# Formation of crystalline tripalmitin-rich spicules in isolated hepatocytes

**Joseph A. Ontko, David L. Stiers, and William F. Woodside**[1]

Cardiovascular Biology and Electron Microscopy Laboratory, Oklahoma Medical Research Foundation, and Department of Biochemistry and Molecular Biology, University of Oklahoma College of Medicine, Oklahoma City, OK 73104

**Abstract** Conditions were developed for rapid deposition of triglyceride in isolated rat hepatocytes. Liver cells from fasted rats were incubated for 90 min at 37°C with 3.0 mM palmitic or oleic acid, 4% bovine serum albumin, 20 mM glucose, 10 mM lactate, and 1 mM pyruvate. When oleic acid was used, numerous cytoplasmic lipid droplets were produced. When hepatocytes were incubated with palmitic acid, similar amounts of triglyceride were synthesized but instead of lipid droplets, a vast accumulation of peculiar spicules permeated the cytoplasm. These inclusions appeared in myriads of swirled threads, thick elongated angular plates, and needles, some of which exhibited longitudinal osmiophilic bands of 250 Å thickness. These structures were associated with smooth endoplasmic reticulum. The cells appeared otherwise normal. Polarized light microscopy at 37°C revealed a multiplicity of brilliant white inclusions between crossed polars in cells incubated with palmitic acid. These birefringent structures exhibited 90° periodicity between both maximum brilliance and extinction, indicative of anisotropic crystalline deposits. Molecular species analysis of triglycerides in cells incubated with palmitic acid, together with data on [1-$^{14}$C]palmitic acid incorporation, demonstrated an almost exclusive synthesis of tripalmitin. Spicules isolated from homogenized hepatocytes displayed needles containing longitudinal single and double osmiophilic bands of 110 Å and 260 Å thickness, repectively, and lipid spicular aggregates. The isolated spicules were almost pure tripalmitin by analysis. These observations document the formation and development of crystalline triglyceride in living cells and may provide a unique system for the study of cellular lipid synthesis, transport and deposition. —**Ontko, J. A., D. L. Stiers, and W. F. Woodside.** Formation of crystalline tripalmitin-rich spicules in isolated hepatocytes. *J. Lipid Res.* 1989. **30:** 1375–1384.

**Supplementary key words** birefringence • endoplasmic reticulum • fatty acids • lipid droplets • liver • oleic acid • palmitic acid • triglyceride synthesis/deposition

Triglycerides and cholesteryl esters are extremely hydrophobic lipids that deposit in a wide variety of cellular systems in the form of cytoplasmic lipid droplets (1–7). The relative abundance of triglycerides and cholesteryl esters in these droplets is a function of the cell type and the species of lipid, lipoproteins, and other substrates in the extracellular fluid environment. Lipid droplet subpopulations of different solvent density have been isolated from rat liver (8). In this organ triglycerides and cholesteryl esters are also present in nascent very low density lipoprotein particles in the secretory pathway (7, 9–12).

In the course of studies on the metabolism of triglyceride-rich lipid droplets in isolated rat hepatocytes, we noticed a plethora of peculiar crystalline-like structures dispersed throughout the cytoplasm when the cells were incubated with palmitic acid under conditions of rapid triglyceride synthesis. This synthetic process occurs in the outer leaflet of the endoplasmic reticulum bilayer (13). The present communication describes the formation, ultrastructure, and chemistry of these unusual lipid deposits.

## MATERIALS AND METHODS

### Animals

Male Holtzman rats were from Sasco (Omaha, NE). The animals weighed 300–400 g and were maintained on Purina Laboratory Chow ad libitum. These animals were housed at 22°C with lights on at 6 AM and off at 6 PM. They were fasted for the final 17–19 h prior to isolation of hepatocytes.

### Isolation and incubation of hepatocytes

Hepatocytes were isolated by perfusion of the liver with collagenase by the procedure of Berry and Friend (14) as modified by Seglen (15). The basic perfusion and incubation medium was Krebs-Henseleit bicarbonate (KHB) solution (16). Glucose was added at a concentration of 25 mM to all perfusion and other media employed in the

Abbreviations: TG, triglyceride.

[1]Present address: Department of Pharmacology, University of New Mexico Medical School, Albuquerque, NM 87131.

hepatocyte isolation. It was found that this substrate maintained the hepatic triglyceride concentration at in vivo levels during the cell isolation procedure.

Hepatocytes were incubated at a concentration of 4–6 mg dry wt/ml in plastic flasks with a gas phase of 95% $O_2$/5% $CO_2$ for 90 min at 37°C in 4% bovine serum albumin (Fraction V, fatty acid-free, Armour Pharmaceutical Co., Kankakee, IL) containing 3 mM palmitic or oleic acid. The medium also contained 20 mM glucose, 10 mM lactate, and 1 mM pyruvate in order to decrease the rate of fatty acid oxidation in the fasted cells and thereby promote triglyceride synthesis. In some experiments [1-$^{14}$C]palmitic or [1-$^{14}$C]oleic acid (New England Nuclear, Boston, MA) at a specific radioactivity of 0.1 μCi/μmol was used to measure incorporation of the fatty acids into cellular lipids and their subsequent mobilization. For the measurement of mobilization, the cells were washed and incubated 90 min at 37°C in 2-ml volumes at cell concentrations of 5–7 mg dry wt/ml in substrate-free KHB containing 2% fatty acid-free albumin. The decline in radioactivity in labeled lipids and the concurrent production of $^{14}CO_2$ and labeled perchloric acid-soluble oxidation products were measured (17) to assess the rate of lipid mobilization.

