Studies on the compartmentation of lipid in adipose cells. I: Subcellular distribution, composition, and transport of newly synthesized lipid: liposomes

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ABSTRACT The subcellular distribution and composition of endogenously synthesized lipid in isolated white adipose cells were studied to determine the nature and extent of lipid compartmentation. After brief incubation of cells with labeled glucose, acetate, or palmitic acid, over 90% of newly synthesized triglyceride was localized in the bulk-lipid phase, indicating rapid intracellular transport and storage. From 13 to 20% of the newly formed lipid was diglyceride, and over 95% of it was localized in the central lipid-storage vacuole rather than in organelle systems concerned with esterification, thus indicating intracellular segregation of newly synthesized partial glycerides. Most of the newly synthesized phosphatides partitioned with membranous organelles. Synthesis of cholesterol or cholesteryl ester was negligible.

After brief incubation of cells with labeled glucose, the relative specific activity of organelle triglyceride was mitochondria $\gg$ microsomes $>$ liposomes $>$ soluble supernatant $>$ bulk lipid. In pulse-chase studies the specific activity of organelle triglyceride decreased and that of the bulk fraction increased reflecting intracellular lipid transport. The data suggest that a significant proportion of newly formed lipid is transferred from mitochondrial membranes into the storage vacuole by direct lipid–lipid interaction. Liposomes, which consist of small enclosed lipid droplets resembling chylomicrons, contained triglycerides of specific activity similar to microsomal triglyceride. While the evidence that liposome triglyceride may be microsomal in origin is indirect, the results do indicate that the liposome fraction represents a phase in the transport and(or) storage of new glyceride.

At least two forms of compartmentation of newly synthesized lipids occurred. The first, termed "structural," refers to localization of lipids to organelle fractions. The second type of compartmentation, termed "chemical," concerns the intracellular segregation of a specific lipid class. The accumulation and segregation of newly synthesized diglyceride in the bulk storage pool are examples of the latter form of compartmentation.

SUPPLEMENTARY KEY WORDS lipid synthesis · diglyceride · lipid pools · compartmentation · adipose organelles · intracellular lipid transport · lipid storage

GLYCERIDES stored in adipose tissue probably exist in more than one anatomic or functional pool. On the basis of nutritional studies in man, Hirsch, Farquhar, Ahrens, Peterson, and Stoffel (1) proposed that adipose cells contain at least two pools of lipid: a large reservoir of stored fat and a small, reactive pool of lipid, which equilibrates rapidly with plasma lipids but only very slowly with the larger pool of metabolically inert lipids. More direct evidence or intracellular compartmentation was reported by Kerpel, Shafrir, and Shapiro (2). They showed that fatty acids, which were released from prelabeled adipose tissue incubated with epinephrine, had a higher specific activity than the total tissue glyceride fatty acid. Furthermore, mixed particulate sediments from homogenates of prelabeled adipose tissue were enriched with newly formed esters, indicating significant inhomogeneity in the cellular distribution of newly synthesized glycerides. While these observations have been amply confirmed (3–5), detailed information about the nature and extent of structural and functional segregation of lipids in the adipose cell is lacking. For this

Abbreviations: TLC, thin-layer chromatography.

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reason, a study of the subcellular distribution and composition of endogenously synthesized lipid in isolated adipose cells was undertaken.

MATERIALS AND METHODS

Male Wistar rats weighing 170–220 g and raised on Purina chow were used throughout the study. The animals were killed by a sharp blow to the head. In each experiment epididymal fat pads from 3 to 10 rats were treated with bacterial collagenase (Worthington Biochemical Corp., Freehold, N.J.) to obtain the adipose cells (5, 6). The free cells were suspended in 12 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% bovine serum albumin (Fraction V; Nutritional Biochemicals Corporation, Cleveland, Ohio) and 1.27 mM Ca^{++}. Glucose was added to a final concentration of 16 μmoles/ml followed by the addition of the isotopic substrates (see below). 1 or 2 ml of the cell–medium mixture was added to 1-oz plastic bottles (Nalgene Labware Div. Nalge/Sybron Corp., Rochester, N.Y.) which were then incubated in a Dubnoff metabolic shaker, oscillating at 90 cycle/min, at 37°C with 95% O_2–5% CO_2 as the gas phase. 1 ml of the incubation mixture contained 35–60 μmoles of cell triglyceride, and corresponded to about 35–60 mg of intact adipose tissue. Unless stated otherwise, each experiment was carried out at least twice.

Isolation of Organelle Fractions (Fig. 1)

Following incubation with isotopic substrate, the contents of several vials were pooled in a 15 ml plastic centrifuge tube and centrifuged at 300 *g* for 15 sec. The infranatant medium was removed by suction through a Pasteur pipette, and the floating cells were resuspended and washed three times in fresh buffer-albumin medium, and three times with 0.25 M sucrose. They were then transferred to a 15 ml glass centrifuge tube, and disrupted by mechanical agitation for 1 min on a Vortex type mixer. This mixture was centrifuged at 300 *g* for 30

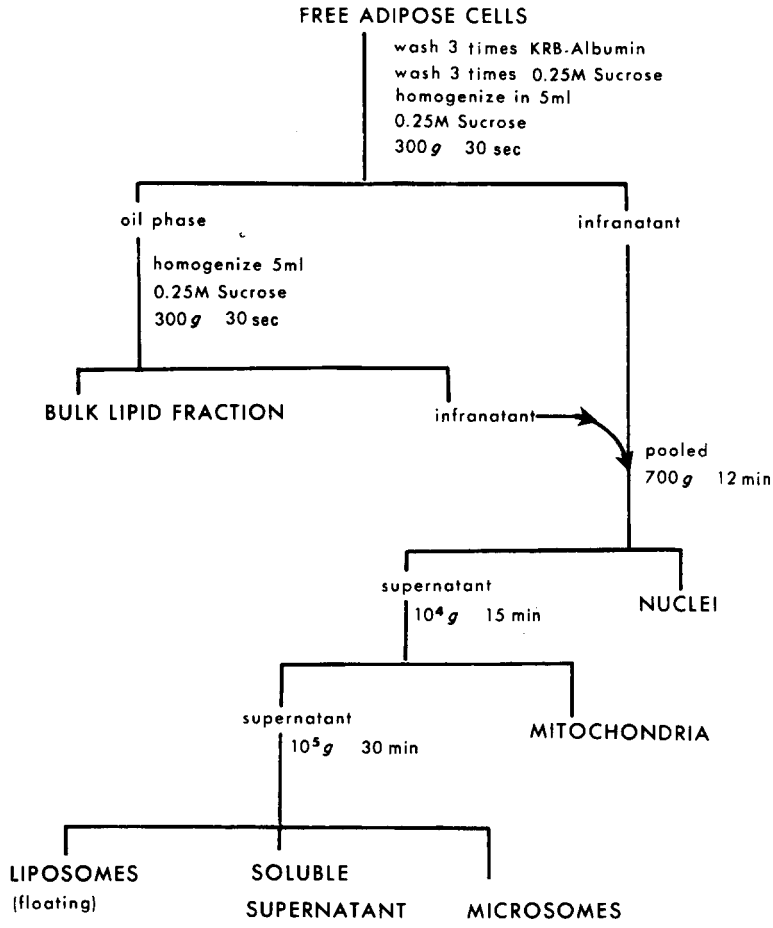

FIG. 1. Scheme for isolating adipose cell organelle fractions. Isolated adipose cells were obtained after collagenase digestion of adipose tissue (7). The cells were washed repeatedly and homogenized by agitation in a glass centrifuge tube. The bulk fraction was obtained at room temperature; the remaining fractions were isolated at 4°C by preparative ultracentrifugation. KRB, Krebs-Ringer bicarbonate buffer.

