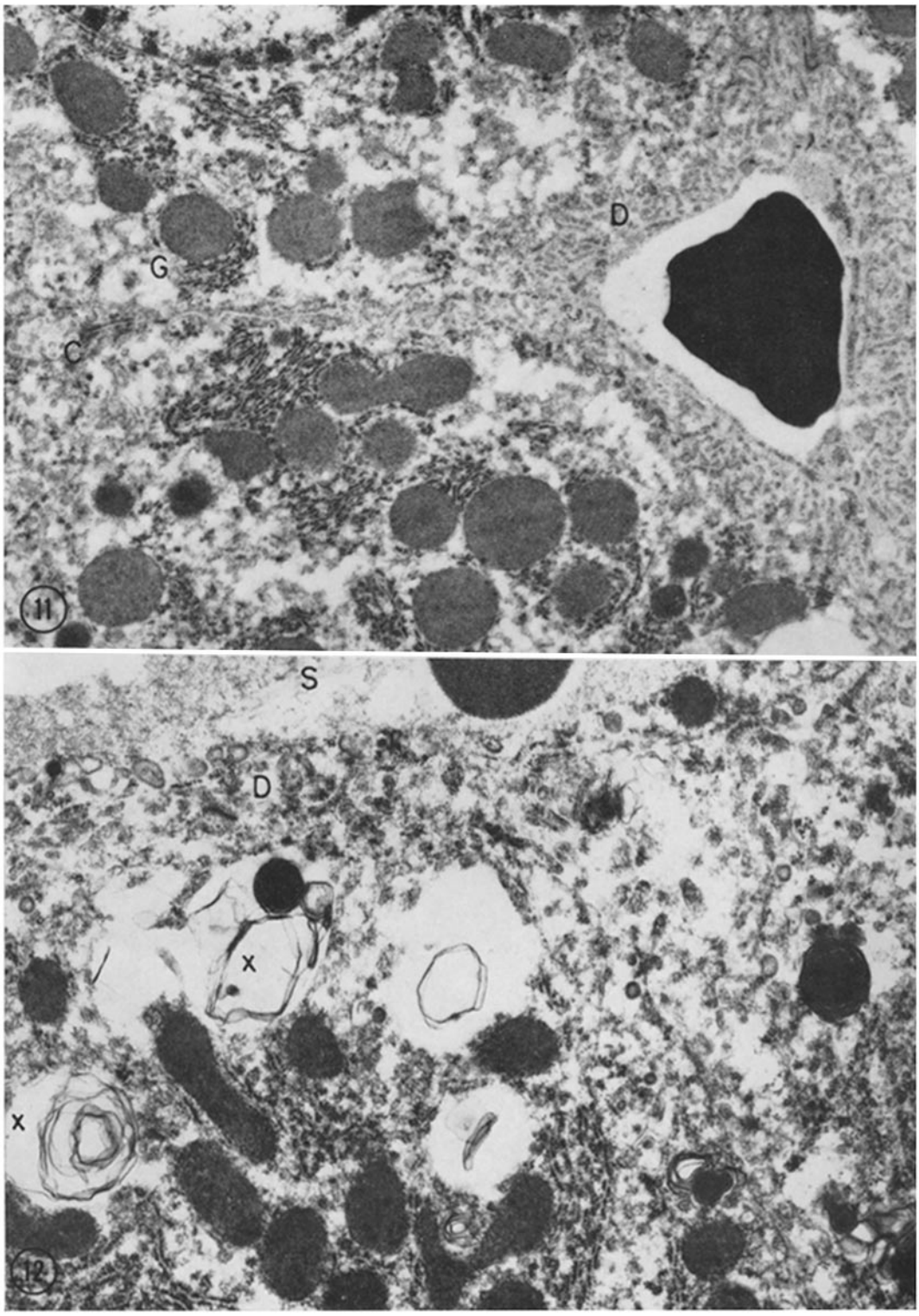
D
G
C
11
S
D
X
X
12

TABLE II

Effects of Lipid Solvents on C^{14}-Labeled Lipids and Proteins in Formaldehyde-Fixed and OsO_4-Fixed Hepatic Tissue. Radioactivity Expressed as Counts per Minute (CPM) from Entire Liver Sample Tested. Tissue Samples Were Thin Slices Amounting to Approximately ½ to 1 g in Each Experiment

Animal No.	C^{14} label used	Fixative used	Radioactivity (RA) in fixed tissue	% Loss of RA fixative fluid	% Loss of RA in ethanol	% Loss of RA in acetone	% Loss of RA in methanol-chloroform
			CPM				
1	C^{14}-l-leucine	Formaldehyde	51,340	10.6	2.5	3.4	0.7
2	l-Leucine	Formaldehyde	38,400	10.5	3.9	2.2	3.0
3	l-Leucine	Formaldehyde	48,040	11.6	2.0	5.8	2.6
4	l-Leucine	Formaldehyde	43,460	10.7	2.7	5.7	1.5
1	l-Leucine	OsO_4	15,720	8.6	0.7	0.7	0.2
2	l-Leucine	OsO_4	15,740	8.0	0.7	0.3	0.2
5	Triolein-C^{14}	Formaldehyde	2,780	2.2	67.4	43.8	58.9
6	Triolein	Formaldehyde	8,540	0.8	89.0	68.2	85.4
7	Triolein	Formaldehyde	12,800	2.1	92.6	74.7	87.2
8	Triolein	Formaldehyde	5,800	1.9	83.9	74.2	79.8
5	Triolein	OsO_4	3,180	23.9	0	7.2	5.4
6	Triolein	OsO_4	6,880	10.7	2.4	2.2	2.6

