Hepatic phosphatidylcholines: evidence for synthesis in the rat by extensive reutilization of endogenous acylglycerides

George M. Patton,[1] Joan M. Fasulo, and Sander J. Robins

Lipid Metabolism Laboratory, Veterans Administration Medical Center, Boston, MA 02130, and Department of Medicine, Boston University School of Medicine, Boston, MA 02118

Abstract Studies were performed to compare the extent of fatty acid incorporation into liver phosphatidylcholines (PCs) by acyl remodeling and by de novo synthesis. To this end, isolated rat livers were first perfused with palmitoleic acid (16:1) and [2-^{3}H]glycerol and then with 17:1 fatty acid and nonradiolabeled glycerol that resulted in the formation of new molecular species of radiolabeled PCs containing 16:1 and 17:1 acyl groups. The specific activities of newly formed molecular species of the de novo precursors of acylglyceride synthesis, phosphatidic acids (PAs) and diglycerides (DGs), and the products of synthesis, PCs and triglycerides (TGs), were measured at periods during both the labeling period of perfusion with 16:1 (first 15 min) and the more prolonged chase period with 17:1 (up to 120 min). At the end of the labeling period, the specific activity of all the 16:1-containing PAs, DGs, and 16:1-16:1-16:1 and 16:1-16:1-18:2 TGs were the same and were much higher than any molecular species that did not contain 16:1. The specific activities of these molecular species are indicative of the specific activity of molecular species synthesized exclusively by de novo synthesis (i.e., by acylation of glycerol 3-phosphate) during the labeling period. In contrast, the specific activity of 16:1-16:1 PC was only ⅔ that of the other 16:1-16:1 glycerides, and the specific activities of the other 16:1-containing PCs were only about ⅓ that of the corresponding 16:1-containing PAs, DGs, and 16:1-16:1 TGs. After the labeling period and during the chase period with perfusion of 17:1 and nonradiolabeled glycerol, the specific activities of major 16:1 PCs exceeded the specific activities of their corresponding PAs and DGs and remained considerably higher than these precursors of de novo synthesis for the duration of perfusions. However, during this period, the specific activities of major 16:1 PCs were less than their corresponding molecular species of TGs. During the chase period, new 17:1 molecular species of PCs were formed that were also radiolabeled. The specific activity of 16:1-17:1 PC, the 17:1 PC with the highest specific activity, always exceeded its corresponding PA and DG precursors. During the chase period, non-16:1 and non-17:1 molecular species of PCs that comprised the bulk of hepatic PCs were also radiolabeled and the specific activities of these molecular species progressively increased during this period. These results suggest that ⅓ of the 16:1-16:1 and ⅔ of the other 16:1-containing molecular species of PC were synthesized by acyl remodeling of pre-existing PCs, that most of the 16:1-17:1 PC was synthesized by acyl remodeling of 16:1 PCs synthesized during the labeling period, and that most of the PA and DG molecular species that did not contain a 16:1 fatty acid were derived from hydrolysis of endogenous acyl glycerides rather than by de novo synthesis.—**Patton, G. M., J. M. Fasulo, and S. J. Robins.** Hepatic phosphatidylcholines: evidence for synthesis in the rat by extensive reutilization of endogenous acylglycerides. *J. Lipid Res.* 1994. **35:** 1211–1221.

Supplementary key words phosphatidic acid • diglyceride • triglyceride • molecular species • acyl remodeling • de novo phosphatidylcholine synthesis

Phosphatidylcholines (PCs) in the liver, as in most mammalian tissues, exist in many molecular forms, and changes in the population of PCs may take place by new synthesis and by acyl group remodeling. Usually, PC synthesis has been measured by the extent of incorporation of exogenous isotopic precursors into PC (primarily using fatty acids (FAs), glycerol, phosphorus, methionine, and choline). However, this approach does not accurately account for the PCs that are formed by remodeling, in which endogenous acylglycerides are the substrates for lipases and provide the FAs, glycerol and/or (phospho)choline precursors for new molecular species of PCs. The extent to which PC remodeling occurs, as well as the specific kinds of PC intermediates that are utilized for remodeling, will clearly influence the extent to which PC synthesis can be accurately measured by the incorporation of an exogenous precursor.

With the use of highly reliable HPLC methods to iso-

Abbreviations: FA(s), fatty acid(s); DG(s), diglyceride(s); TG(s), triglyceride(s); PA(s), phosphatidic acid(s); PC(s), phosphatidylcholine(s); PE(s), phosphatidylethanolamine(s); HPLC, high performance liquid chromatography. The fatty acids are: 16:0, hexadecanoic acid; 18:0, octadecanoic acid; 16:1, *cis*-9-hexadecenoic acid; 17:1, *cis*-10-heptadecenoic acid; 18:1, *cis*-9-octadecenoic acid and/or *cis*-7-octadecenoic acid; 18:2, *cis*-9,12-octadecenoic acid; 20:4, *cis*-5,8,11,14-eicosatetraenoic acid; 22:6, *cis*-4,7,10,13,16,19-docosahexaenoic acid.

[1]To whom correspondence should be addressed at: Research Building, Room B-8, Boston VA Medical Center, 150 South Huntington Avenue, Boston, MA 02130.

late individual PC molecular species (1), it is possible to radiolabel and trace the turnover of individual PCs (2–8). In addition, with HPLC, it is possible to isolate the acylglyceride precursors of specific PC molecular species (9). The present studies were performed using the isolated perfused rat liver to trace the turnover of newly synthesized molecular species of PCs along with their acylglyceride precursors to determine the extent to which hepatic PC synthesis occurs by acyl group remodeling or by conventional synthetic pathways. In the liver, de novo PC synthesis takes place by the acylation of glycero 3-phosphate to phosphatidic acid (PA) that is dephosphorylated to diglyceride (DG). In the presence of CDP-choline, DG is then converted to PC (10). A smaller amount of hepatic PC is synthesized by the N-methylation of phosphatidylethanolamine (PE) (11) and even smaller amounts by base exchange (12) or by lysoPC transesterification (13). The amount of PC that is actually synthesized by acyl group remodeling, i.e., by the utilization of endogenous acylglycerides for new synthesis rather than by the utilization of exogenous (radiolabeled) precursors, is largely unknown, as in previous studies (2–8) only the composition and turnover of the (PC) products of synthesis have been determined. Thus, it has been impossible to distinguish PCs that have been initially synthesized by the incorporation of endogenous acylglycerides from PCs that have undergone a change in composition or in specific activity by a deacylation-reacylation reaction(s) after synthesis has occurred. The present studies were performed not only to isolate the PCs that were newly synthesized after perfusion of the rat liver with FAs and [^{3}H]glycerol but to compare the specific activities of the newly synthesized PCs with the specific activities of acylglyceride precursors of PCs, including individual PAs, DGs, triglycerides (TGs), and PEs with the same acyl group composition as the newly synthesized PCs. The methods used provide clear evidence that reutilization of endogenous acylglycerides provides the major source of precursors for new PCs that are synthesized in the rat liver.

EXPERIMENTAL PROCEDURES

Materials

[2-^{3}H]glycerol (11.5 Ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA), fatty acid free bovine serum albumin was from Sigma (St. Louis, MO), sodium taurocholic acid (A grade) was from Calbiochem (La Jolla, CA), *cis*-9-hexadecenoic acid and *cis*-10-heptadecenoic acid were from Nu-Chek-Prep (Elysian, MN), and di-*cis*-9-octadecenoyl PC was from Avanti Polar Lipids (Birmingham, AL). Analytical and HPLC grade solvents were obtained from Fisher (Medford, MA).

Animals

Male Sprague-Dawley rats (Taconic Animal Farm, Germantown, NY) weighing 250–300 g were fed Purina rat chow ad libitum.