### Isolation of lipid spicules

The lipid inclusions from hepatocytes were isolated by homogenization (18) and centrifugation in sucrose gradients (8). Following incubation for 90 min in 3 mM palmitic acid as described above, the cells were sedimented by low speed centrifugation and resuspended in 0.25 M sucrose buffered with 10 mM imidazole, pH 7.4. The cells were then lysed with a Dounce homogenizer. Cell rupture was verified by light microscopy. The homogenate was adjusted to a sucrose concentration of 52%, overlaid with 35% sucrose, and centrifuged at 25,770 *g* for 30 min. Lipid droplets and deposits were recovered at the surface of the 35% sucrose layer. This fraction was overlaid with 25% sucrose, 10% sucrose, and water, which was centrifuged at 25,770 *g* for 30 min. Layers of lipid were prominent at the top of the water zone (layer A), at the water–10% sucrose interface (layer B), at the 10%–25% sucrose interface (layer C), and at the 25%–35% sucrose interface (layer D). These layers were individually resuspended in 35% sucrose and recentrifuged under the same conditions in the water–10% sucrose–25% sucrose–35% sucrose gradient. They migrated predominantly to their respective locations in the first gradient. These lipid fractions were examined by electron microscopy, as described below. In the isolation of lipid spicules from hepatocytes, cell homogenization and gradient centrifugation were done at 0–5°C and at room temperature for comparison. Results were similar at both temperatures. However, the various bands of lipid droplets and spicules in the sucrose gradients appeared with somewhat greater definition at 0–5°C.

### Lipid extraction and analysis

Aliquots of the hepatocyte suspensions were extracted at the beginning and end of the incubation with chloroform–methanol 2:1 according to the procedure of Folch, Lees, and Sloan Stanley (19). It was possible to obtain a fluffy precipitate of protein when 6.7 volumes of methanol were first added to the cell suspension with swirling and heated in a water bath at 40°C for 30 min, followed by the addition of 13.3 volumes of chloroform. After 1 h the mixture was filtered, washed (19), and the final lipid extract was used for triglyceride assay (20) and, in some experiments, thin-layer chromatographic separation of phospholipids, diglycerides, free fatty acids, triglycerides, and cholesteryl esters (21, 22). In the assay of triglyceride (20) a Zeolite mixture (#990-2, Sigma Chemical Co., St. Louis, MO) was used instead of silicic acid to remove phospholipids.

### Triglyceride molecular species

Triglycerides were analyzed by gas–liquid chromatography to quantify molecular species which contained total fatty acyl chain contents of 48, 50, 52, 54, and 56 carbons (23, 24). For these analyses the hepatocytes and lipid fractions from the sucrose gradients were initially extracted with 10 volumes of heptane–isopropanol 3:7 which contained cholesterol butyrate, 2.5 μg/ml, as internal standard.

### Electron microscopy

At the beginning and end of the 90-min incubation period, the isolated hepatocytes were fixed by adding an equal volume of half-strength Karnovsky's fixative (25) at 37°C or at room temperature and pH 7.2 to the cell suspension. Primary fixation continued for 2 h, after which the cells were rinsed in 0.1 M cacodylate buffer, pH 7.2, and then post-fixed in 2% cacodylate-buffered $OsO_4$ for 1 h. After post-fixation, the cells were rinsed in cacodylate buffer, dehydrated through an ascending series of alcohol, and infiltrated and embedded in a low viscosity epoxy resin. After oven curing, the samples were thin-sectioned on a Reichert Ultracut E, contrasted with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX electron microscope. At both fixation temperatures spicules were prominent in cells incubated with palmitic acid.

For examination of the isolated lipid spicules, grids were floated carbon-coated side down on drops containing the spicules for 2 min, excess fluid was wicked off using filter paper, and grids were suspended sample side down over 2% $OsO_4$ for 5 min at a height of 1 mm. After vapor fixation, the grids were allowed to air dry for 1 h to

remove traces of $OsO_4$ vapor prior to examination in the microscope cited above.

### Polarized light microscopy

At the end of the 90-min period of incubation at 37°C with and without long chain fatty acid substrates, hepatocyte suspensions were immediately transferred to a portable 37°C water bath and carried to a constant temperature room maintained at 37°C for microscopic examination with transmitted polarized light. A Zeiss binocular polarizing microscope with a graduated rotary stage, 10 × eyepieces, and both 10 × and 40 × objectives, was used. This microscope contained a swing-out polarizer carrier with fixed polarizer below the condenser and an analyzer slider with an adjustable analyzer above the objective lens. Since this microscope was not equipped with a heating–cooling stage, microscopic examination of the liver cells between crossed polars was conducted in the constant temperature room to insure maintenance of the cells at 37°C. The polarizing microscope was placed in this room for a period sufficient to achieve temperature stability prior to use. The cells were first focused with the 10 × objective with the polarizer carrier out. The polarizer was then swung into the beam of transmitted light.