sec at room temperature, and the following three phases appeared: (*a*) a supernatant oil layer, (*b*) an aqueous infranatant phase containing most of the organelles, and (*c*) a white emulsion at the interphase. The opaque infranatant fluid was aspirated into a chilled syringe through a long needle. The oil layer was thoroughly mixed with 5 ml of 0.25 M sucrose (Vortex mixer) and centrifuged. The first and second infranatant fractions were pooled. The remaining oil layer was assumed to represent the stored lipid and will be referred to as the bulk-lipid fraction. The pooled aqueous extracts were centrifuged at 700 *g* for 12 min at 4°C in order to sediment nuclei and float any oil carried over in earlier transfers. 7 ml of the supernatant fluid was centrifuged at 10,000 *g* for 15 min at 4°C in a Spinco Model L ultracentrifuge (No. 40 rotor) to obtain a mitochondrial pellet. A thin gray-white film of material floated at the top of the 10,000 *g* supernatant fluid and was partly adherent to the side wall of the tube. On electron microscopy, this fraction was shown to consist of small, membrane-enclosed lipid droplets called liposomes (7, 8). Using a Pasteur pipette, this material was dispersed into the supernatant fluid which was then quantitatively removed and centrifuged at 100,000 *g* for 30 min at 4°C. The following three fractions were evident: (*a*) a pellet of microsomes, (*b*) the soluble supernatant, and (*c*) a floating film of liposomes which dispersed like a cloud into the supernatant fluid upon the slightest agitation.

2 ml of the soluble supernatant was drawn into a syringe through a needle that was passed through the floating film to a point in the supernatant fluid that was optically clear. The floating liposome fraction was then carefully aspirated with a Pasteur pipette. About 2 ml of the soluble phase was carried along with the liposome fraction; in the analysis of data, appropriate corrections were made for the presence of this material. In some experiments mitochondria and microsomes were isolated together by centrifuging the 700 *g* supernatant at 100,000 *g* for 30 min. This fraction was designated "M–M." Before extracting the mitochondrial and microsomal pellets with lipid solvents, the tube walls were carefully wiped with Kleenex moistened with chloroform to minimize contamination. The lipid ester content or lipid radioactivity content of each fraction was corrected back to the starting volume of the whole homogenate.

In studies on intracellular transport of newly synthesized lipid, the centrifuge tube was cut with a tube slicer (Spinco), and the liposomes, with about 0.5 ml soluble supernatant solution, were removed. This fraction was resuspended in 11 ml of 0.25 M sucrose and centrifuged at 100,000 *g* for 30 min. This procedure minimized contamination of liposome particles by soluble and microsomal membranes. The triglyceride/phospholipid (w/w) ratios of liposomes, soluble, and microsomal lipids were 16.2 ± 3.3, 3.8 ± 0.5, 0.42 ± 0.07, respectively (Mean ± SEM, n = 8). The differences between all groups were statistically significant ($P < 0.001$). While these compositional differences do not exclude overlap at the enzymatic level, they nevertheless show that the fractions are not simply derivatives of one another.

Electron microscopy of the subcellular fractions has been reported (5), and they were shown to be free of intact adipose cells. A representative electron micrograph of the liposome fraction is shown in Fig. 2. The mitochondria and microsomal fractions were enriched with the respective membrane systems, however, the nuclear pellet was significantly contaminated with a variety of extraneous membrane systems (5).

Lipid Extraction and Thin-Layer Chromatography

Incubates or cell fractions were extracted with chloroform–methanol 2:1 as described by Folch, Lees, and Sloane Stanley (11). The extracts were washed three times with pure solvents upper phase (11) to ensure complete removal of labeled substrate and polar metabolic intermediates. Aliquots of the washed chloroform extracts were evaporated and counted (see below). For separation of lipids into major classes, separate aliquots were evaporated and applied to thin-layer plates coated with Silica Gel H (E. Merck A. G., Darmstadt, Germany) in a small volume of chloroform–methanol 2:1. The plates were developed by ascending chromatography in petroleum ether (bp 30°–60°C)–ethyl ether–glacial acetic acid 80:20:1 as previously described (12). With this system, cholesteryl esters, triglycerides, free fatty acids, 1,3-diglycerides, and 1,2-diglycerides are separated, and monoglycerides and phospholipids remain at the origin. Since no significant activity was found in monoglycerides, radioactivity remaining at the origin was assumed to be present in mixed phospholipids. The lipids were visualized by exposure of the plates to iodine vapors, and they were identified from parallel reference standards (Hormel Foundation, Austin, Minn.). The spots were decolorized by sublimation of the I_2 for 1.5–2 hr in air, and the areas of silica gel containing the lipids were scraped directly into counting vials for radioactive assay.

To determine the specific activity of triglycerides associated with each of the various subcellular fractions, lipid extracts were separated by TLC, and the silica gel scrapings containing triglyceride were vacuumed into a 5 × 0.5 cm (I. D.) glass column plugged at one end with a small wad of glass wool. The glass wool prevented loss of silica gel into the vacuum line and acted as a support during subsequent elution. Triglyceride was eluted with 8 ml of chloroform; the recoveries were 95% or better. Aliquots of the chloroform eluate were taken for radio-