Liver perfusion

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the livers, with the bile duct cannulated, were perfused in situ with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 25 mM glucose at a flow rate of approximately 35 ml/min as previously described (14). Sodium taurocholate was infused directly into the portal vein cannula at a rate of 60 μmol/h. After 15 min of equilibration, the liver was perfused in a recirculating system for 15 min with fresh buffer (100 ml) containing 100 mmol of 16:1 FA (as the potassium salt) complexed to 3% albumin. A constant infusion of 16:1 FA (90 μmol/h) and [2-^{3}H]glycerol (1.09 μmol/h, 370 μCi/μmol) was maintained during this 15-min perfusion. After 15 min, the liver was either removed or washed for 5 min with fresh buffer containing 3% FA-free albumin and nonradiolabeled glycerol (1.09 μmol/h). After the 5 min washout perfusion, the liver was either removed or perfused in a recirculating system for up to 2 h with fresh buffer containing 17:1 FA complexed to 3% albumin. A constant infusion of 17:1 FA (90 μmol/h) and non-radiolabeled glycerol (1.09 μmol/h) was maintained during this perfusion period. Livers were removed at 15, 30, and 120 min after the beginning of the perfusion with 17:1 FA. There was one liver perfusion at each time point except at the end of the labeling period (−5 min) which was the average of two perfusions. At the end of each perfusion, the liver was immediately homogenized in cold chloroform–methanol 2:1.

Lipid analysis

The lipid extract was partitioned with saline as described by Folch, Lees, and Sloane Stanley (15). The lower phase was separated into neutral lipids and individual phospholipid classes by HPLC as previously described (16), except that the mobile phase contained 0.01% acetic acid. In this mobile phase, PA eluted after phosphatidylserine and together with dimethyl-PE and an unidentified component. The PA was separated from these components by rechromatography in the same system except that the mobile phase contained 0.06% acetic acid. Under these conditions the PA coeluted with phosphatidylinositol. DGs and TGs were isolated from the neutral lipid fraction by HPLC as previously described with a mobile phase of hexane–tetrahydrofuran–acetic acid 500:50:0.1. The DG, PA, PE, and PC fractions were converted to their benzoyl esters and separated into molecular species by reversed phase HPLC as previously described (1) using two 5-μm Ultrasphere ODS columns (2 × 250 mm) (Beckman, San Ramon, CA). As most of the fractions contained multiple components, the frac-

tions of interest were further separated by reversed phase chromatography using two 3-μm Spherisorb S3 ODS2 columns (2 × 250 mm) (Phase Separations, Norwalk, CT) with acetonitrile as the mobile phase at a flow rate of 0.3 or 0.5 ml/min. The amount of each molecular species was determined by digital integration of peak areas detected at 230 nm. Known amounts of benzoylated dimyristate were used to determine peak area to mass ratios. The fractions of interest were collected in counting vials and their tritium radioactivity was determined by liquid scintillation spectrometry.

The TG fraction was separated into molecular species by reversed phase HPLC on a 5-μm Ultrasphere ODS column (4.6 × 250 mm) (Beckman, San Ramon, CA) with methanol as the mobile phase at a flow rate of 1 ml/min. The fractions of interest were further separated by reversed phase HPLC on two 3-μm Spherisorb ODS columns (2 × 250 mm) (MetaChem, Rodondo Beach, CA) with acetonitrile–2-propanol 8:2 as the mobile phase at a flow rate of 0.3 ml/min. The separations of 16:1-16:1-16:1, 16:1-16:1-18:2, 16:1-16:1-16:0, and the 16:1-16:1-18:1 TGs are shown in **Fig. 1.** The TG fractions were collected and 18:1-18:1 PC was added as an internal standard. An aliquot was taken for liquid scintillation spectrometry and the remainder was transesterified with sodium methoxide. The purity of the TG fractions was confirmed and the amount of the TG molecular species was quantitated by capillary gas chromatography of the FA methyl esters on an SP2330 column (Supelco, Bellefonte, PA) as previously described (17).

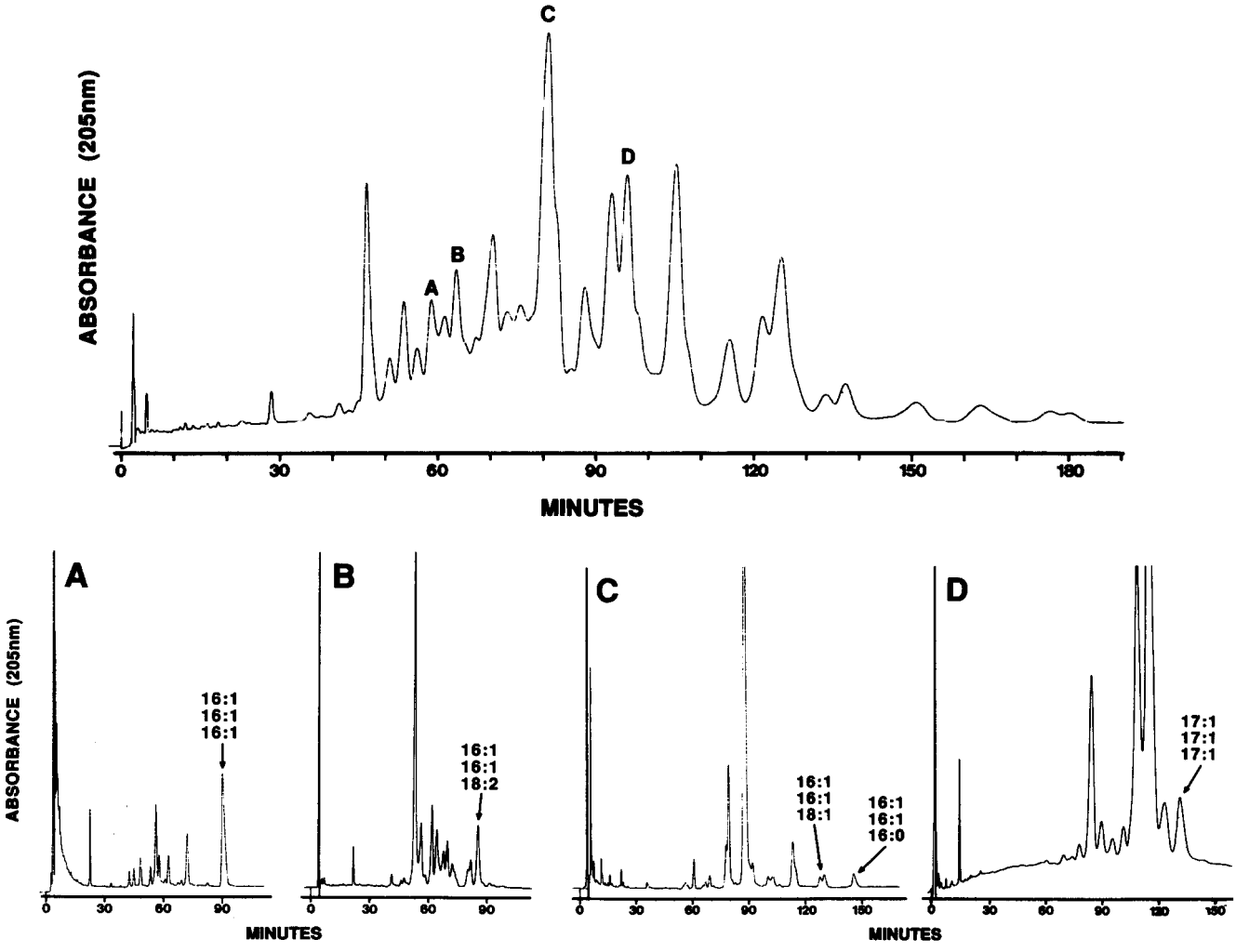

Fig. 1. Separation of TG molecular species by reversed-phase HPLC. Liver TGs were separated into molecular species on a 5-μm Ultrasphere ODS column (4.6 × 250 mm) with methanol as the mobile phase (upper panel). The flow rate was 1 ml/min and the effluent was monitored at 205 nm. In order to further purify the major 16:1-16:1 TGs and 17:1-17:1-17:1 TG, the peaks marked A, B, C, and D in the upper panel that contained 16:1-16:1-16:1, 16:1-16:1-18:2, 16:1-16:1-18:1 + 16:1-16:1-16:0, and 17:1-17:1-17:1, respectively, were collected and rechromatographed on two 3-μm Spherisorb S3 ODS2 columns (2 × 250 mm) with acetonitrile–2-propanol 8:2 as the mobile phase. The flow rate was 0.3 ml/min and the effluent was monitored at 205 nm.