### Hepatocyte conversion factors

Cell concentrations and results are reported in units of dry weight. For approximate conversion to other units the following factors may be useful. The dry weight may be multiplied by 3.7 to estimate wet weight (26). For approximate conversion to protein content, we have observed that hepatocytes from fasted rats contain an average of 0.57 mg of protein/mg dry wt by parallel assays of protein (27) and dry weight. This value is in the range of that reported by Quistorff, Bondesen, and Grunnet (28), specifically 140–180 mg protein/g wet wt, when we used the above factor of 3.7 (26) for interconversion of dry weight and wet weight. In terms of cell number, a value of $128 \times 10^6$ hepatocytes/g wet wt has been found (29). This was obtained with cells from fasted rats. It should be considered that since acute fasting causes a considerable reduction in liver size (approximately 30%) without cell destruction, hepatocytes from fed rats are larger and therefore fewer in number/g wet wt. A value of $138 \times 10^6$ was calculated by Krebs et al. (30) based on a hepatocyte volume of 7250 $\mu^3$ reported by Greengard, Federman, and Knox (31). It is apparent that the rats used in these studies (31) were fed animals. These values, $128 \times 10^6$ and $138 \times 10^6$/g wet wt, are closer than anticipated, based on differences in liver size in fed and fasted rats.

### Chemicals

Chemicals were obtained from sources described previously (8, 9, 20, 32, 33). Bovine albumin, fraction V, fatty acid-free, was supplied by ICN Immuno Biologicals (#82-002-04, Lisle, IL). This was formerly Pentex bovine albumin #82-002-4 from Miles Laboratories (Elkhart, IN). The tripalmitin was obtained from Sigma Chemical Co. (St. Louis, MO).

## RESULTS

### Triglyceride contents of hepatocytes

In the course of experiments designed to cause the acute accumulation of lipid droplets in hepatocytes, we incubated isolated liver cells with high concentrations of long chain fatty acids together with antiketogenic substrates to inhibit fatty acid oxidation and consequently promote triglyceride synthesis. Palmitic acid at a level of 3 mM in the presence of 4% bovine serum albumin together with 20 mM glucose, 10 mM lactate, and 1 mM pyruvate were used. In six experiments the initial concentration of triglyceride in the cells was 17.4 ± 2.3 (SD) nmol/mg dry wt and after 90 min of incubation with palmitic acid, as described above, the cellular triglycerides were elevated to 71.3 ± 7.2 (SD) nmol/mg dry wt. Similar results were obtained in five experiments with 3 mM oleic acid substrate, with triglycerides starting at 18.9 ± 3.9 (SD) nmol/ml dry wt and increasing to 68.5 ± 9.3 (SD) nmol/mg dry wt in 90 min. When the cells were incubated 90 min in the absence of the added substrates, the triglyceride content declined 4.8 ± 2.0 (SD) nmol/mg dry wt (four experiments), equivalent to an average loss of 27% of the cellular triglyceride.

### Ultrastructure of hepatocytes

The freshly isolated hepatocytes suspended in the incubation medium were examined at the beginning (zero time) and at the end of 90 min of incubation with the fatty acid substrates. The hepatocytes were from fasted rats and exhibited normal appearance at zero time (**Fig. 1A**) with a few lipid droplets. After 90 min of incubation with 3 mM palmitic acid, a 16-carbon saturated fatty acid, numerous arrays of crystal-like deposits ramified the cytoplasm (Fig. 1B). These clearly defined structures, termed spicules herein, displayed numerous configurations, most prominently aggregates of swirled threads and crystal-like plates and needles. When the hepatocytes were incubated with oleic acid, an 18-carbon monounsaturated fatty acid, instead of palmitic acid, an abundance of typical spherical lipid droplets was observed (Fig. 1C). Organelles and plasma membranes appeared normal in cells incubated with either of the fatty acids. The unusual lipid deposits (Fig. 1B) are shown at higher magnification in Fig. 1D to stretch prominently from sites of termination of the rough endoplasmic reticulum. Some of these inclusions exhibited multiple branches (**Fig. 2A**) and some contained thin osmiophilic ribbon-like longitu-