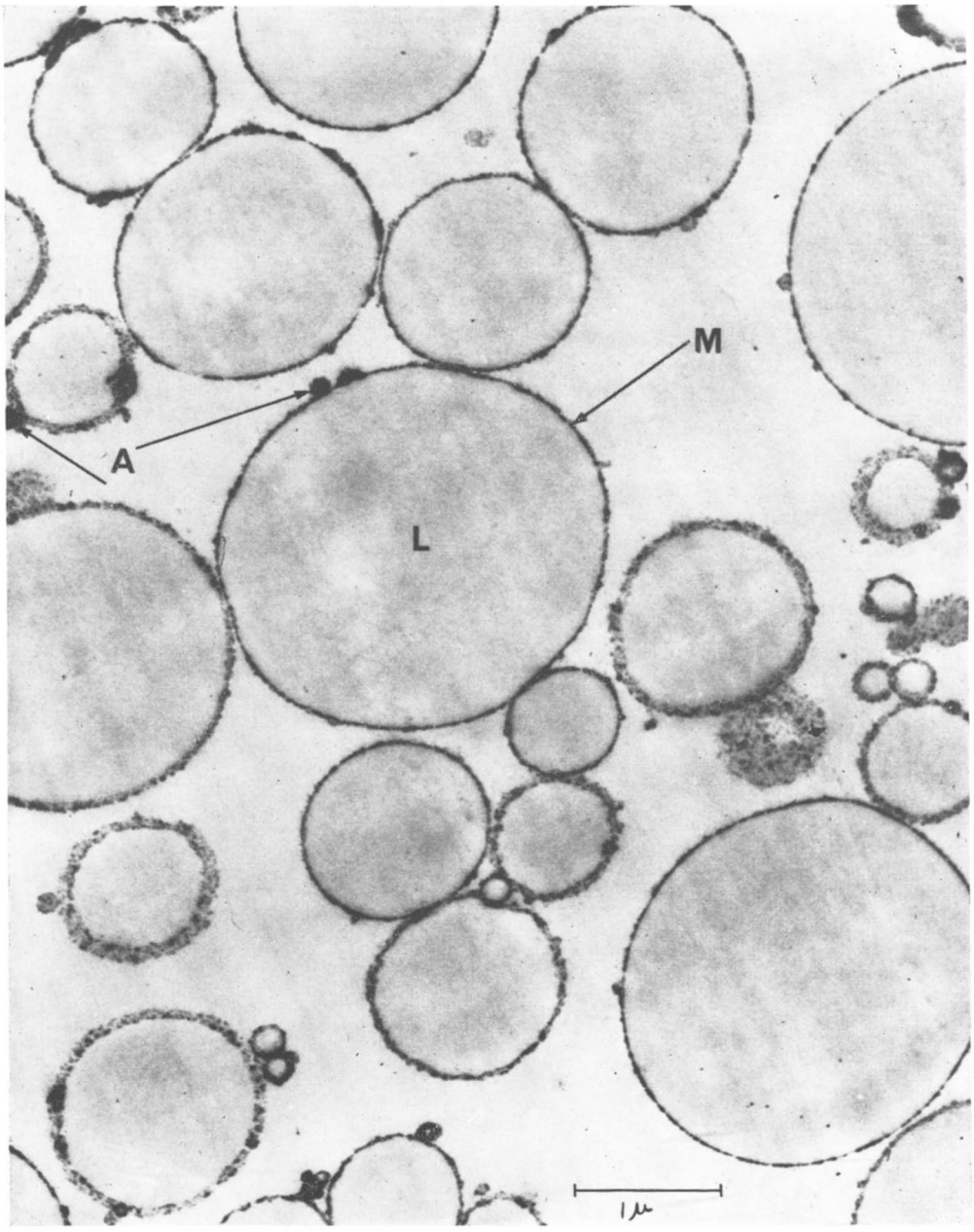

FIG. 2. Electron microscopy of liposomes. These particles were isolated as a floating fraction from adipose cell homogenates as outlined in Fig. 1. They vary in diameter from less than 0.5 to 2.0 μ and contained neutral lipid (L); each was surrounded by a single-layer electron-dense membrane (M). Dense osmophilic aggregates (A) were intimately associated with the limiting material. Some liposomes had a granular membrane. Adipose liposomes resemble giant liposomes obtained from liver (8) and also have a morphology similar to chylomicrons (9, 10). × 19,650: fixation 2 hr in 2% osmium textroxide (5).

assay and ester determinations (13, 14) using tripalmitin as a standard. Mixed phospholipids were similarly isolated except that a stepwise gradient of chloroform–methanol ending in 100% methanol was used to elute the columns. Aliquots were taken for counting and lipid-phosphorus determinations (15). Recovery of organelle phospholipid by this isolation procedure was better than 85%.

Materials

Glucose-U-^{14}C was purchased from International Chemical & Nuclear Corporation (ICN), (Burbank, Calif.) and was greater than 99% radiopure according to manufacturer's specifications. Acetate-1-^{14}C, 98% pure, was obtained from Schwarz Bio Research Inc., Orangeburg, N.Y. Palmitic acid-9,10-^{3}H was purchased from Nuclear-Chicago Corporation (Des Plaines, Ill.) and

purified by TLC. A 20 mM stock solution of potassium palmitate was prepared by heating the acid with one-third excess KOH. The solvents, unlabeled substrates, and inorganic materials were reagent grade. Solutions were made up in double distilled water (glass).

Radioactive Assay

Radioactivity was assayed in a Packard Model 3000 liquid scintillation spectrometer. The residues of lipid extracts, column eluates, or silica gel scrapings were counted at 5°C in 15 ml of scintillation mixture containing 0.4% PPO, 0.01% POPOP, 10% naphthalene, and 10% methanol (by volume) in dioxane. Counting efficiency was 50%, and corrections for self-absorption and quenching were not necessary since care was taken to ensure complete sublimation of iodine. Silica gel did not seriously interfere with the radioassay. While it did reduce efficiency of counting by 5–8% in the dioxane scintillation mixture employed, more recent experience has shown that with Bray's solution (16) silica gel had no untoward effect.

RESULTS

Lipid Synthesis from Acetate and Glucose, and Esterification of Palmitic Acid

Initially experiments were carried out to establish ideal conditions for the study of lipid transport in adipose cells. For this purpose a steady state of linear glyceride synthesis was deemed optimal. In earlier studies (5, 7) and in preliminary experiments, 16 mM glucose was found to support a nearly linear rate of synthesis of lipid from added acetate or free fatty acid during brief periods of incubation.

In Table 1 the rates of glyceride synthesis from various substrates are compared, and it is apparent that isolated cells readily converted acetate, glucose, and free fatty acids to esterified lipids. Glyceride synthesis from exogenous fatty acid exceeded *de novo* synthesis from glucose or acetate.

Fatty acid synthesis from acetate frequently displayed an initial lag (Fig. 3), presumably due either to the time taken for isotopic equilibration or to changing activities of fatty acid synthesizing enzymes. Lipid formation from glucose displayed a more rapid initial velocity, followed by a slower yet progressive phase of lipid biosynthesis (Fig. 4). On examining the distribution of radioactivity between glyceride–glycerol and glyceride–fatty acids after incubation with glucose-U-^{14}C, a lag in entry of glucose carbon into the fatty acid moiety was apparent (Fig. 5). This would suggest that exogenous acetate and acetyl CoA derived from glucose were similarly metabolized in the adipose cell.

TABLE 1 GLYCERIDE SYNTHESIS IN ISOLATED WHITE ADIPOSE CELLS

Exp. No.	Incubation Time	Substrate*	Rate of Lipid Synthesis†	
	min		*nmoles/μmole of cell TG per hr*	*mμatoms substrate carbon/ μmole of cell TG per hr*
4	60	Acetate-1-^{14}C	0.40 ± 0.04‡	0.8
6	30	Glucose-U-^{14}C	2.42 ± 0.29	14.5
4	30	Palmitic acid-9,10-^{3}H	7.46 ± 1.58	119.0

* All experiments contained glucose (16 μmoles/ml) in addition to the isotopic substrate. Acetate concentration was 0.56 μmoles/ml; palmitate concentration was 2.0 μmoles/ml.

† Following incubation with acetate-1-^{14}C or glucose-U-^{14}C, chloroform–methanol extracts were prepared, washed repeatedly, and analyzed for total lipid radioactivity. In experiments with palmitic acid-9,10-^{3}H, lipid extracts were evaporated, dissolved in heptane, washed with alkaline ethanol to remove unreacted fatty acid, and then analyzed for radioactivity.

‡ Mean ± SEM.

Palmitic acid was rapidly and nearly completely esterified by isolated cells under the experimental conditions used here (Fig. 6). The rate of fatty acid esterification was linear for 30 min, and then plateaued in a manner typical of a first-order type reaction. Within 1 hr, three-fourths of the medium fatty acid was esterified, and in 2 hr well over 90% of the available substrate was converted to glyceride esters.

The Composition of Newly Synthesized Lipid

In some studies lipid extracts were separated by TLC into component major lipid classes, and the various spots were assayed for radioactivity. The composition of lipids produced from labeled acetate, glucose, and palmitate are shown in Figs. 3, 7, and 8, respectively. In each instance within 5 min from the start of incubation, well over 80% of the lipid synthesized by the cell was in glyceride esters, and within 15 min 90% was in this form. The patterns obtained from labeled acetate and labeled glucose were generally similar (Figs. 3 and 7). Certain differences were evident at earlier incubation times. At 5 min incubation with labeled glucose, a high proportion (8–18%) of lipid radioactivity was found in phospholipid; with acetate-1-^{14}C, free fatty acid, rather than phospholipid, contained up to 18% (6–18%) of the lipid radioactivity. This probably reflects the fact that labeled glucose reached isotopic equilibrium with intracellular glycerophosphate more rapidly than with acyl CoA (17), so that radioactivity in phosphatidic acid would appear sooner than in free fatty acid. With acetate as substrate, radioactivity would be expected first to enter a fatty acid pool. After 15 min of incubation, the