RESULTS

Isolated rat livers were perfused for 15 min with [2-^{3}H]glycerol and 16:1 FA and then, during a washout period, perfused for an additional 5 min with FA-free albumin and nonradiolabeled glycerol. At that point, approximately two-thirds (68.7%) of the [^{3}H]glycerol incorporated into liver lipids was in the neutral lipid fraction and 90% of the ^{3}H in the neutral lipid fraction was in TG. Most of the remainder (9%) was in DG. Of the one-third (31.2%) of the [^{3}H]glycerol incorporated into the phospholipids, more than three-quarters (77.8%) was in PC. Appreciable [^{3}H]glycerol was also incorporated into PE and to a lesser extent phosphatidylinositol (i.e., 17.8% and 3.6% of the [^{3}H]glycerol incorporated into the phospholipids, respectively). There was essentially no incorporation of [^{3}H]glycerol into phosphatidylserine (0.02%) or diphosphatidylglycerol (0.03%).

After the 15-min labeling period and 5-min washout period, livers were subsequently perfused with nonradiolabeled glycerol and 17:1 FA for up to 2 h. During this chase period, there was a progressive decrease in the proportion of radiolabel in TG and DG and, with the exception of PA, there was an increase in the proportion of radiolabel in the phospholipid fractions (**Fig. 2**).

Most of the radiolabeled glycerides contained a 16:1 FA. In the case of TG, at the end of the washout period, 63.1% of the [^{3}H]glycerol was incorporated into four molecular species, i.e., 16:1-16:1-16:1, 16:1-16:1-18:2, 16:1-16:1-16:0, and 16:1-16:1-18:1[2] (**Fig. 3**). Likewise, at the end of the labeling period, approximately 90% of the [^{3}H]glycerol incorporated into PC was in a molecular species that contained a 16:1 FA (i.e., 16:1-16:1, 16:1-18:2, 16:0-16:1, 16:1-18:1), even though these four molecular species accounted for only 3.2% of the PCs in the liver. These same four molecular species also accounted for 38.7% of the radiolabeled PEs although these molecular species comprised only 0.67% of the PEs in the liver. During the chase with 17:1 FA and unlabeled glycerol, the proportion of ^{3}H in the 16:1-containing molecular species decreased and there was an increase in the proportion of radioactivity in both the 17:1-containing molecular species and in those molecular species that contain neither a 16:1 nor a 17:1 FA (data not shown).

At the end of the 15-min labeling period, nearly 40% of the PA and DG molecular species contained a 16:1 FA (**Fig. 4** and **Fig. 5**). During the washout period and the beginning of the chase period, the proportion of 16:1-containing molecular species of both PA and DG decreased rapidly, i.e., by a third during the 5-min washout period and by two-thirds at 15 min into the chase period. The proportion of 16:1-containing molecular species of PA and DG continued to decrease slowly until, at the end of the chase period, the 16:1-containing molecular species accounted for only about 10% of the total PA and DG molecular species. Conversely, after 15 min of the chase with 17:1 FA, 17:1-containing molecular species accounted for about a third and a quarter of the total molecular species of PA and DG, respectively, and the

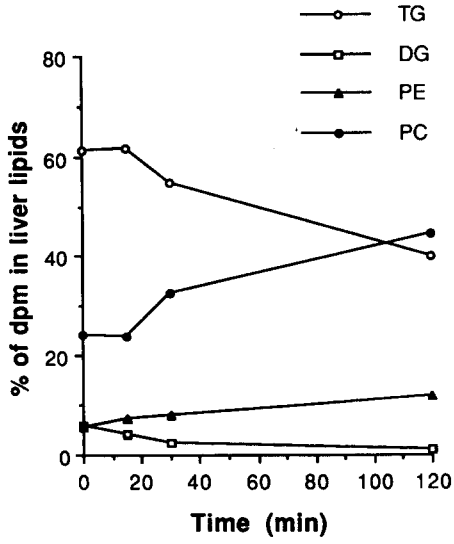

Fig. 2. Distribution of [2-^{3}H]glycerol radioactivity incorporated into liver acylglycerides. Livers were perfused with 16:1 FA and [2-^{3}H]glycerol for 15 min, washed free of FA and glycerol for 5 min, and then chased for up to 2 h with 17:1 FA and nonradiolabeled glycerol as described in Methods. Zero time represents the end of the washout period. Results are expressed as the percentage of the total radioactivity recovered in the indicated lipid class at each individual time point. At 0 time, PA contained 0.24% of the radioactivity, but less than 0.1% thereafter (not shown). Diphosphatidylglycerol and phosphatidylserine each contained less than 0.1% and phosphatidylinositol contained between 1% (0 time) and 2% (120 min) of the radioactivity (not shown).

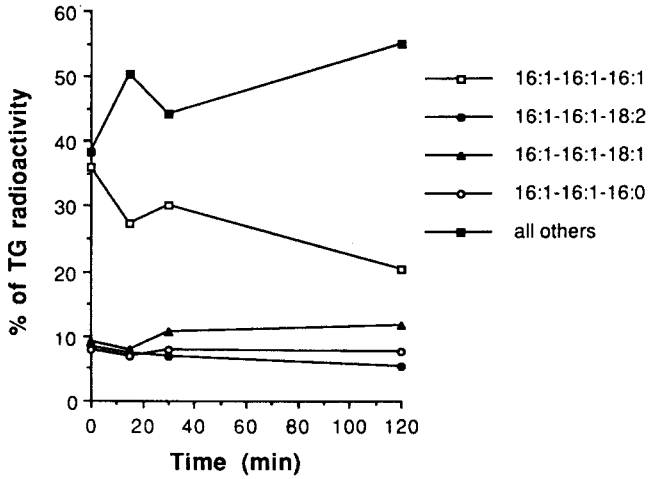

Fig. 3. Distribution of radioactivity among the major radiolabeled molecular species of TG. Experimental details are described in the legend to Fig. 2. TG molecular species were isolated as described in the legend to Fig. 1. Results are expressed as the percentage of total radioactivity in the TG fraction.

[2]This notation is only intended to indicate the fatty acid composition of the molecular species and is not intended to indicate the position of the fatty acid on the glycerol.

proportion of 17:1-containing molecular species did not increase appreciably thereafter. Although the proportion of molecular species of PA and DG that did not contain a 16:1 or 17:1 FA varied during the course of the experiment, the ratio of these molecular species to each other did not show any consistent variation. In contrast to PA and DG, the proportion of 16:1-containing molecular species of PC remained relatively constant but did increase slightly during the chase period (**Fig. 6**), rising most rapidly at first as the proportion of 16:1-containing molecular species of PA and DG decreased most rapidly. The ratio of the individual 16:1-containing molecular species of PE was highly variable but in aggregate, the total proportion of 16:1-containing molecular species of PE was essentially constant at about 0.7% (data not shown).

At the end of the labeling period, the specific activities of the 16:1-containing molecular species of PA and DG were all similar and were much higher than the specific activity of any molecular species that did not contain a 16:1 FA (**Fig. 7**). The specific activity of all the radiolabeled molecular species of PA decreased rapidly during the 5 min washout period and the beginning of the chase

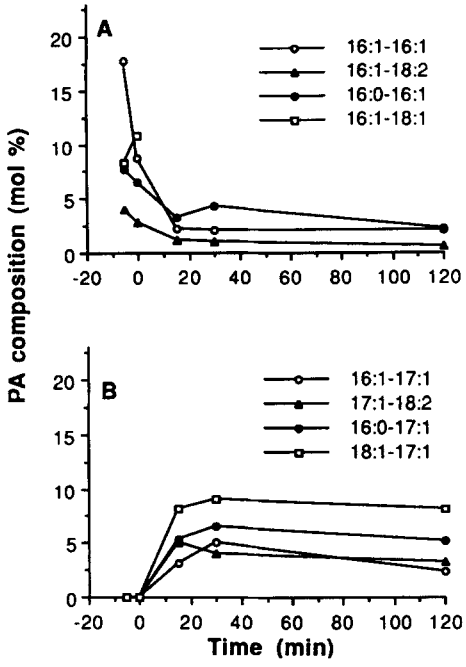

Fig. 4. The composition of the 16:1- and 17:1-containing molecular species of liver PAs. Livers were perfused for 15 min with 16:1 FA and [2-^{3}H]glycerol, washed for 5 min, and then chased with nonradiolabeled glycerol and 17:1 FA for up to 2 h as described in Methods. The minus 5 min time (−5) is at the end of the labeling period and the 0 min time is the end of the 5-min washout period. The PAs, isolated by normal phase HPLC, were converted to the benzoyl DGs and the benzoyl DGs were separated into individual molecular species by a two-step HPLC procedure described in Methods. Individual molecular species were quantitated by integration of the peak areas detected at 230 nm. Results are expressed as the percentage of total PA molecular species. A: 16:1-containing molecular species; B: 17:1-containing molecular species. The 16:1-18:1 and 17:1-17:1 molecular species could not be separated from each other at 15, 30, and 120 min and are not shown.