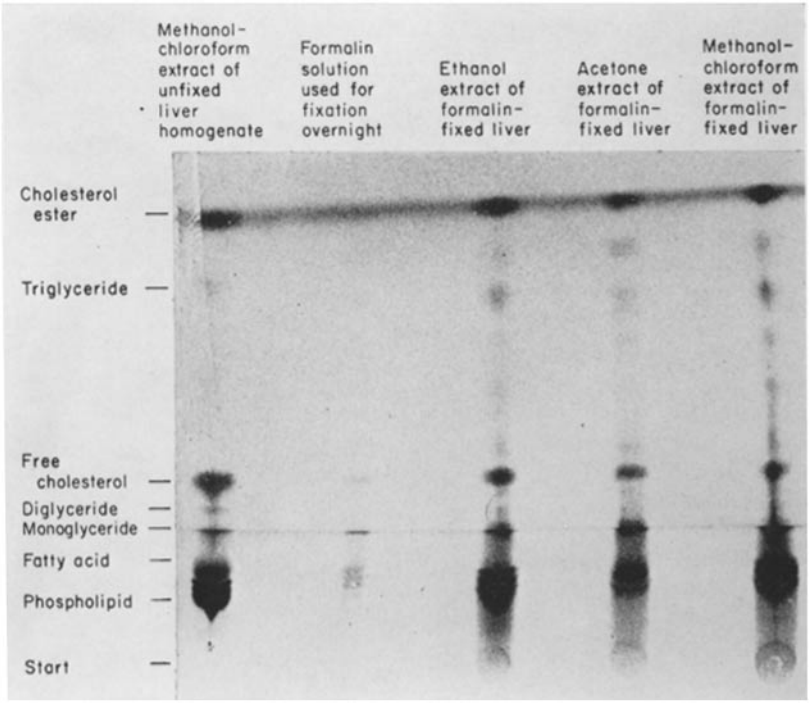

FIGURE 13 Phosphomolybdic acid–stained thin layer chromatographs of lipid extracts of unfixed liver and of formaldehyde-fixed liver. The lipid solvent extracts of formaldehyde-fixed liver produce a pattern of lipid classes similar to that of the methanol-chloroform extract of unfixed liver. The phospholipid zone is less prominent in the chromatograph of acetone extract of fixed liver than in that of ethanol or methanol-chloroform extracts. The methanol-chloroform extract of the formaldehyde solution in which the liver was fixed shows only a small amount of lipid, predominantly phospholipid.

in nongranular membrane-lined vesicles. These were identified as lipid-containing bodies by their removal with lipid solvents. Such bodies, therefore, may represent lipoprotein particles which are being synthesized and secreted by the liver cells.

The role of lysosomes in intracellular hepatic lipid metabolism has received little attention (36). Our studies have shown that lysosomes of hepatic cells of rats contain dense osmiophilic droplets which are more abundant during fat absorption, and that they disappear following lipid solvent effect. What bearing this content of lipid droplets in lysosomes may have upon the processes of intracellular lipid metabolism remains to be determined.

One other observation on the effect of pretreatment with lipid solvents upon liver cell ultrastructure is the complete disappearance of mitochondrial dense granules in the same manner that outer mitochondrial membranes and cristae disappear, while the mitochondrial matrix is unaffected. The composition of these dense intramitochondrial granules has been variously assessed to be artifact (37), iron (38), and calcium or other cations (39, 40). The presently favored concept is that they represent accumulations of cations, especially calcium (5, 40). That mitochondria sequester calcium and phosphate has been shown by direct chemical analysis (41), but the identity of these cations with mitochondrial dense granules has not been established. Furthermore, Reynolds (42) has recently shown that the deposits of calcium phosphate which occur in the mitochondria of hepatocytes of rats treated with carbon tetrachloride are distinct from the normal, dense intramitochondrial granules. The disappearance of the dense granules within mitochondria following lipid solvent extraction, as we have shown, indicates that they contain lipid which accounts entirely for their electron opacity. The role of mitochondria in the oxidation of fatty acids is well known (43), and a lipid component of mitochondria which has a rapid metabolic turnover has been described (44). Obviously, the exact chemical form of lipid in which the droplets exist cannot be ascertained from these studies. Further studies are needed to determine the metabolic role of these intramitochondrial lipid droplets.

The technical assistance provided by M. D. Glass and N. J. Arnold in this study is gratefully acknowledged.

This study was supported by United States Public Health Service Grant No. HE 08676-02. Dr. Leonard and Dr. Eigenbrodt are Fellows supported in part by Research Training Grant No. 5 T1 GM 227-04.

Received for publication 17 December 1965.

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