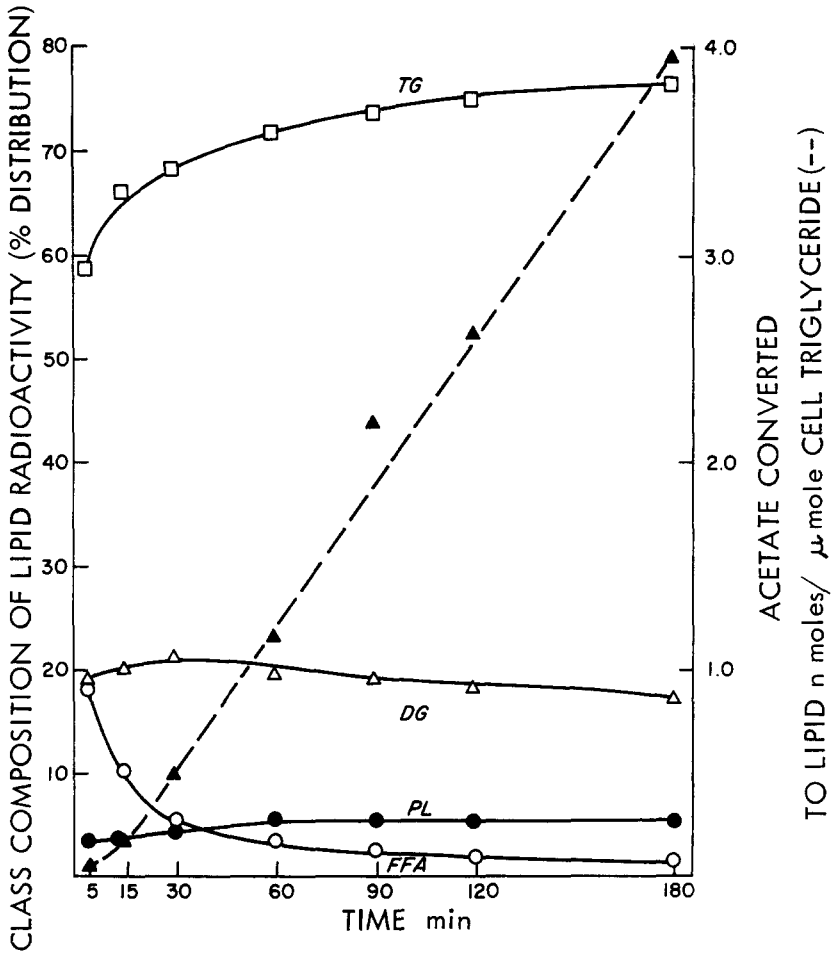

FIG. 3. Lipogenesis from acetate-1-^{14}C in isolated white adipose cells. The composition of newly synthesized lipids in rat adipocytes. Isolated cells were incubated in Krebs-Ringer bicarbonate 5% albumin buffer with acetate-1-^{14}C 0.56 μmoles/ml, and glucose 16 μmoles/ml for the times shown. Lipid extracts of cells plus medium were analyzed for radioactivity, and aliquots were separated by TLC into component major classes. The distribution of radioactivity was determined by liquid scintillation assay of silica gel scrapings. Lipid synthesis from acetate (right-hand ordinate) is described by the broken line (- - - -). An initial lag in entry of substrate label into cell lipid was frequently observed. Within 5 min over 80% of the lipid radioactivity was in ester form. Diglyceride contained about 20% of the lipid radioactivity. Cholesterol and cholesteryl ester contained less than 1% of the lipid radioactivity and are not shown. After 30 min the composition of the newly synthesized lipid remained quite constant in spite of continued synthesis of total lipid. Thus a new steady state was achieved, and the profile of endogenously synthesized lipid was revealed. Each point is the percentage of total lipid radioactivity in a given major lipid class at a given time. TG, triglyceride (□); DG, diglyceride (△); FFA, free fatty acid (○); PL, phospholipids (●).

proportion of lipid radioactivity in phospholipid was usually between 3 and 5%, and for free fatty acids, 1.5–5%. With both labeled glucose and labeled acetate about 20% of the lipid radioactivity was in diglyceride, and this leveled off at around 17% and remained relatively constant throughout the incubation. In other experiments not shown, the proportion of newly synthesized lipid in diglyceride was 12–26%.

The labeled lipids produced from palmitate-9,10-^{3}H (Fig. 8) differed somewhat from those from labeled glucose or acetate. Phospholipids did not accumulate a significant proportion of label. The proportion of newly synthesized lipid in diglyceride was also less than that obtained with glucose and amounted to 10% of the total ester pool during the first 45 min of incubation; thereafter, it decreased progressively to 5% at 150 min. It is important to point out, however, that comparisons on a percentage basis alone are misleading since the total amount of lipid synthesized from fatty acid precursor exceeds that produced from glucose or acetate substrate by a factor of 8 (Table 1). Therefore, the total chemical mass of diglyceride produced using fatty acid as substrate would be greater than that produced by *de novo* synthesis from acetate or glucose.

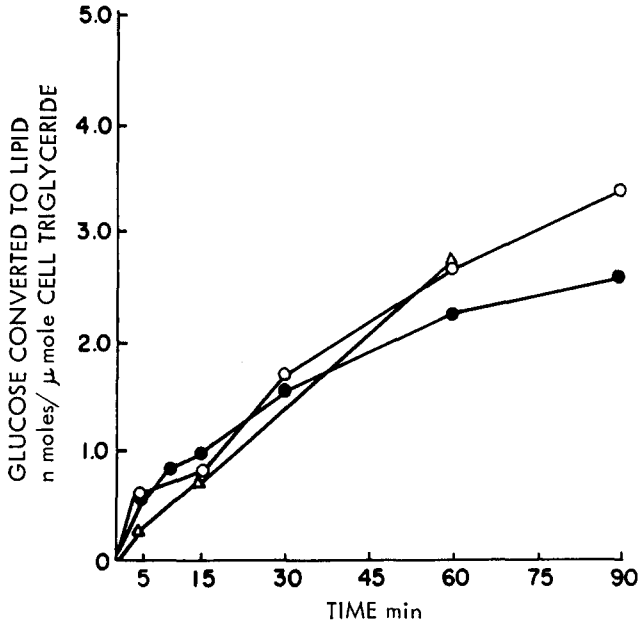

FIG. 4. Lipid synthesis from glucose in isolated white adipose cells. Cells were incubated with glucose-U-^{14}C 16 μmoles/ml for various periods of time. The reaction was stopped by addition of organic solvents for lipid extraction. Each point represents a single incubation, and each symbol (○, ●, △) is a separate experiment.

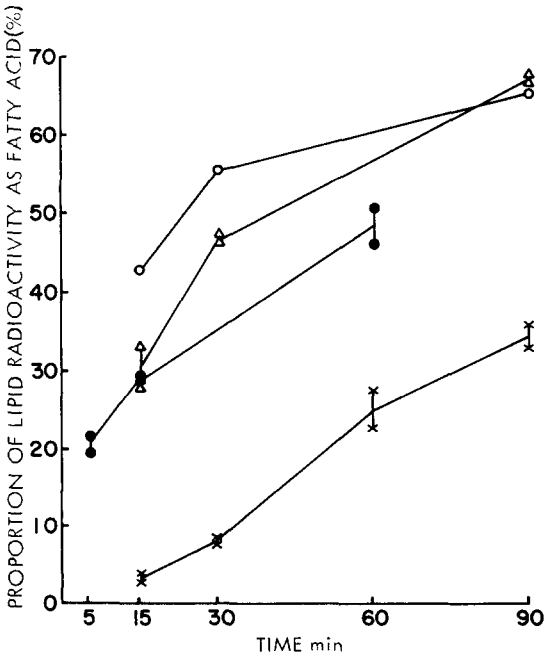

FIG. 5. The effect of duration of incubation on the portion of radioactivity in the fatty acid moiety of total cell lipid after incubation with glucose-U-^{14}C. Following incubation with 16 μmoles/ml radioglucose, total cell lipids were extracted and counted. Aliquots were then saponified (13) with alcoholic KOH, and the liberated fatty acids were isolated and counted. Each symbol (○, ●, ×, △) represents a separate experiment. Double symbols represent duplicate incubations.