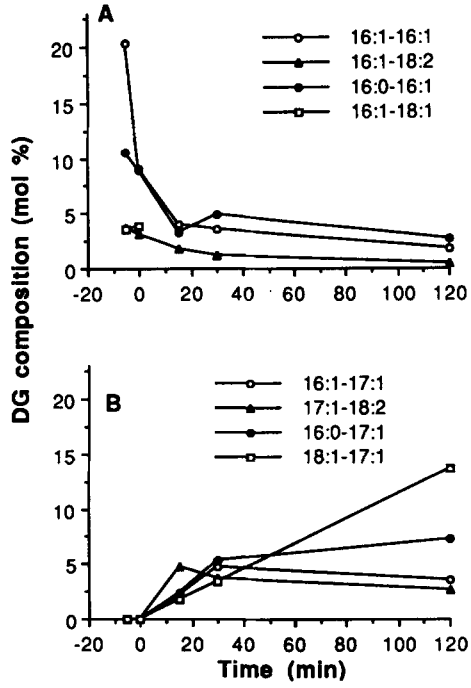

Fig. 5. The composition of the 16:1- and 17:1-containing molecular species of liver DGs. Experimental details are given in the legend to Fig. 4. DGs were isolated from the neutral lipid fraction by normal phase HPLC as described in Methods and converted to the benzoyl DGs. Individual benzoyl DG molecular species were separated and quantitated as described in the legend to Fig. 4. Results are expressed as the percentage of total DG molecular species. A: 16:1-containing molecular species; B: 17:1-containing molecular species.

period (i.e., by 15 min) and continued to decrease slowly thereafter. A similar pattern was observed for the DGs (**Fig. 8**) although the decrease in the specific activity of the radiolabeled DG molecular species did not proceed to the same extent as the corresponding PA molecular species.

In contrast to the PAs and DGs, at the end of the labeling period the specific activities of the 16:1-containing molecular species of PC (**Fig. 9**) were not all the same and they all had a specific activity considerably lower than the specific activity of the corresponding PA and DG molecular species (compare Figs. 7 and 8 with Fig. 9). In particular, the specific activity of 16:1-16:1 PC was about two-thirds that of 16:1-16:1 PA and DG, and of 16:1-16:1-16:1 TG (**Fig. 10**). The specific activities of the other 16:1-containing molecular species of PC (16:1-18:2, 16:0-16:1 and 16:1-18:1) were approximately half that of 16:1-16:1 PC and a third that of the corresponding PA and DG molecular species. During the washout period, the specific activity of all the 16:1-containing PCs increased slightly, except for 16:1-16:1 PC which decreased slightly. During the chase period, the specific activities of all the 16:1-containing molecular species[3] of PC decreased, and

[3]After the introduction of 17:1 FA, the specific activity of the 16:1-18:1 molecular species could not be determined because 16:1-18:1 could not be separated from the 17:1-17:1 molecular species.

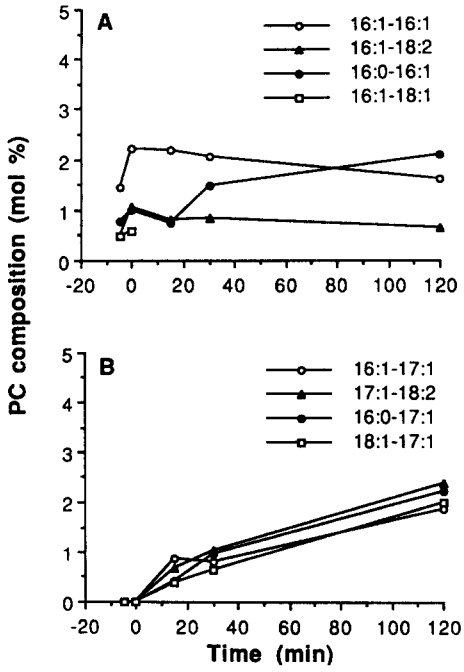

Fig. 6. The composition of the 16:1- and 17:1-containing molecular species of liver PCs. Experimental details are given in the legend to Fig. 4. PCs were isolated by normal phase HPLC and converted to the benzoyl DGs. The benzoyl DGs were separated into individual molecular species and quantitated as described in the legend to Fig. 4. Results are expressed as the percentage of total PC molecular species. A: 16:1-containing molecular species; B: 17:1-containing molecular species.

at the end of the chase period (120 min) the specific activities of all the 16:1-containing molecular species were about half of what they were at the end of the labeling period. The specific activity of 16:1-16:1-16:1 TG and 16:1-16:1-18:2 TG declined in parallel with the 16:1-containing molecular species of PC and at the end of the experiment were also half of what they were at the end of the labeling period.

The specific activity of 16:1-16:1 PE (**Fig. 11**) at the end of the labeling period was higher than 16:1-16:1 PC but less than 16:1-16:1 PA and DG. On the other hand, the specific activities of the 16:1-18:2, 16:0-16:1, and 16:1-18:1 PE were all about the same and were similar to the corresponding PC molecular species. During the washout and chase periods, the specific activity of 16:0-16:1 and 16:1-18:2 PE decreased in parallel with the corresponding molecular species of PC. On the other hand, the specific activity of 16:1-16:1 PE decreased more rapidly than 16:1-16:1 PC and at 30 min had a lower specific activity than 16:1-16:1 PC. The specific activities of those molecular species of PE that were radiolabeled but did not contain a 16:1 FA were generally similar to the corresponding PC molecular species. In contrast to PA and DG, the specific activity of the molecular species of PC and PE that contained neither a 16:1 nor a 17:1 FA continued to rise throughout the chase period and, in general, at 120 min they had a specific activity higher than the corresponding PA or DG molecular species.

At 15 min of the chase period, the specific activity of the 17:1-containing molecular species of PA and DG were virtually all the same, were very low and remained low throughout the experiment. At 15 min the specific activities of 17:1-18:2, 16:0-17:1, and 18:1-17:1 PC (13–19 dpm/nmol) were similar to the corresponding PA (12–21 dpm/nmol) and DG (18–23 dpm/nmol) molecular species, and decreased slowly thereafter. The specific activity of