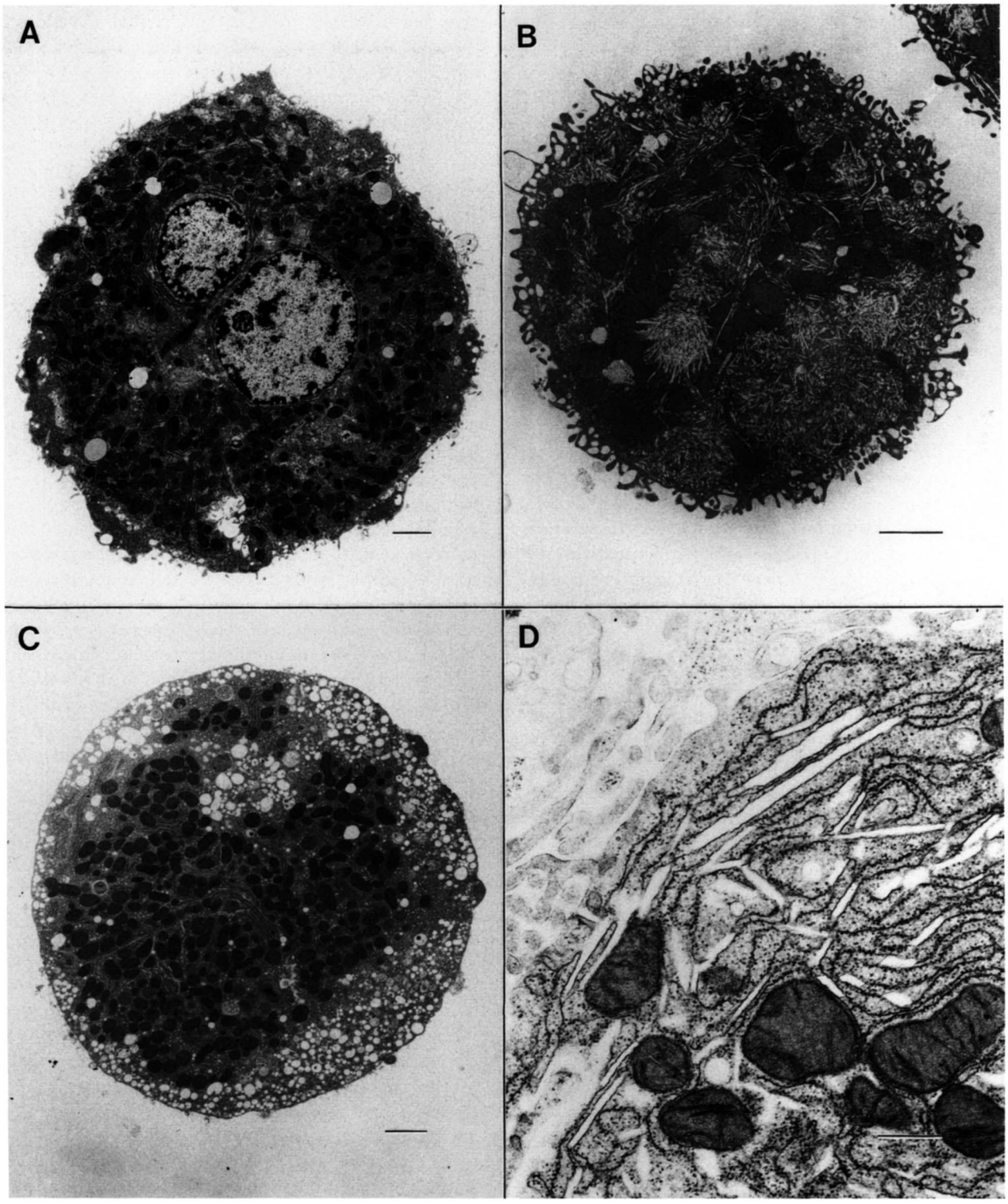


**Fig. 1.** Transmission electron micrographs of isolated rat hepatocytes prior to and following incubation with long chain fatty acid substrates. Hepatocytes were isolated from a fasted rat and incubated 90 min at 37°C under conditions favoring rapid triglyceride synthesis. A high concentration of fatty acids (3.0 mM) together with 4% bovine albumin and antiketogenic substrates (20 mM glucose, 10 mM lactate, and 1.0 mM pyruvate), pH 7.2–7.3, in Krebs-Henseleit medium with a gas phase of 95% $O_2$/5% $CO_2$ were employed. The cells were fixed in formaldehyde/glutaraldehyde, post-fixed in osmium tetroxide, dehydrated, infiltrated and embedded, thin-sectioned, and contrasted with uranyl acetate and lead citrate. Cells shown are representative of many observations. A: Hepatocyte prior to incubation contains a few mature lipid droplets. B: Hepatocyte after 90 min of incubation at 37°C with 3.0 mM palmitic acid. Myriads of needles and swirled threads permeate and dominate the cytoplasm. C: Hepatocyte after 90 min of incubation at 37°C with 3.0 mM oleic acid. Minute lipid droplets are exhibited in abundance. D: Hepatocyte after 90 min of incubation at 37°C with 3.0 mM palmitic acid is shown at higher magnification. Crystalline structures project from terminal sites of rough endoplasmic reticulum. In all panels A–D mitochondria appear normal and the maintenance of cell integrity is evident. Bars: A, B, C, 2 μm; D, 500 nm.