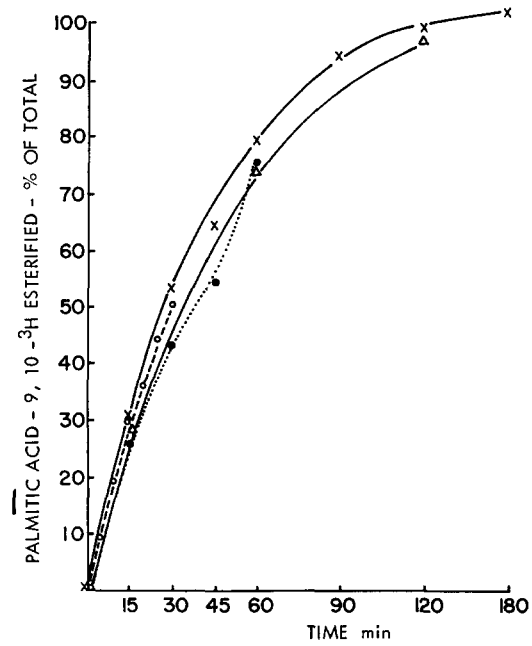

FIG. 6. Esterification of free fatty acid by adipose cells. Isolated cells were incubated in Krebs-Ringer bicarbonate 5% albumin buffer containing 2.0 μmoles/ml of potassium palmitate plus 16 μmoles/ml glucose for varying times. The results of four experiments are shown (○, ●, ×, △). Each point represents a single incubation flask.

It is interesting that regardless of substrate, less than 1% of the lipid radioactivity was recoverable in free cholesterol or cholesteryl esters (Figs. 3, 7, and 8). This is consistent with published work (18) showing that *de novo* synthesis of cholesterol in adipose tissue is negligible.

The Subcellular Distribution of Newly Synthesized Lipid

In order to determine the intracellular disposition of newly synthesized lipid, adipose cells were incubated with isotopic precursors, homogenized, and then fractionated by preparative ultracentrifugation. Lipid extracts of each subcellular fraction were prepared and analyzed for total lipid esters and total lipid radioactivity. In these experiments (Fig. 9), a significant difference between the subcellular distribution of newly synthesized lipid compared with the distribution of preexisting lipid esters was observed. The bulk fraction contained 98% of the unlabeled lipid and 92% of the lipid radioactivity. About 8% of the newly synthesized lipid was localized in the organelle fractions compared with 2% of total cell esters. Thus, the specific activity of organelle lipids was consistently greater than that of the bulk fraction, indicating an enrichment of organelles with newly formed lipid. Since contamination of other compartments by glyceride from the bulk-storage pool probably occurs during cellular fractionation, it is possible that the dif-

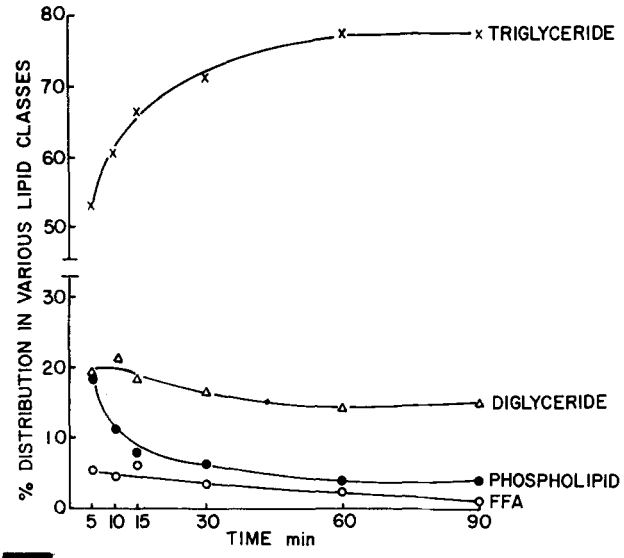

FIG. 7. The composition of newly synthesized lipid in adipose cells. Isolated adipocytes were incubated in Krebs-Ringer bicarbonate 5% albumin buffer with 16 mM glucose-U-^{14}C for various times. Lipid extracts were prepared and separated by TLC into major classes for radioassay. ×, triglyceride; △, diglyceride, ●, phospholipid; ○, FFA.

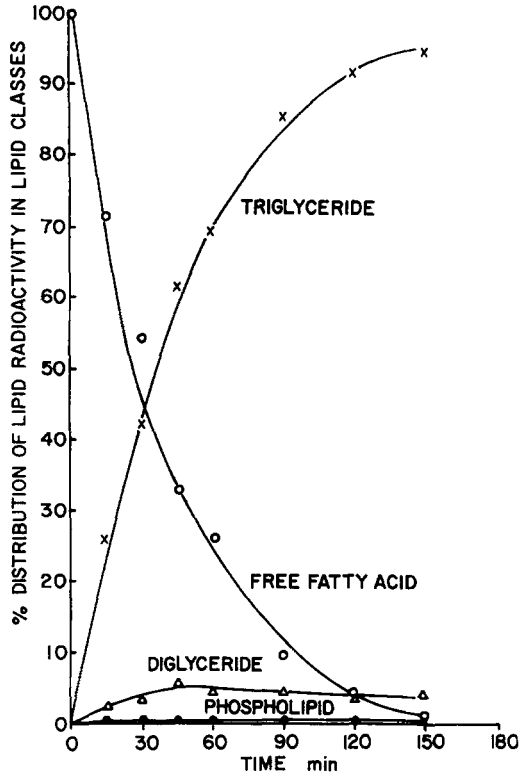

FIG. 8. The distribution of lipid radioactivity during incubation of isolated cells with labeled palmitate. Adipose cells were incubated in Krebs-Ringer bicarbonate 5% albumin buffer containing 2.0 μmoles/ml of potassium palmitate-9,10-^{3}H plus 16 μmoles/ml of glucose for the times shown. Symbols as in Fig. 7.

ferences in specific activity observed may have been much greater had dilution not occurred.

The subcellular distribution pattern of lipid synthesized from labeled glucose was similar (Table 2A) to that obtained with labeled acetate (Fig. 9). The pattern obtained with palmitic acid-^{3}H differed somewhat in that a greater proportion (97.5%) of the lipid radioactivity was in the bulk lipid fraction after 90 min of incubation (Table 2B). This is particularly significant since the total amount of glyceride synthesized from fatty acid substrate is many times greater than that produced from glucose alone (Table 1).

TABLE 2 SUBCELLULAR DISTRIBUTION OF NEWLY SYNTHESIZED LIPID IN ISOLATED WHITE ADIPOSE CELLS INCUBATED WITH GLUCOSE-U-^{14}C OR PALMITIC ACID-9,10-^{3}H

	Experiment A: Glucose-U-^{14}C		Experiment B: Palmitic Acid-9,10-^{3}H	
Incubation Time	15 min	60 min	5 min	90 min
	%		%	
Fractions				
Bulk	94.2	95.0	95.0	97.8
Nuclei	0.3	0.3	0.9	0.1
Mitochondria	0.8	0.8	1.4*	0.4*
Microsome	1.1	1.3		
Liposome	1.8	1.2	2.2	1.4
Soluble	1.9	1.3	0.9	0.5

Isolated cells were incubated with labeled glucose (16 μmoles/ml) (A) or labeled palmitate (1.8 μmoles/ml) plus 16 μmoles/ml of glucose (B) for the times shown. Following incubation the cells were washed repeatedly to remove unreacted substrate, homogenized in 0.25 M sucrose, and separated into subcellular fractions as described in Fig. 1. Lipid extracts of each fraction were then assayed for radioactivity.