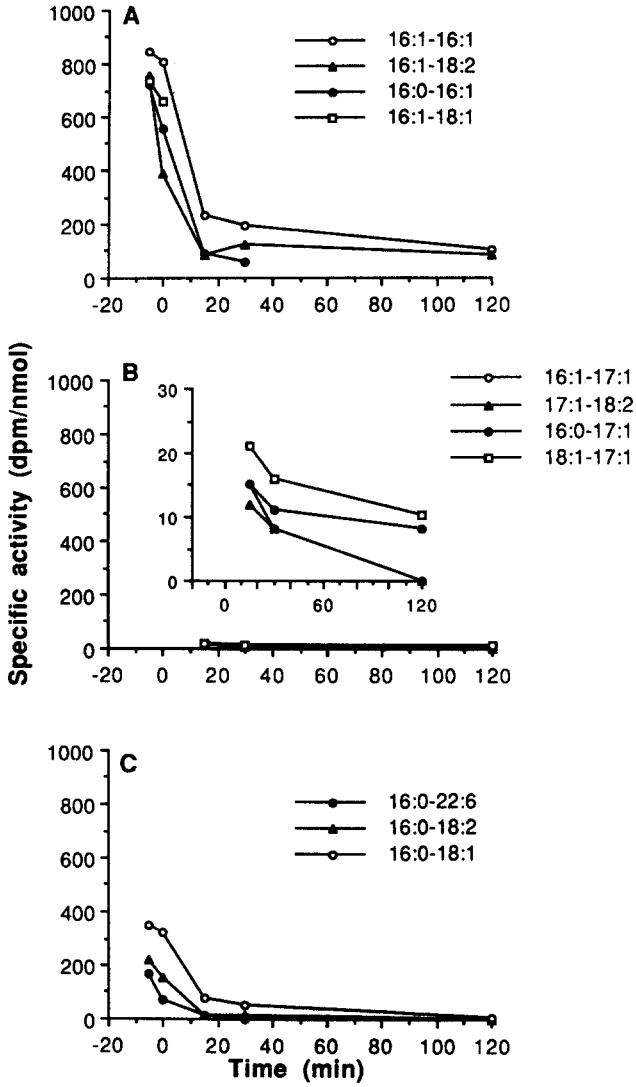

Fig. 7. The specific activity of individual molecular species of liver PAs. The experimental design and the isolation and quantitation of individual molecular species of PA are presented in the legend to Fig. 4. Radioactivity was determined by liquid scintillation spectrometry of fractions collected from the second reversed phase HPLC system. A: 16:1-containing molecular species; B: 17:1-containing molecular species; C: the major 16:0-containing molecular species. There was no radioactivity in the major 18:0 molecular species (18:0-22:6, 18:0-20:4, or 18:0-18:2). The insert in panel B shows the same data replotted on an expanded scale.

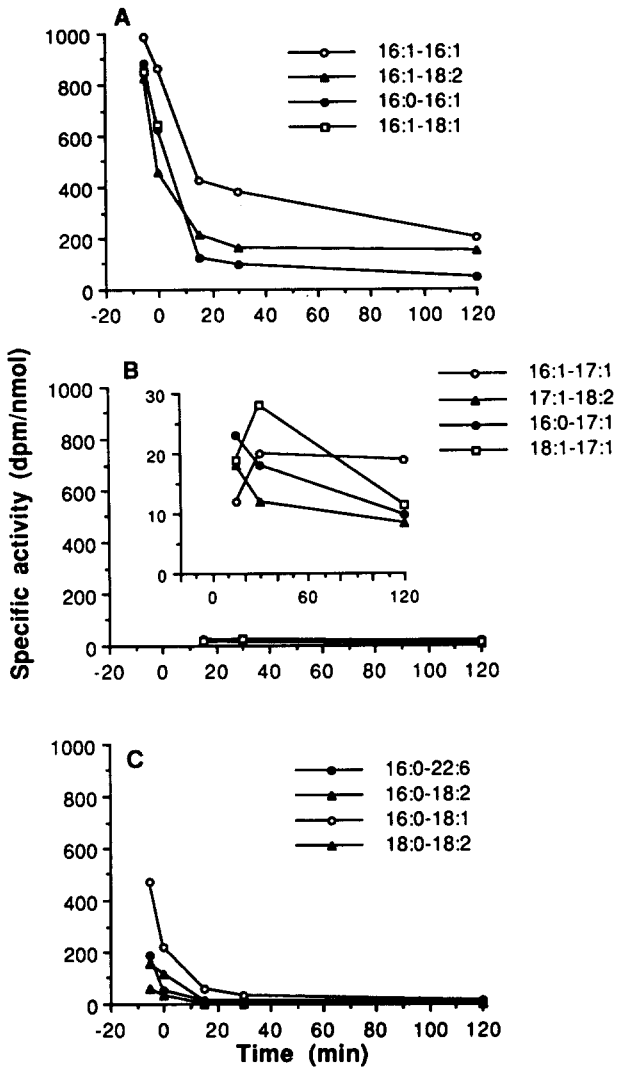

Fig. 8. The specific activity of the 16:1-, 17:1-, and major 16:0- and 18:0-containing molecular species of liver DGs. The experimental design and the procedures for the isolation and quantitation of the individual molecular species are presented in the legend to Fig. 4. A: 16:1-containing molecular species; B: 17:1-containing molecular species; C: major 16:0- and 18:0-containing molecular species (16:0-20:4, 18:0-22:6, and 18:0-20:4 contained little or no radioactivity). The insert in panel B shows the same data replotted on an expanded scale.

17:1-17:1-17:1 TG (47 dpm/nmol) at 15 min of the chase period was higher than the 17:1-containing molecular species of PA, DG, or PC at that time. The specific activity of 17:1-17:1-17:1 TG subsequently decreased by about half. As was the case with the 16:1-containing molecular species of PE, the proportion of 17:1-containing molecular species of PE was very small and they all had a low specific activity. Generally, the 17:1-containing molecular species of PE did not contain sufficient radioactivity for a reliable determination of their specific activity.

The specific activities of 16:1-17:1 PA and DG were similar to each other and to the other 17:1-containing molecular species. However, the specific activity of 16:1-17:1 PC after 15 min of the chase period was twice that of the other 17:1-containing molecular species of PC and 3 times that of 16:1-17:1 PA or DG, and was comparable to the specific activity of 17:1-17:1-17:1 TG. By 30 min into the chase period the specific activity of 16:1-17:1 PC actually increased, and was higher than any other 17:1-containing glyceride. Although the specific activity of 16:1-17:1 PC subsequently decreased, at 120 min the specific activity of 16:1-17:1 PC was still twice that of any other 17:1-containing glyceride, including 17:1-17:1-17:1 TG.

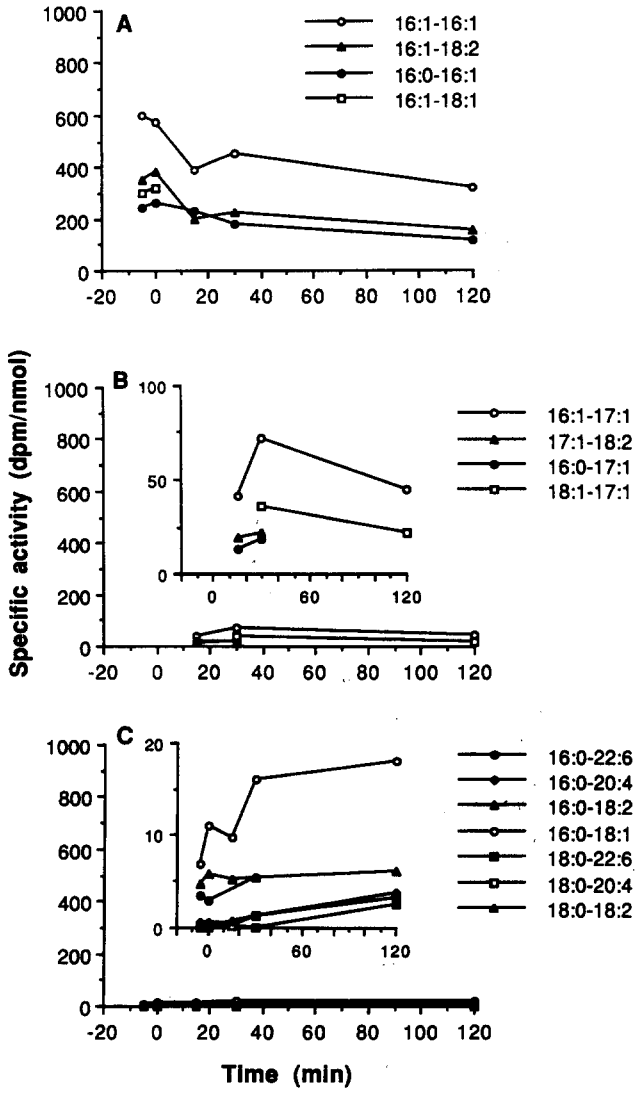

Fig. 9. The specific activity of the 16:1-, 17:1-, and the major 16:0- and 18:0-containing molecular species of liver PCs. The experimental design and the procedures for the isolation and quantitation of the individual molecular species are presented in the legend to Fig. 4. A: 16:1-containing molecular species; B: 17:1-containing molecular species; C: 16:0- and 18:0-containing molecular species. The inserts in panels B and C show the same data replotted on an expanded scale.