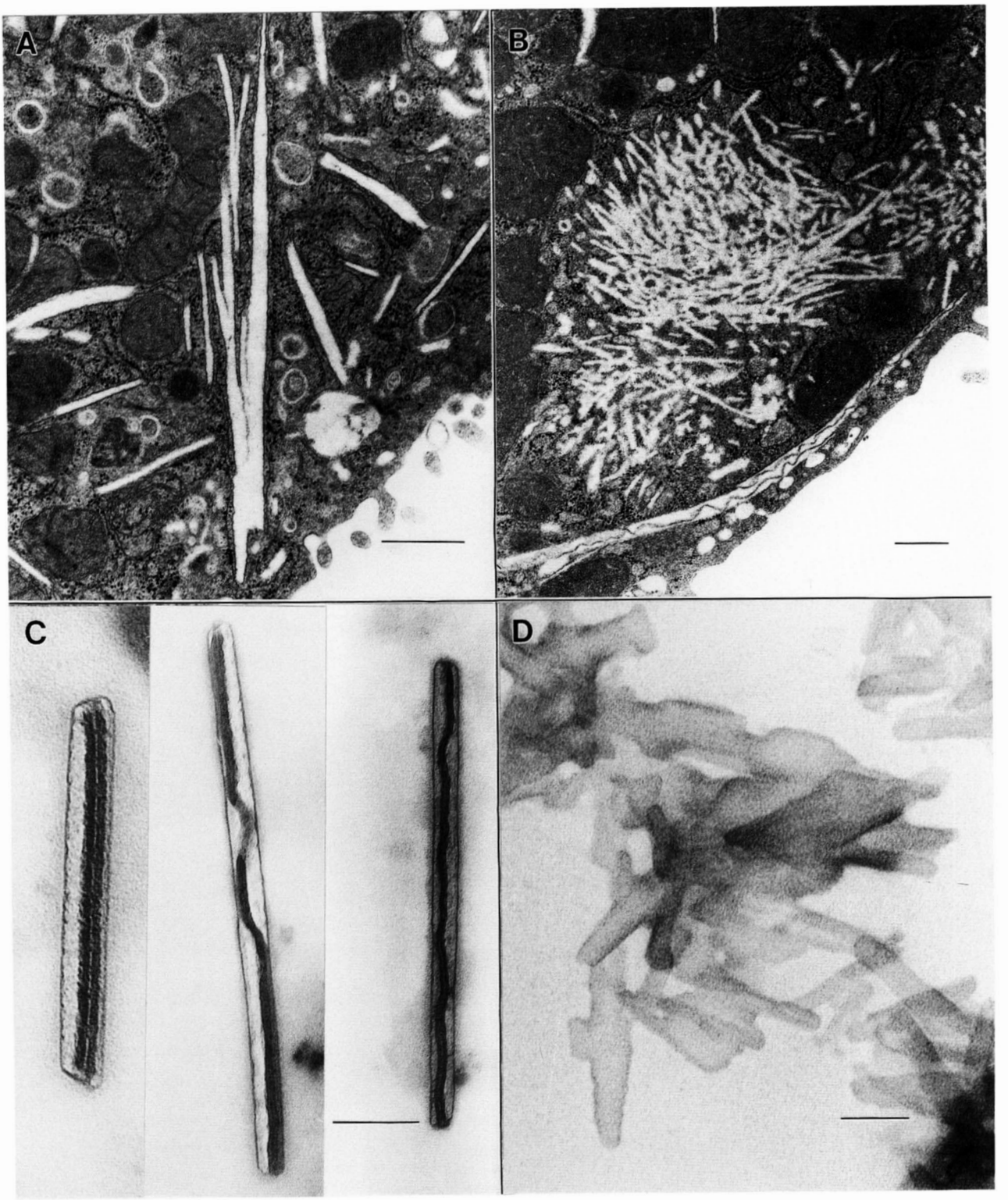


**Fig. 2.** Transmission electron micrographs of rat hepatocytes incubated with palmitic acid (A and B), and of lipid spicules isolated from the same cell population (C and D). Hepatocytes from a fasted rat were incubated 90 min at 37°C with 3.0 mM palmitic acid and processed for electron microscopy as described in Fig. 1. A: The presence of branched crystals is clearly demonstrated. B: A giant curved crystal, with interiorized longitudinal osmiophilic bands of 250 Å thickness, parallels the cell periphery. Clusters of swirled threads are prominent. After incubation, some of the cells were disrupted with a Dounce homogenizer and crystalline inclusions were harvested from the lipid-rich band between the water and 10% sucrose layers of the discontinuous sucrose density gradient. Spicules were adsorbed on carbon-coated grids by flotation and fixed in $OsO_4$ vapor. C: Membranes appear to be longitudinally embedded within isolated triglyceride crystals. Single osmiophilic bands are uniformly 110 Å thick (right and center) and the double band in the left crystal exhibits a uniform total width of 260 Å. D: Isolated spicules are displayed in complex clusters. Bars: A and B, 500 nm; C, 100 nm; D, 200 nm.

dinal bands, as visible in the large needle-like deposit near the periphery of the cell in Fig. 2B. This electron-dense ribbon has a uniform thickness of 250 Å. Two such ribbons are adjacent in some regions of this large crystal-like deposit.

### Electron microscopic examination of isolated spicules

Hepatocytes incubated 90 min with 3 mM palmitic acid were homogenized (18), adjusted to a sucrose concentration of 52%, and centrifuged in a discontinuous sucrose gradient designed for the isolation of lipid droplet subpopulations (8) (see Materials and Methods). Prominent layers of material at the surface of the water zone (layer A) and at the water–10% sucrose interface (layer B) were harvested, and examined by electron microscopy. Layer A contained lipid droplets and a few spicules and layer B was found to contain an abundance of needle-like spicules of variable thickness and aggregates thereof (Figs. 2C and 2D). Many of the needles contained osmiophilic longitudinal bands (Fig. 2C). The center and right needle-shaped spicules in Fig. 2C exhibit bands with a distinct and uniform thickness of 110 Å. The left spicule in Fig. 2C contains a double band with a combined thickness of 260 Å.