* Combined mitochondria-microsome pellet.

The Composition of Newly Synthesized Lipid in Subcellular Fractions

Since organelles are composed of complex lipoprotein membranes containing a variety of polar and nonpolar lipids (19), it was of interest to study the composition of newly synthesized lipid in each organelle fraction. The data in Tables 3 and 4 and Fig. 10 show such studies. It is apparent that the composition of newly formed lipid in each subcellular fraction differs significantly from the class distributions as determined on total cell extracts (Figs. 3, 7, and 8).

While most of the newly synthesized triglyceride and diglyceride were rapidly localized to the bulk-storage pool, most of the phospholipid radioactivity was with the various particulate fractions. Mitochondria and microsomes usually contained the highest proportion of labeled phospholipids, followed by soluble supernatant and then liposomes. Organelle fractions also had a proportionately greater amount of radioactivity in free fatty acids than did bulk fractions. This may have been attributable to the high affinity of adipose cell organelles for free fatty acids (20).

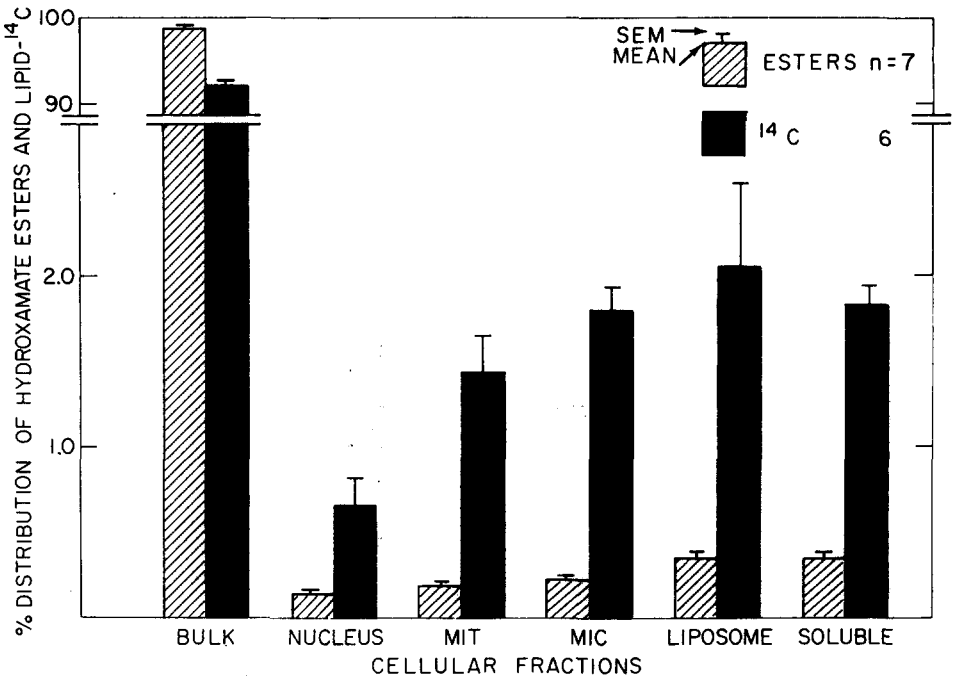

FIG. 9. The subcellular distribution of newly synthesized and preexisting lipids in adipose cells. Isolated adipose cells were incubated in Krebs-Ringer bicarbonate 5% albumin buffer containing acetate-1-^{14}C, 1.76 μmoles/ml, and 16 μmoles/ml glucose for 5 min, 15 min, or 60 min. Following incubation the cells were washed repeatedly, homogenized in 0.25 M sucrose, and separated by preparative ultracentrifugation into organelle fractions as shown in Fig. 1. Lipid extracts of each fraction were prepared and assayed for ester content and radioactivity. The values at each time interval were very similar, and to simplify presentation, the results of six experiments were combined. The bulk fraction contained over 98% of the lipid esters and 92% of the lipid radioactivity. This localization of new lipid in the bulk fraction is assumed to indicate rapid transfer of newly synthesized lipid into the storage compartment. The ratio of lipid radioactivity (black bars) to the amount of lipid ester (hatched bars) is an index of specific activity. It is apparent that the specific activity of organelle lipids exceeds that of the bulk fraction. MIT, mitochondria; MIC, microsomes.

The Specific Activity and Transport of Lipid in Various Organelle Fractions

During incubation of adipose cells with labeled substrate, the specific activity of organelle lipids always exceeded that of the bulk fraction (Fig. 9 and Table 3). In order to further characterize this apparent enrichment of organelles with newly synthesized lipid, the specific activities of component triglyceride and mixed phospholipids were determined. Additionally, to obtain more definitive information on the lability and movement of lipid at the subcellular level, pulse-chase type experiments were performed (Figs. 11 and 12). Following incubation of cells prelabeled with glucose-U-^{14}C, the specific activity of organelle triglycerides exceeded that of the bulk glyceride pool at all times, and the specific activity of mitochondrial triglyceride exceeded that of all other fractions (Fig. 11). Microsomal triglyceride was next highest in specific activity, but because of a large variance it was not statistically greater than the specific activities of liposome or soluble supernatant triglyceride. The specific activity of liposome triglyceride was significantly greater than that of the soluble fraction at zero time and after 60 min of chase, but they were not significantly different after 240 min of incubation. It is apparent that the specific activity of organelle triglycerides approached but did not reach that of the bulk lipid pool. The pattern of fall of mitochondrial triglyceride activity was characterized by a rapid initial decline during the first 60 min of incubation, followed by a much slower rate of decline over the next 3 hr, whereas triglyceride in the rest of the fractions showed a slower and more gradual decline during the entire incubation period. The rapid fall in mitochondrial triglyceride specific activity corresponds in time to a rise in bulk triglyceride specific activity which was the only triglyceride pool to increase in specific activity during incubation of prelabeled cells (see legend to Fig. 11).

The specific activity of organelle phospholipids exceeded that of the bulk glyceride pool and, with the exception of mitochondria the specific activity of phospholipids in each organelle system, exceeded that of triglyceride in the same fraction as shown in Fig. 12. The high specific activity of total microsomal fatty acids following incubation of adipose cells with labeled acetate

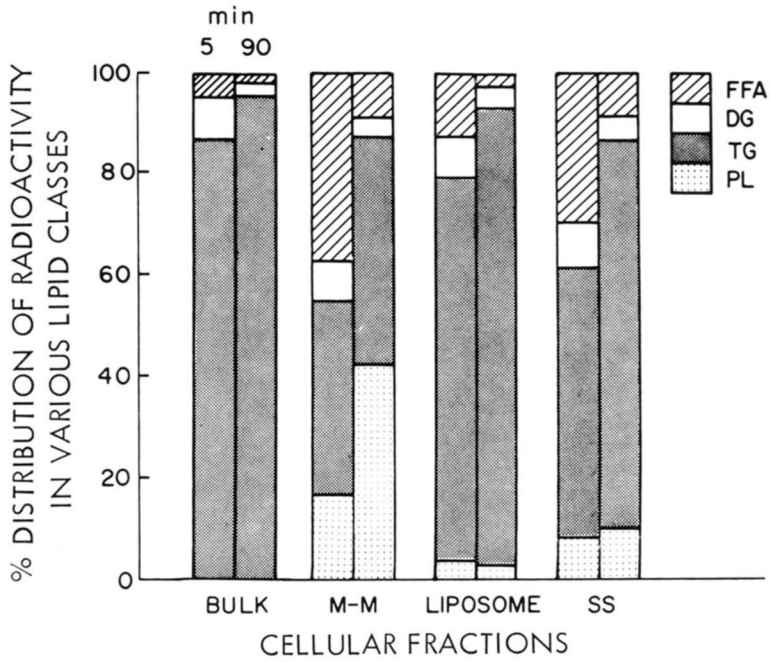

FIG. 10. The profile of lipid radioactivity in isolated adipose subfractions following incubation of adipose cells with palmitate-9,10-^{3}H. Lipid extracts from experiment B, Table 2, were separated by TLC, and the distribution of lipid radioactivity was determined by assay of the silica gel scrapings. Most of the phospholipid radioactivity was localized to the organelle fraction, especially mitochondria and microsomes (M–M), whereas neutral glyceride radioactivity was mainly in the bulk lipid fraction.