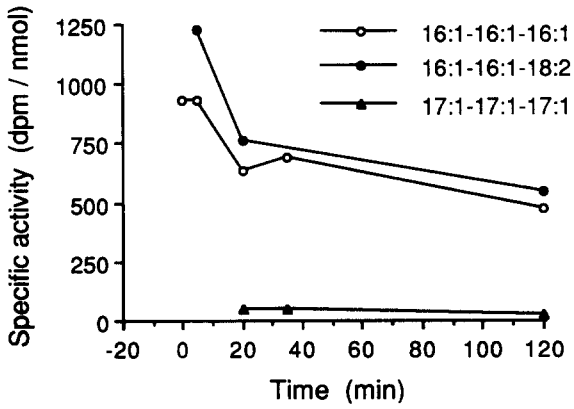

Fig. 10. The specific activity of 16:1-16:1-16:1, 16:1-16:1-18:2, and 17:1-17:1-17:1 molecular species of liver TGs. The experimental design is given in the legend to Fig. 4, and procedures for the purification of the TG molecular species are given in the legend to Fig. 1. TG molecular species were quantitated by gas chromatography of their FA methyl esters with 18:1-18:1 PC as the internal standard.

DISCUSSION

A large number of previous studies have been performed using radiolabeled precursors to trace the synthesis of liver glycerolipids. Specifically, with exogenous radiolabeled monounsaturated FA (18:1) and/or glycerol, it has been demonstrated that most of the precursor is incorporated into TGs with the remainder largely being incorporated into PCs (18–20). The present study was performed with the infusion of radiolabeled glycerol and large amounts of FAs that are ordinarily not present in liver PCs (i.e., 16:1 and 17:1 FAs) to define, by isolating individual molecular species of newly formed (and largely unique) PCs and their precursors, the pathways of synthesis of new PCs. In previous studies, when 16:1 glycerides were fed to rats, 16:1 FA was incorporated into intestinal PCs (21) and these PCs were secreted in intestinal lymph lipoproteins to the same extent as 18:1 (21), 18:2 (22), or 18:3 (21) FA. The major 16:1-containing PC in lymph lipoproteins after administration of 16:1 glycerides was 16:1-16:1 PC. A number of studies have been performed comparing the metabolism of model lipoproteins prepared with various pure PCs including 16:1-16:1 PC. These studies demonstrated that in vivo the fractional clearance rate of cholesteryl ester, cholesterol, and TG from emulsion particles prepared with 16:1-16:1 PC was not significantly different than from emulsions prepared with a more usual PC, 16:0-18:2 PC (23). Likewise, the uptake by the liver both in vivo (24) and in vitro (25) of cholesteryl ester from reconstituted HDL prepared with 16:1-16:1 PC was the same as from reconstituted HDL prepared with 16:0-18:2 or 18:0-18:2 PC.

Previous studies have also shown that a wide variety of exogenous FAs including 16:1 FA are taken up by the perfused rat liver at essentially the same rate (14, 26). In one study (14), we demonstrated that, after the infusion of 16:1 and 17:1 FAs, the increase in the amount of 16:1 and 17:1 FAs in liver PCs was similar to the increase of 18:1 and 18:2 FAs in liver PCs after the infusion of 18:1 and 18:2 FAs. Moreover, these 16:1 and 17:1 PCs are secreted into bile at a rate comparable to 18:2 PCs, the major unsaturated FA in bile. In that same study (14), we also observed that there was an identical increase of 16:1, 17:1, and 18:1 FAs in liver TGs after the infusion of the corresponding FA (data not published). Similar results had previously been reported by others for perfused rat livers (27) and for cultured hepatocytes (28) incubated with various FAs. In those studies, 16:1 FA was incorporated into both TG and phospholipids at rates comparable to 16:0 and 18:1 FAs and the resulting TGs (and phospholipids) were also secreted in lipoproteins at rates similar to 18:1 and 18:2 (perfused liver) or 16:0 and 18:1 (hepatocytes). In the one previous study (19) in which the turnover of molecular species of PC was determined after incubation of hepatocytes with exogenous FAs and radiolabeled glycerol (using a pulse chase protocol), the turnover of 16:0-16:0 PC and of 18:2-18:2 PC was comparable to that observed in the present study for glycerol labeled 16:1-16:1 PC.

Because there were no 16:1-16:1 molecular species or other 16:1-containing molecular species (except for a small amount of 16:0-16:1) in the liver prior to the simultaneous infusion of 16:1 FA and [^{3}H]glycerol, and because 16:1-16:1 PA, DG, and 16:1-16:1-16:1 TG all had nearly the same specific activity at the end of the 15-min labeling period, it is likely that the specific activity of these molecular species represents the specific activity of those molecular species synthesized exclusively by de novo synthesis from glycerol 3-phosphate. If that assumption is correct, then the extent to which a 16:1-containing molecular species had a lower specific activity than 16:1-16:1 PA and DG, and 16:1-16:1-16:1 TG at the end of the labeling period is

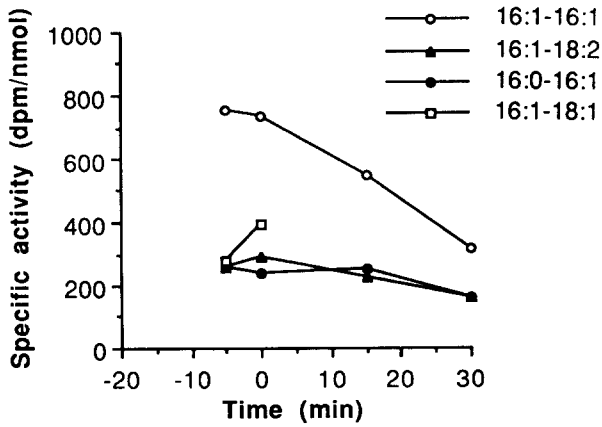

Fig. 11. The specific activity of the 16:1-containing molecular species of liver PEs. The experimental design and the procedures for the purification and quantitation of the molecular species are given in the legend to Fig. 4. The specific activity of the PEs was not determined at 120 min.

a measure of the extent to which that molecular species arose by a mechanism other than de novo synthesis from glycerol 3-phosphate. For instance, all the molecular species of PA that contained a single 16:1 FA were very highly labeled but on average had a specific activity that was only 85% of the specific activity of the 16:1-16:1 molecular species. This difference in specific activities indicates that molecular species with a single 16:1 FA were formed in part by a pathway other than acylation of glycerol 3-phosphate. Among the possibilities are the acylation of lyso-PA arising from nonradiolabeled (pre-existing) lysophospholipids; by deacylation of PA and reacylation with 16:1 FA; or by the recycling of these molecular species from the corresponding PC or PE molecular species (which have a lower specific activity) through the action of a phospholipase D. If the recycling explanation is correct, then at the end of the labeling period, about a third of the 16:1-18:2, 16:0-16:1, and 16:1-18:1 PA arose by recycling of existing phospholipid molecular species, i.e., a third had a specific activity of approximately 300 dpm/nmol (weighted average of the corresponding PC and PE molecular species) and two-thirds had a specific activity of approximately 950 (the specific activity of the de novo synthesized molecular species 16:1-16:1 PA).