### Appearance of hepatocytes in polarized light

Hepatocytes incubated 90 min at 37°C in Krebs-Henseleit medium containing 4% bovine serum albumin with either 3.0 mM palmitic or oleic acid were examined at 37°C between crossed polars with a polarizing microscope. The cells incubated with oleic acid appeared in uniform dark greyish outlines. They were barely visible and contained no bright inclusions. However, at the same degree of illumination, the cells incubated with palmitic acid exhibited intense brilliant white inclusions in a multiplicity of sizes and shapes. Bright lights intermittently flashed at different sites in these hepatocytes when focused at different cytoplasmic levels at a magnification of 400. While many of these cells radiated a myriad of bright particles, others contained only a few. All of the bright particles, many of which appeared as needles, alternated between greatest brightness and disappearance at 45° intervals when the microscope stage was rotated. Maximum brilliance and extinction therefore both exhibited a 90° periodicity. Light transmission through some of the particles, while sharply displaying this 90° oscillation, was not totally extinguished at the minimum, possibly the consequence of adjacent inclusions, branched structures, and/or formation of complex clusters. Polarized light microscopic examination therefore demonstrated an abundance of intracellular birefringent crystals in these hepatocytes, which were carefully maintained at 37°C throughout both incubation and morphological study. Microcrystals of commercial tripalmitin, recrystallized from ethanol–diethyl ether 1:2, possessed the same birefringent properties, namely bright white with 90° periodicity, as did the hepatocyte inclusions.

### Molecular species composition of hepatocyte triglycerides and isolated spicules

The incubation of hepatocytes for 90 min with palmitic acid and oleic acid markedly enriched the triglyceride with 48 and 54 fatty acyl carbon atoms, respectively (**Table 1**). The marked accumulation of TG-48 and TG-54 in the cells incubated with palmitic and oleic acids, respectively, reflects high rates of synthesis of tripalmitin and triolein in these cells. Triglyceride molecular species analysis of layer B from the sucrose gradient (above) demonstrated that this lipid spicule-rich fraction contained 88% TG-48, 9% TG-50, 2% TG-52, and 1% TG-54. The spicules probably contain an even higher percentage of TG-48 (tripalmitin) but in their isolation the presence of a few lipid droplets which contain higher molecular weight triglycerides is probable.

### Incorporation of [1-$^{14}$C]palmitic and [1-$^{14}$C]oleic acids into cellular lipids

Over 90% of the fatty acids that were incorporated into hepatocyte lipids during the 90-min incubation period were found in the triglyceride fraction, while 3–4% entered cellular phospholipids (**Table 2**). Small amounts were also present in diglycerides, free fatty acids, and cholesteryl esters. Under the conditions used, 40–60% of the added fatty acid was utilized by the hepatocytes in the various experiments during the 90-min period of triglyceride accumulation. When the lower concentration of 0.5 mM palmitate or oleate of the same specific radioactivities was incubated with the hepatocytes, the amounts of fatty acid incorporation into the cellular lipids were only 15.8% as great and the ratio of radioactivity incor-

TABLE 1. Triglyceride molecular species in isolated hepatocytes

| Hepatocytes | TG-48[a] | TG-50 | TG-52 | TG-54 | TG-56 |
|---|---|---|---|---|---|
| Control[b] | | | | | |
| μg TG/mg dry wt | 0.07 | 1.10 | 5.97 | 4.23 | 1.66 |
| % of total | 0.5 | 8.4 | 45.8 | 32.5 | 12.7 |
| Palmitic acid | | | | | |
| μg TG/mg dry wt | 46.23 | 13.59 | 5.03 | 2.99 | 0.78 |
| % of total | 67.4 | 19.8 | 7.3 | 4.4 | 1.1 |
| Oleic acid | | | | | |
| μg TG/mg dry wt | 0.41 | 1.46 | 12.57 | 56.32 | 1.86 |
| % of total | 0.6 | 2.0 | 17.3 | 77.6 | 2.6 |

Analysis following incubation for 90 min with a 3 mM concentration of the fatty acid plus 20 mM glucose, 10 mM lactate, and 1 mM pyruvate.

[a]TG-48, TG-50, TG-52, TG-54, and TG-56 refer to molecular species of triglyceride that contain fatty acyl chains with a total of 48, 50, 52, 54, and 56 carbon atoms.

[b]Hepatocytes were extracted for analysis prior to incubation with fatty acids.

TABLE 2. Lipid radioactivities in hepatocytes incubated with [1-$^{14}$C]palmitic and [1-$^{14}$C]oleic acids

| Lipids[a] | [1-$^{14}$C]Palmitic Acid | [1-$^{14}$C]Oleic Acid |
|---|---|---|
| | % | % |
| Triglycerides | 92.84 | 93.87 |
| Phospholipids | 3.41 | 3.85 |
| Diglycerides | 2.53 | 0.75 |
| Free fatty acids | 0.86 | 0.48 |
| Cholesteryl esters | 0.36 | 1.05 |

[a]Lipids were extracted from hepatocytes, after incubation for 90 min with 3 mM [1-$^{14}$C]palmitic acid or 3 mM [1-$^{14}$C]oleic acid, and separated by thin-layer chromatography.

porated into triglycerides to that into phospholipids decreased from 24–27 (Table 2) to 5–7. In this situation net triglyceride accumulation was less than 5 nmol/mg dry wt and lipid spicules were not observed in the hepatocytes when examined by electron microscopy.

In three experiments in which hepatocytes were incubated 90 min with 3 mM [1-$^{14}$C]palmitic acid (235,610 dpm/$\mu$mol in all experiments) together with 20 mM glucose, 10 mM lactate, and 1 mM pyruvate, the average accumulation of cellular triglyceride was 61 nmol/mg dry wt. The observed accumulation of triglyceride radioactivity averaged 45,590 dpm/mg dry wt, which demonstrates incorporation of 193 nmol of palmitic acid. This is equivalent to 64 nmol of triglyceride, in close agreement with the observed increase in triglyceride mass. These results indicate the predominant synthesis of tripalmitin.