TABLE 3 THE COMPOSITION OF NEWLY SYNTHESIZED LIPID IN ADIPOSE CELL SUBFRACTIONS AFTER INCUBATION WITH ACETATE-1-^{14}C*

	Subcellular Distribution of Lipid Radioactivity	Specific Activity	Composition†			
			TG	FFA	DG	PL
	%	*cpm/μmole of fatty acid ester*	%			
5 min						
Bulk	91.7	51	77.0	5.8	16.1	0.9
Mitochondria	0.9	348	49.7	11.4	8.2	30.7
Microsomes	1.6	540	37.4	10.7	8.0	43.0
Liposomes	4.2	392	74.6	5.0	7.7	12.3
Soluble	1.2	297	43.5	18.9	7.6	30.0
60 min						
Bulk	93.8	628	80.0	1.6	17.2	1.0
Mitochondria	1.1	2640	61.0	5.8	6.7	26.0
Microsomes	2.0	6520	26.7	7.8	4.2	61.0
Liposomes	0.7	2450	52.4	7.6	10.4	29.4
Soluble	1.7	3610	28.9	7.1	11.8	51.7

* Adipose cells were incubated with acetate-1-^{14}C (1.76 μmoles/ml, 1.33 μCi/mole) and glucose (16 μmoles/ml) for 5 min and 60 min, and processed as described in Table 2.

† Lipid extracts of each subcellular fraction were separated by TLC and assayed for radioactivity. Cholesterol and cholesteryl ester contained negligible activity and are not tabulated. TG, triglyceride; DG, diglyceride; FFA, free fatty acid; PL, phospholipids.

(Table 3) undoubtedly reflects enrichment of this fraction with high specific activity phospholipids.

DISCUSSION

Separation of adipose cell homogenates into component centrifugal fractions facilitated the direct demonstration of compartmentation of endogenously synthesized lipid within the adipose cell. Well over 90% of the newly synthesized tri- and diglycerides were localized in the bulk lipid pool, whereas newly synthesized phospholipids were found mainly in membranous fractions. This compartmentation was independent of the choice of substrate or the duration of incubation and attests to the

TABLE 4 THE COMPOSITION OF NEWLY SYNTHESIZED LIPID IN ADIPOSE CELL SUBFRACTIONS AFTER INCUBATION WITH GLUCOSE-U-^{14}C*

	Subcellular Distribution	Class Composition			
		TG	FFA	DG	PL
	%			%	
Bulk	93.2	79.0	1.4	18.6	1.0
Mitochondria	2.2	66.9	3.5	11.0	18.1
Microsomes	1.2	63.3	1.4	14.9	20.4
Liposomes	1.6	56.4	2.2	20.0	21.4
Soluble	0.9	47.6	2.2	21.1	29.0

* Adipose cells were incubated with glucose-U-^{14}C (16 μmoles/ml) for 60 min and were processed as described in Tables 2 and 3.

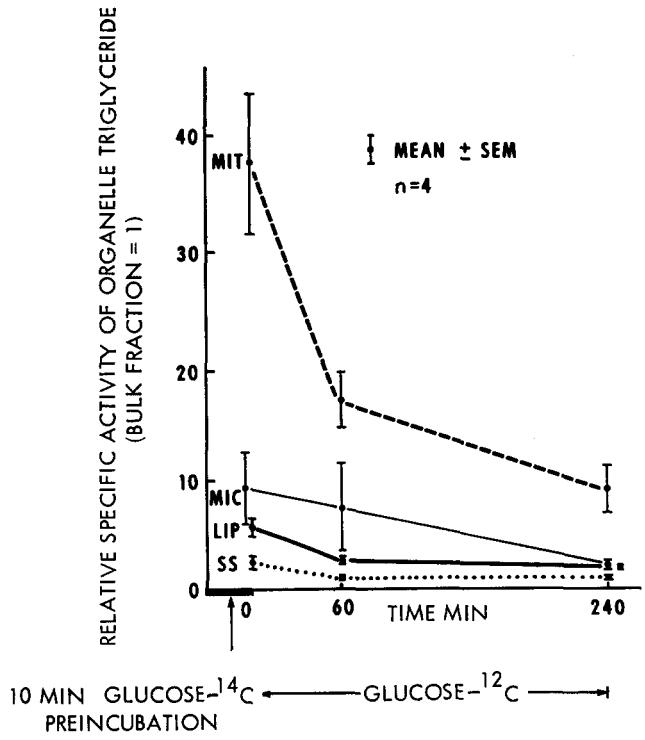

FIG. 11. Pulse-chase of newly synthesized triglyceride in white adipose cell organelles. Isolated adipose cells were preincubated for 10 min with glucose-U-^{14}C (16 mM, 1.1 × 10^6 cpm/μmole) and then washed three times with fresh buffer medium to remove labeled substrate. A portion of the cells was then taken (zero time) for subcellular fractionation, and the remainder was resuspended in fresh Krebs-Ringer bicarbonate buffer–5% albumin containing unlabeled glucose, 16 mM, and incubated for 60 or 240 min prior to fractionation. The results are expressed in relative terms with the specific activity of bulk fraction triglyceride designated 1. The actual bulk triglyceride specific activity (cpm/μmole of triglyceride) for these experiments are: zero time, 589 ± 99; 60 min, 912 ± 115; 240 min, 1026 ± 131 (Mean ± SEM, n = 4). The specific activity of mitochondrial triglyceride exceeded that of all other pools at all times.

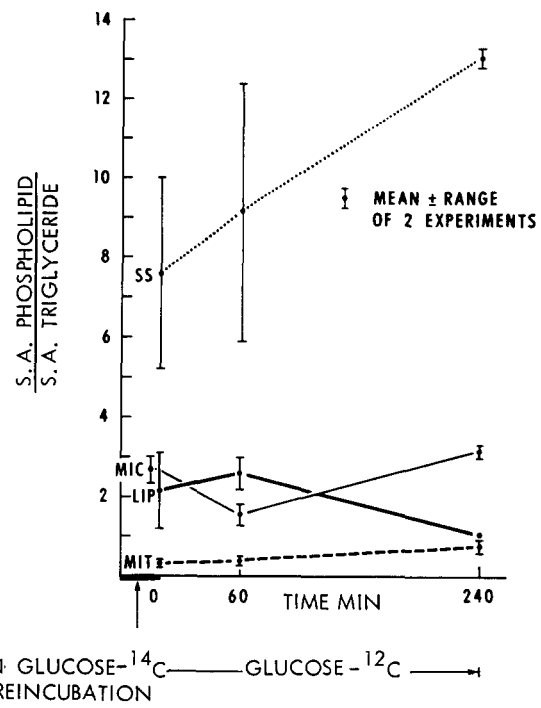

FIG. 12. Appearance of newly synthesized phospholipid in white adipose cell organelles. Prelabeled adipose cells were incubated without isotopic substrate for various times, and total phospholipids were isolated from TLC scrapings for assay of mass and radioactivity (see legend to Fig. 11 for experimental details). The results are expressed in relation to the specific activity of triglyceride of each organelle fraction. Bulk fraction phospholipids were not measurable. Each point is the average of two experiments, and the range is described by the vertical bars.

speed and polarization of the lipid-storage process in the cell. The extraordinary rapidity and extent to which newly synthesized glyceride entered the storage compartment suggest that the entry of lipid into the storage phase occurs immediately upon or very shortly after its synthesis. This idea is consistent with electron microscopic radioautographic evidence in which almost all the radiographic reaction is localized over the central lipid droplet following incubation of intact adipose tissue slices or isolated adipose cells with labeled long-chain fatty acids (7).