With the exception of 16:0-20:4, the 16:0-containing molecular species of PA were also labeled to an appreciable extent. However, their specific activities were much less than the 16:1-containing molecular species. Thus, by reasoning analogous to that for the 16:1-containing molecular species of PA, because only a third, a fifth, and an eighth of the 16:0-18:1, 16:0-18:2, and 16:0-22:6 PA, respectively, appear to have arisen by acylation of glycerol 3-phosphate, the majority of these molecular species of PA were also probably formed by the action of a phospholipase D on pre-existing phospholipids. That there is appreciable de novo synthesis of these molecular species even in the presence of an overwhelming supply of exogenous 16:1 FA indicates that there was also considerable turnover of 16:0, 18:1, 18:2, and 22:6 molecular species through the action of a phospholipase A or by the action of a TG lipase(s). During the chase period, the specific activity of these PA molecular species decreased and eventually reached a specific activity comparable to that of the corresponding PC or PE molecular species. This is what would be expected if these PA molecular species were formed largely by the action of a phospholipase D on preexistent PC (and PE). Because there was virtually no radiolabel in the 18:0 molecular species of PA, no definitive conclusion can be drawn about the metabolism of the 18:0-containing molecular species, except that there was virtually no acylation of glycerol 3-phosphate with 18:0 FA, at least under the conditions of this study.

The pool of PA in the liver was very small (36 nmol/g wet weight liver), and based upon the amount of [^{3}H]glycerol incorporated into liver lipids, the flux through PA was very large. Therefore, when the infusion of 16:1 FA was discontinued, and the liver was washed free of this FA with fatty acid-free albumin, and a second FA (17:1) was infused, the proportion of 16:1-containing molecular species of PA, especially 16:1-16:1 PA, was expected to decline very rapidly. Indeed this is what was observed. The proportion of 16:1-16:1 PA quickly decreased to about a tenth of what it was at the end of the 16:1 FA perfusion and remained at that level (about 2% of total PA molecular species) for the duration of the perfusion. This residual 16:1-16:1 PA could be expected to reflect the recycling of 16:1-16:1 phospholipids by phospholipase D. If so, then the specific activity of 16:1-16:1 PA should have rapidly approached the specific activity of the 16:1-16:1 PC (and PE) as did the 16:0-containing PAs. However, the specific activity of 16:1-16:1 PA declined to about a third of the specific activity of the 16:1-16:1 phospholipids. This suggests that there was ongoing synthesis of 16:1-16:1 PA from 16:1 FA and nonradiolabeled glycerol 3-phosphate. The extent of this new synthesis was surprising because, with the infusion of 17:1 FA, it would be expected that the major 16:1-containing molecular species of PA would be 16:1-17:1. However, the proportion of 16:1-17:1 PA was at most only twice that of 16:1-16:1 PA and at 120 min was only slightly greater than 16:1-16:1 PA. Moreover, the specific activity of the 16:1-17:1 PA that was synthesized was very low (15 dpm/nmol at 15 min) and dropped to zero by 120 min indicating that 16:1-17:1 PA was being synthesized de novo from nonradiolabeled glycerol 3-phosphate. The ongoing synthesis of 16:1-16:1 PA at a rate comparable to 16:1-17:1 in the presence of a large amount of exogenous 17:1 FA suggests that much of the 16:1 FA incorporated into PA during the chase period was sequestered either in a location or in a chemical form that did not allow it to equilibrate with the exogenous FA pool. In this regard, the behavior of 16:1-16:1-16:1 TG was particularly interesting. As has been mentioned above, at the end of the labeling period, 16:1-16:1-containing TGs had the same specific activity as 16:1-16:1 DG. Moreover, the fact that 16:1-16:1-18:2 and 16:1-16:1-16:1 TG had the same specific activity suggests that acyl remodeling was not a significant pathway of 16:1 FA incorporation into TG. However, like the other 16:1-16:1 glycerides including PC and PE, the specific activity of 16:1-16:1-16:1 TG decreased over time and at a rate similar to PC. This implies that there was ongoing synthesis of 16:1-16:1-16:1 TG from 16:1-16:1 DG even though large amounts of 17:1 FA were being administered. This strongly supports the notion that there was recycling of 16:1 FA and that the recycling process was sequestered in some manner.

Although there is much more DG than PA (about 30-fold), the amount of DG in the liver was also very low. In general, however, the DG molecular species responded

essentially identically to the corresponding PA molecular species throughout the course of this experiment except that the DGs consistently had a slightly higher specific activity than the corresponding PA molecular species (compare Fig. 7 with Fig. 8). This is what would be expected for a larger, more slowly turning over pool. Like the PAs, at the end of the labeling period the specific activities of those molecular species of DG that contained a single 16:1 FA were about 85% of the specific activity of 16:1-16:1 DG. During the chase period, the specific activity of all the molecular species declined rapidly and the specific activity of the 16:1 DGs dropped below that of the corresponding PC or PE molecular species. Unfortunately, because neither the magnitude of the flux through PA of recycled 16:1 FA nor the extent to which glyceride from TG is recycled into DG are known, it is impossible to estimate the extent to which phospholipids are recycled into DG via phospholipase C. However, because the DG molecular species paralleled the behavior of PA so closely, it does not appear that phospholipase C hydrolysis of phospholipids made a major contribution to DG synthesis under the condition of this experiment.

If the specific activity of 16:1-16:1 DG and of 16:1-16:1-16:1 TG at the end of the labeling period is indicative of the specific activity of glycerides synthesized de novo, then because the specific activities of the PC molecular species with a single 16:1 FA were only about a third of what would be expected if they were synthesized de novo, it is clear that the vast majority of these newly formed 16:1-containing PCs arose by mechanisms other than de novo synthesis from DGs. Presumably these molecules arose primarily by remodeling of pre-existing PCs by deacylation and reacylation with 16:1 FA. This even includes 16:1-16:1 PC which had a specific activity only two-thirds of what would be expected for a de novo synthesized PC.

Our studies also provide additional evidence for PC formation by remodeling. In particular, the specific activity of 16:0-18:1, 16:0-18:2, and 16:0-22:6 PC increased slowly throughout the chase period. Because there was not enough of these molecular species of PA and DG at the end of the labeling period to sustain the observed increase of radiolabel in these molecular species of PC (and PE), it is likely that some of the increase in [^{3}H]glycerol in these molecular species of PC and PE was due to remodeling of high specific activity 16:1-containing molecular species of PC and PE. Likewise, because there is virtually no radiolabel in the 18:0-containing PA and DG at the end of the labeling period, the increase in the specific activity of the 18:0-containing molecular species of PC and PE was almost certainly due to remodeling of radiolabled 16:0 and 16:1-containing PC and PE. Evidence of PC remodeling can also be seen by looking at the specific activity of the 16:1-17:1 molecular species. At the earliest time examined (15 min after the beginning of the perfusion of 17:1 FA), the specific activity of 16:1-17:1 PA was about the same as the specific activity of the other 17:1-containing molecular species and was comparable to the specific activity of 16:1-17:1 DG (and the other 17:1-containing DGs). At that point the specific activity of 16:1-17:1 PC was about the same as would be expected for a de novo synthesized PC, i.e., it had a specific activity similar to 17:1-17:1-17:1 TG. By 30 min, however, when the specific activity of the other 17:1-containing molecular species of PC had decreased, the specific activity of 16:1-17:1 PC increased and its specific activity at that point was 50% higher than any other 17:1-containing glyceride including 17:1-17:1-17:1 TG. While the specific activity of 16:1-17:1 PC declined over the next 90 min, at the end of the experiment (120 min) it still had a specific activity twice that of the de novo synthesized 17:1-containing glycerides (17:1-17:1-17:1 TG). Moreover, the specific activities of 16:1-17:1 PA and DG were always much less than that of 16:1-17:1 PC. The 16:1-17:1 PA and DG would have had a specific activity comparable to 16:1-17:1 PC if the 16:1-17:1 PC was synthesized by recycling of high specific activity 16:1-containing TGs or PCs. This clearly indicates that much of the 16:1-17:1 PC was formed by remodeling of radiolabeled 16:1-containing PCs.

It has been suggested that newly synthesized PE is preferentially converted to PC (8). The results of this experiment are consistent with that proposition. At the end of the labeling period, the specific activity of 16:1-16:1 PE was intermediate between the specific activity of the 16:1-16:1 DG and PC molecular species. During the chase period, as the specific activity of 16:1-16:1 DG rapidly decreased, the specific activity of 16:1-16:1 PE also decreased more rapidly than 16:1-16:1 PC. This is exactly the pattern that would be expected if 16:1-16:1 DG was being converted to PE and then that newly synthesized PE was preferentially being converted into PC. However, from this experiment it was not possible to determine the extent to which 16:1-16:1 PC arises by direct conversion of 16:1-16:1 DG to 16:1-16:1 PC and how much by the conversion of 16:1-16:1 DG to 16:1-16:1 PE followed by the conversion of that PE to PC. The specific activities of the other 16:1-containing molecular species of PE were similar to that of the corresponding PC molecular species, indicating that there is also substantial remodeling of PE, i.e., about two-thirds of the 16:1 fatty acid incorporated into these molecular species was through remodeling of pre-existing PEs.