### Mobilization of triglycerides accumulated by hepatocytes

Following the incubation of hepatocytes for 90 min with 3 mM [1-$^{14}$C]palmitic and [1-$^{14}$C]oleic acids, the cells were washed and incubated for another 90 min in the absence of added substrates. The decline in radioactivity in the labeled lipids and the concurrent production of labeled oxidation products were measured to assess the rate of lipid utilization. When cellular triglycerides were labeled by incubation with 3 mM [1-$^{14}$C]oleic acid, resulting in the accumulation of triglycerides in lipid droplets (Fig. 1C), approximately 12% of the triglyceride was mobilized during the subsequent 90-min period of incubation. When the same cells were labeled with 3 mM [1-$^{14}$C]palmitic acid, causing deposition of triglycerides in the form of lipid spicules (Fig. 1B), the same percentage of cellular triglycerides (12%) was mobilized during 90 min of additional incubation. It was observed by electron microscopy that an abundance of lipid spicules remained in the palmitic acid-labeled cells at the end of the mobilization period.

## DISCUSSION

Triglyceride accumulates in the liver under a variety of conditions such as alcohol consumption (34), diabetes (35), genetic obesity (32), carbon tetrachloride poisoning (36), tetradecylglycidic acid (20), orotic acid (37), a sucrose-rich fat-free lipogenic diet (33), excess dietary cholesterol (38), and in many other circumstances. The underlying metabolic alterations include inhibition of fatty acid oxidation, suppressed lipoprotein synthesis or secretion, and excessive triglyceride synthesis from an elevated supply of plasma free fatty acids or from an elevated rate of de novo fatty acid synthesis. All of these situations promote triglyceride deposition in the form of lipid droplets. Lipid droplets rich in cholesteryl ester also accumulate in cellular systems provided with appropriate forms of cholesterol (39).

Although triglyceride synthesis is known to occur on the outer leaflet of the endoplasmic reticulum (13), the mechanisms involved in the formation and turnover of lipid droplets remain obscure. The present observation of an unusual form of cellular triglyceride deposition resulted from studies designed to produce cells with a high triglyceride content in vitro. A combination of factors apparently contributed to lipid spicule formation. The use of the saturated fatty acid palmitic acid was of central importance and the addition of glucose, lactate, and pyruvate minimized the oxidation of the fatty acid, thereby elevating its conversion to triglyceride. The utilization of hepatocytes from fasting rats depressed the activity of hepatic fatty acyl-CoA desaturase (40), and thereby minimized the conversion of palmitic acid to the corresponding monounsaturated fatty acid. The introduction of double bonds in the hydrocarbon chain of the fatty acid lowers the melting point to that of a liquid at 37°C. The formation of tripalmitin crystals is accordingly favored by low desaturase activity. The high concentration of palmitic acid was also a key factor, as it elevated triglyceride synthesis to rates which apparently exceeded its potential for transfer to and solution in the hydrophobic fluid phase of preexisting lipid droplets (9).

The analysis of triglyceride molecular species following incubation of hepatocytes with palmitic acid, which contains 16 carbons, showed that 70% of the triglyceride molecules contained fatty acid chains with 48 carbon atoms (Table 1). Prior to incubation this molecular species was almost nil. Concurrently, the triglyceride mass increased from 20 to 70 nmol/mg dry wt. Thus 50 nmol of the 70 nmol total, or 70%, was newly synthesized. These observations together with the data on the incorporation of [1-$^{14}$C] palmitic acid of known specific radioactivity into cellular triglycerides (Table 2 and text) conclusively demonstrate the rapid and almost exclusive synthesis of tripalmitin under the conditions used. Despite its pres-

ence in the solid state, the newly synthesized tripalmitin could still undergo metabolic turnover, as evidenced by its mobilization (text). The greatly increased surface area of this tripalmitin, in the form of swirled threads and needles in contrast to spherical droplets, may have influenced this activity.

The formation of tripalmitin at the cytosolic surface of the endoplasmic reticulum apparently occurs so rapidly that triglyceride molecules saturate the environment adjacent to the membrane, leading to the development of multilayered structures of solid tripalmitin as a combined consequence of low solubility and high melting point. The $\alpha$, $\beta'$ and $\beta$ forms of tripalmitin melt at 44.7, 56.6, and 66.4°C, respectively (41). Proximity of the endoplasmic reticulum is supported by the presence of osmiophilic bands of appropriate size imbedded in some of the tripalmitin spicules (Figs. 2B and 2C). Thus, the bands of 110 Å thickness (center and right panels in Fig. 2C) may be endoplasmic reticulum membranes. Those of approximately 250 Å thickness, which contain a uniform core of low staining intensity (left panel of Fig. 2C) are suggestive of adjacent membranes of the tubular endoplasmic reticulum with narrowed cisternae. Compression of the cisternal compartment could result from continued synthesis and deposition of tripalmitin after solidification of the triglyceride was initiated.