The localization of high specific activity triglyceride to mitochondria is significant and probably represents binding of newly formed glyceride at sites of fatty acid esterification. This interpretation is entirely consistent with earlier observations that glyceride synthesis in broken cell preparations of adipose tissue is almost entirely dependent on the presence of mitochondrial particles rather than microsomal membranes (21). The rapid fall in triglyceride specific activity in mitochondria during the first hour of incubation of prelabeled cells after exposure to labeled glucose, probably reflects transfer of new lipid from sites of synthesis into the bulk-storage pool which increased in activity rather than to other organelles such as microsomes, liposomes, or soluble fraction since the triglyceride specific activity of these latter fractions did not increase during the chase (Fig. 11).

In considering the process of transport of newly synthesized glycerides from sites of fatty acid esterification on mitochondrial and microsomal membranes to areas of lipid storage, it is important to recall that these membranous organelles are everywhere distributed about the large surface of the central lipid droplet (5). This contiguity could provide newly synthesized lipid on membrane elements direct access to the lipid-storage phase and would allow transfer of neutral lipid to the storage area by lipid–lipid interaction at a rate close to or almost identical with that of glyceride formation. The speed of entry of newly synthesized lipid into the bulk-storage compartment, while suggestive of a direct route does not of itself preclude the possibility that intermediate stages

might exist between synthesis and storage of neutral lipid. It has been suggested that submicroscopic aggregations of newly synthesized lipid might coalesce to form lipid droplets which can accumulate, coalesce further, or dissolve into the large central lipid vacuole (22). The possibility that constituents of the liposome fraction represent such a stage in the storage or transport process is supported by the findings that these isolated droplets contained glycerides of specific activity intermediate between the stored pool (bulk fraction) and the newly synthesized pool (mitochondria) following incubation of cells with labeled glucose (Fig. 11) and that its specific activity equilibrated with the storage pool in pulse-chase studies (Fig. 11). If the liposome fraction actually represents an interval between glyceride synthesis and storage, and if this system were the predominant pathway of intracellular lipid transport, the turnover rate of this fraction would have to be high since at any one time less than 3% of the newly synthesized lipid was localized in this centrifugal fraction. While the available data do not demonstrate the quantitative significance of the liposome system compared with the direct entry mechanisms for transport of lipid within the adipose cell, it nevertheless shows that liposomes constitute an important form of "structural" compartmentation of newly synthesized lipid.

The close grouping of microsomal, liposomal, and soluble fraction triglyceride specific activities (Fig. 11) and their similar patterns of decline during incubation of prelabeled cells suggest that these pools are related. While the mean specific activity of liposome triglyceride was less than that of microsomal triglyceride, the difference was not statistically significant. However, since the total lipid content of liposomes is about 30% greater than microsomal lipid (Fig. 9), and the triglyceride/phospholipid ratio of liposome lipid is over 30 times greater than that of microsomal particles (see Methods), it is unlikely that the labeled glyceride in liposomes can represent contamination by microsomes. These observations are all consistent with but do not prove that liposomes constitute a structurally distinct pool of glyceride that might originate from microsomal bound triglyceride. The same reasoning applies to newly synthesized lipid in the soluble fraction. These data suggest that the mitochondrial and microsome–liposome-soluble pools of newly formed triglyceride are separate, and imply structural segregation of glyceride synthesis and(or) transport in adipose cells. In this regard it is worth noting that separation of glyceride synthesis into two distinct functional pathways has been demonstrated in the small gut mucosal cell (23).

Little is known about the origin of adipose cell membrane lipid, its composition, or turnover. The present study indicates that adipose cells synthesize phospholipid *de novo* from acetate or glucose but not to a significant extent from exogenous free fatty acid. Further, adipose cells did not synthesize significant amounts of cholesterol or its ester from labeled substrate, implying a dependence in vivo on an exogenous supply (24). While these observations are interesting, a better appreciation of their significance must await further information on factors that control membrane synthesis and composition in this tissue.

Following incubation of isolated adipose cells with labeled glucose or acetate, up to 20% of the lipid radioactivity was in diglyceride, and this proportion remained quite constant during linear synthesis of lipid (Figs. 3 and 7). Similar class patterns of newly synthesized lipid have been observed in a variety of in vivo and in vitro preparations of rat as well as human adipose tissue (25–27). This suggests that the profile of newly synthesized lipid observed in isolated adipose cells accurately reflects that produced under physiological circumstances. Since less than 1% of native adipose cell lipid is diglyceride (28), the striking difference between the composition of newly formed lipids and those preexisting must be reconciled. One explanation for the high levels of diglyceride radioactivity is the accumulation of substrate at a rate-limiting step in triglyceride biosynthesis. This seems unlikely since esterification of added fatty acid was eight times greater than that occurring by *de novo* synthesis; yet with fatty acid as substrate, diglyceride contained a lower proportion of activity than in *de novo* synthesis studies. Another mechanism that could account for the high proportion of newly synthesized diglyceride activity might relate to the fact that most of the diglyceride radioactivity was in the bulk-storage compartment at all times during active lipid synthesis. This is interpreted to mean that a relatively constant proportion (approximately $^1/_5$ to $^1/_6$) of the total diglyceride produced is somehow displaced from esterification sites on mitochondria or microsomal membranes and enters the large storage pool. It follows, therefore, that most of the labeled diglyceride may represent a segregated pool of newly formed glycerides removed from the mainstream of triglyceride synthesis. If this is true, then this diglyceride would not serve as an immediate precursor to triglyceride but should display a retarded half-life in pulse-chase studies. This has been shown to occur in epididymal fat labeled by in vivo–in vitro incubation (25) as well as in isolated adipose cells (29).

The possibility that newly synthesized diglyceride was secondarily transferred to the bulk-storage pool during the homogenization procedure must be mentioned since it has not been excluded with absolute certainty. However, considerable indirect evidence exists which argues against a significant artifactual redistribution of lower glycerides during the preparative procedures. First, in radioautography studies of adipose tissue and isolated

adipose cells following incubation with labeled long-chain fatty acids virtually all the radiographic reaction is over the central lipid droplet, and negligible activity is localized to organelle systems (7). Secondly, recovery experiments on the fractionation procedure have shown no selective loss of any of the organelle fractions, thus excluding spurious redistribution of activity that might be attributable to systematic deletion of a specific organelle fraction (7). Finally, as mentioned above, a prolonged half-life of labeled diglyceride in pulse-chase studies suggest segregation of this glyceride, presumably in bulk-storage pool.

These findings suggest that the sequestration of newly synthesized diglyceride could represent a special form of lipid compartmentation within the adipose cell. Since this pool of lipid is distinguished by its class composition rather than by its geographic distribution, it is appropriately referred to as "chemical" compartmentation of lipid in contrast with "structural" compartmentation of lipid.

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