On the basis of this study, it appears that the major pathway for the incorporation of 16:1 and 17:1 FA into PC and PE is remodeling of pre-existing PCs and PEs. On the other hand, there is no evidence for acyl group remodeling of TGs. Moreover, the vast majority of the precursors of de novo phospholipid synthesis, appear to derive from endogenous sources as opposed to acylation of glycerol 3-phosphate. It also appears that there is exten-

sive preferential reutilization of previously esterified glyceride FA for de novo glycerolipid synthesis. i.e., the 16:1 FA incorporated into glycerides during the 15-min labeling period were reutilized for the acylation of non-radiolabeled glycerol 3-phosphate without extensive mixing with exogenous 17:1 FA.

This study was supported by the General Medical Research Service of the Veterans Administration and National Institutes of Health Grant AM-28640.

Manuscript received 15 September 1993 and in revised form 6 January 1994.

REFERENCES

1. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1990. Analysis of lipids by high performance liquid chromatography. Part II: Phospholipids. *J. Nutr. Biochem.* **1:** 549–556.
2. Schmid, P. C., S. B. Johnson, and H. H. O. Schmid. 1991. Remodeling of rat hepatocyte phospholipids by selective acyl turnover. *J. Biol. Chem.* **266:** 13690–13697.
3. Scagnelli, G. P., P. S. Cooper, J. M. VandenBroek, W. F. Berman, and C. C. Schwartz. 1991. Plasma 1-palmitoyl-2-linoleoyl phosphatidylcholine: evidence for extensive phospholipase A1 hydrolysis and hepatic metabolism of the products. *J. Biol. Chem.* **266:** 18002–18011.
4. Caesar, P. A., M. C. McElroy, F. J. Kelly, I. C. S. Normand, and A. D. Postle. 1991. Mechanisms of phosphatidylcholine acyl remodeling by human fetal lung. *Am. J. Respir. Cell Mol. Biol.* **5:** 363–370.
5. Rostow, B., M. Schlame, R. Haupt, D. Wilhelm, and D. Kunze. 1992. Studies on the formation of dipalmitoyl species of phosphatidylcholine and phosphatidylethanolamine in pulmonary type II cells. *Biochem. J.* **282:** 453–458.
6. Burdge, G. C., F. J. Kelly, and A. D. Postle. 1993. Synthesis of phosphatidylcholine in guinea pig fetal lungs involves acyl remodelling and differential turnover of individual molecular species. *Biochim. Biophys. Acta.* **161:** 251–257.
7. Burdge, G. C., F. J. Kelly, and A. D. Postle. 1993. Mechanisms of hepatic phosphatidylcholine synthesis in the developing guinea pig: contributions of acyl remodelling and of N-methylation of phosphatidylethanolamine. *Biochem. J.* **290:** 67–73.
8. Samborski, R. W., N. D. Ridgway, and D. E. Vance. 1990. Evidence that only newly made phosphatidylethanolamine is methylated to phosphatidylcholine and that phosphatidylethanolamine is not significantly deacylated-reacylated in rat hepatocytes. *J. Biol. Chem.* **265:** 18322–18329.
9. Rustow, B., Y. Nakagawa, H. Rabe, G. Reichman, D. Kunze, and K. Waku. 1988. Comparison of the HPLC separated species patterns of phosphatidic acid, CDP-diacylglycerol and diacylglycerol synthesized de novo in rat liver microsomes. *Biochim. Biophys. Acta.* **961:** 364–369.
10. Kennedy, E. P., and S. B. Weiss. 1956. The function of cytidine coenzymes in the biosynthesis of phospholipids. *J. Biol. Chem.* **222:** 193–214.
11. Bremmer, J., and D. M. Greenberg. 1961. Methyl transferring enzyme system of microsomes in the biosynthesis of lecithin. *Biochim. Biophys. Acta.* **46:** 205–216.
12. Bjerve, K. S. 1973. The Ca^{++} dependent biosynthesis of lecithin, phosphatidylethanolamine and phosphatidylserine in rat liver subcellular particles. *Biochim. Biophys. Acta.* **296:** 549–562.
13. Erbland, J., and G. V. Marinetti. 1965. The enzymatic acylation and hydrolysis of lyso-lecithin. *Biochim. Biophys. Acta.* **106:** 128–138.
14. Robins, S. J., J. M. Fasulo, V. F. Robins, and G. M. Patton. 1991. Utilization of different fatty acids for hepatic and biliary phosphatidylcholine formation and the effect of changes in phosphatidylcholine molecular species on biliary lipid secretion. *J. Lipid Res.* **32:** 985–992.
15. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
16. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1990. Analysis of lipids by high performance liquid chromatography: Part I. *J. Nutr. Biochem.* **1:** 493–500.
17. Patton, G. M., S. Cann, H. Brunengraber, and J. M. Lowenstein. 1981. Separation of methyl esters of fatty acids by gas chromatography on capillary columns, including the separation of deuterated from nondeuterated fatty acids. *Methods Enzymol.* **72:** 8–18.
18. Soler-Argilaga, C., and M. Heimberg. 1976. Comparison of metabolism of free fatty acid by isolated perfused livers from male and female rats. *J. Lipid Res.* **17:** 605–615.
19. Kanoh, H., and B. Akesson. 1977. Phospholipid degradation in isolated rat hepatocytes: metabolism of intracellularly formed dilinoleoyl, dipalmitoyl and dimyristoylglycerophosphocholine. *Biochim. Biophys. Acta.* **486:** 511–523.
20. Anderson, D. B., and B. J. Holub. 1985. Influence of dietary cholesterol on the relative synthesis of hepatic glycerides and molecular classes of 1,2 diglycerides and phospholipids in the gerbil in vivo. *Lipids.* **20:** 167–172.
21. Robins, S. J., and G. M. Patton. 1986. Separation of phospholipid molecular species by high performance liquid chromatography: potentials for use in metabolic studies. *J. Lipid Res.* **27:** 131–139.
22. Patton, G. M., S. B. Clark, J. M. Fasulo, and S. J. Robins. 1984. Utilization of individual lecithins in intestinal lipoprotein formation in the rat. *J. Clin. Invest.* **73:** 231–240.
23. Robins, S. J., J. M. Fasulo, and G. M. Patton. 1988. Effect of different molecular species of phosphatidylcholine on the clearance of emulsion particle lipids. *J. Lipid Res.* **29:** 1195–1203.
24. Leduc, R., G. M. Patton, D. Atkinson, and S. J. Robins. 1987. Influence of different molecular species of phosphatidylcholine on cholesterol transport from lipoprotein recombinants in the rat. *J. Biol. Chem.* **262:** 7680–7685.
25. Kadowaki, H., G. M. Patton, and S. J. Robins. 1993. Effect of phosphatidylcholine molecular species on the uptake of HDL triglycerides and cholesteryl esters by the liver. *J. Lipid Res.* **34:** 180–189.
26. Ide, T., and J. A. Ontko. 1981. Increased secretion of very low density lipoprotein triglyceride following inhibition of long chain fatty acid oxidation in isolated rat liver. *J. Biol. Chem.* **256:** 10247–10255.
27. Wilcox, H. G., G. D. Dunn, and M. Heimberg. 1975. Effects of several common long chain fatty acids on the properties and lipid composition of the very low density lipoprotein secreted by the perfused rat liver. *Biochim. Biophys. Acta.* **398:** 39–54.
28. Lamb, R. G., C. K. Wood, B. M. Landa, P. S. Guzelian, and H. J. Fallon. 1977. Studies of the formation and release of glycerolipids by primary monolayer cultures of adult rat hepatocytes. *Biochim. Biophys. Acta.* **489:** 318–329.