The lipid spicules appear to occur in association with smooth endoplasmic reticulum and to terminate at junctions with rough endoplasmic reticulum (Fig. 1D). Ribosomes are not present at the lipid spicular surfaces (Figs. 1D, 2A, and 2B) nor are they found to be associated with, or imbedded within, isolated spicules (Figs. 2C and 2D). These observations suggest that triglyceride synthesis is localized in the smooth endoplasmic reticulum. This would indicate that in plasma lipoprotein synthesis apolipoprotein and triglyceride are synthesized in different regions of the endoplasmic reticulum and that assembly of triglyceride-rich lipoproteins occurs during sequential passage of the apolipoproteins through the smooth endoplasmic reticulum, since nascent VLDL particles appear in smooth surfaced vesicles. Sequential flow from the rough to the smooth endoplasmic reticulum with subsequent vesicular transport to Golgi compartments is the generally accepted view (42).

The molecular organization of the triglyceride in the hepatocellular spicules is of interest. Triglycerides do not form liquid crystals (43). The tripalmitin deposits appear to occur in two different configurations in the hepatocytes, namely in the form of swirls of minute needles and as thick straight angle-edged geometric projections similar to crystalline inclusions. The swirled structures are abundant in Fig. 1B and prominent in Fig. 2B. The large angular geometric inclusions are present in Figs. 1B, 2A, and 2B. The isolated spicules of Fig. 2C are similar to these geometric inclusions, whereas those in Fig. 2D more closely resemble the swirled needle deposits. It is conceivable that the two different configurations relate to the movement of the endoplasmic reticulum. Thus, if the cell cytoplasm is in motion during triglyceride formation on the smooth endoplasmic reticulum, swirled threads of tripalmitin could be spun from the membrane surfaces. In a more quiescent region of the cell large crystals of tripalmitin may grow and develop throughout a longer period. Alternatively, the smooth endoplasmic reticulum may exist in a tubular configuration in areas of the cytoplasm where swirled needles are formed and in a parallel sheet configuration where the large angular geometric structures are visible. Intracellular gradients in substrate concentration may be contributory. The large crystalline structures may develop in regions of high palmitate concentration. These inclusions seem to be more prevalent at the cell periphery (Figs. 1D, 2A, and 2B), and this was also evident when the cells were viewed in polarized light.

The existence of crystalline structures at 37°C in the living cells during incubation with palmitic acid was established by polarized light microscopy. The display by these inclusions of the anisotropic property of bright birefringence in polarized light and the exhibition of 90° periodicity of both brilliance and extinction between crossed polars were the key observations.

The presence of tripalmitin in the crystalline state in the hepatocytes is supported by observations that triglycerides of long chain saturated fatty acids crystallize in needles (44, 45) in shapes observed in Fig. 1D, 2A, 2B, and 2C. If the isolated needles (Fig. 2C) crystallized only at some terminal stage during their incubation, it is unlikely that they would incorporate osmiophilic bands of membrane dimensions in such a highly uniform manner. The straight, parallel edges of the spicules also indicate their progressive and continuous construction in a molecular lattice. If the newly synthesized tripalmitin was initially present as a super-cooled liquid in the cells at 37°C, and in some manner subsequently solidified during the incubation, the highly organized integration of the lipid spicules in the hepatocyte cytoplasm (Figs. 1B, 1D, 2A, and 2B) would not have been observed. Further, specificity in the location of spicules at sites extending from the rough endoplasmic reticulum (Fig. 1D) would not have been seen. Thus, triglyceride in a liquid state assumes the shape of spherical lipid droplets, as exhibited by the cells incubated with oleic acid (Fig. 1C). If this triglyceride solidified from this state, it would do so in a spherical form, and if conditions favored its slow crystallization, sharp-edged needles would have randomly emerged and penetrated surrounding organelles. However, never were spicules observed to penetrate mitochondria, nuclei, or other membrane-bound organelles, nor did they protrude through the plasma membrane. This is strong evidence

for the gradual and organized growth and development of the tripalmitin crystals during tripalmitin synthesis with concurrent solidification without disturbance of intracellular architecture.

Deposition of cholesterol in crystalline form in tissues has been reported in lipid-laden atheromatous cells (46, 47) and atherosclerotic arterial lesions (48). Cholesterol monohydrate crystallizes in plates and melts above 85°C (41). Cholesterol crystals appear to be present in the livers of cholesterol-fed guinea pigs (49) and rabbits (50). Crystalline cholesteryl esters have also been reported in the livers of diabetic rats fed cholesterol and cholic acid (51). In contrast, triglycerides in the solid state have not been observed in cells or tissues at body temperature. Triglyceride crystals in the form of needles have been described in frozen sections of human liver (52) and in frozen adipose tissue (43). Low temperatures are normally required to induce crystallization, since tissue triglycerides commonly contain substantial amounts of mono- and polyunsaturated fatty acids.

The present study describes conditions that lead to the formation of crystalline triglyceride structures in the hepatocyte. More detailed examination of the physicochemical properties of these lipid deposits will be the subject of future investigation. Triglycerides are normally present in cells in the form of free-floating lipid droplets in the cytoplasm. The observed tripalmitin spicules represent triglyceride trapped at its site of synthesis. This system may provide an approach to gain insight on this process and on related mechanisms of intracellular triglyceride transport and metabolism. These studies may also have bearing on the cellular dynamics of the hydrophobic companion of triglyceride, cholesteryl ester, which, like triglyceride, deposits in lipid droplets and accumulates in various pathological conditions.

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