The Incorporation of Labeled Palmitic Acid into the Phospholipids of Normal and Fatty Livers*  

JOSEPH L. GLENN,† EDWARD OPALKA, AND KONRAD TISCHER  

From the Department of Biochemistry, Albany Medical College of Union University, Albany 8, New York  

(Received for publication, September 7, 1962)  

It has been previously demonstrated that the administration of cerium to female rats resulted in a 2- to 3-fold increase in hepatic lipid (2). The increase of lipid in this type of fatty liver was shown to be due to an elevation of triglyceride, whereas phospholipid content remained normal (3). An additional finding was a pronounced elevation of plasma free fatty acids after the injection of this rare earth (4).

Numerous investigators have often suggested that phospholipids play a role in maintaining a normal lipid content in the liver, although the metabolism of these compounds was reported to decrease (5) as well as increase (6) under conditions when the liver was accumulating lipid. The present study was designed to follow the incorporation of a metabolically active fatty acid, palmitic acid-\(1^\text{C}14\), into the individual phospholipids of liver in normal animals and in animals undergoing fatty degeneration of hepatic tissue.

**EXPERIMENTAL PROCEDURE**

**Materials**—All animals used in this study were 175- to 225-g female rats of the Wistar strain obtained from the Blue Spruce Animal Farms, Altamont, New York. Palmitic acid-\(1^\text{C}14\) with a specific activity of 4.5 \(\mu\)c per \(\mu\)mole was obtained from the ChemTrac Corporation, Cambridge, Massachusetts; cerous chloride from the Fisher Scientific Company; and silicic acid and organic chemicals of Analytical Reagent Grade from the Mallinckrodt Chemical Works. Methyl ester standards were obtained from Applied Science Laboratories, College Station, Pennsylvania, and from the National Institutes of Health, Bethesda 14, Maryland.

**Production of Fatty Liver**—Cerium was administered intravenously to normally fed rats by injection of \(\text{CeCl}_4\) into the tail vein (3.5 mg of cerium, as element, per kg of body weight). This corresponds to 700 \(\mu\)g of cerium per 200-g rat.

**Preparation and Injection of Palmitic Acid-\(1^\text{C}14\)**—The palmitic acid was dissolved in ethanol, neutralized with \(\text{NaOH}\), and then taken to dryness. The sodium palmitate was dissolved in 10% human serum albumin so that the final solution contained 10 \(\mu\)c per ml. This solution was stored in the frozen state. The isotope (5 to 7 \(\mu\)c) was injected into the tail vein under light ether anesthesia to normal animals or to rats that had received cerium previously. The animal was allowed to recover from anesthesia and kept unrestrained for a 10-minute period, after which it was killed by decapitation and the liver was removed

**Total Lipid Extraction and Preliminary Fractionation**—The liver was homogenized in 20 volumes of chloroform-methanol (2:1, volume for volume) for 3 minutes in a micro Waring Blender, filtered through Whatman No. 3 paper under slight vacuum, and washed with 50 ml of the above solvent. To the filtrate was added 0.2 volume of 0.73\% \(\text{NaCl}\), the mixture was stirred, and the aqueous layer was separated by centrifugation (7). The aqueous layer was aspirated and four successive washes with chloroform-methanol-0.73\% \(\text{NaCl}\) (3:48:47 by volume) were performed before the total lipid extract was concentrated to dryness and taken up in light petroleum ether. Insoluble material was removed, and the extract was concentrated to a small volume (10 to 15 ml). A preliminary separation into neutral lipids and phospholipids was made on a 10-g silicic acid column plus 5 g of Hyflo Super-Cel by eluting first with 200 ml of chloroform and then with 200 ml of absolute methanol. Aliquots of the chloroform and methanol fractions were taken to determine the amount of neutral lipids and phospholipids, respectively.

The methanol fraction containing the phosphatides was taken to dryness under vacuum, dissolved in 10 to 15 ml of petroleum ether, and placed in a freezer (\(-15^\circ\)) under nitrogen until the following day.

**Column Chromatography of Phospholipids with Silicic Acid**—The method of Hanahan, Dittmer, and Warashina (8) was used because of its excellent reproducibility, but was modified in one major aspect. The loading ratio of lipid phosphorus to silicic acid was decreased from 0.8 to 1.0, to 0.2 to 0.3 mg of lipid phosphorus per g of silicic acid. The reason for this is explained in “results.” In a typical run, 6.0 mg of lipid phosphorus, isolated in the preliminary silicic acid separation, were placed on a column containing 25 g of silicic acid and 12.5 g of Hyflo Super-Cel. This gave a loading ratio of 0.24. The phosphatides were carefully washed on the column with petroleum ether and then eluted with (a) 300 ml of chloroform-methanol (3:2, volume for volume), (b) 600 ml of chloroform-methanol (3:2, volume for volume), and (c) 250 ml of chloroform-methanol (1:4, volume for volume). The lipids were collected in 7.0-ml volumes with a Gilson automatic fraction collector. As each fraction came off the column, aliquots were taken for phosphate analysis and radioactivity measurements. The remainder of each fraction was stored in the cold for subsequent chemical analysis.

**Chemical Determinations**—Total phosphorus was determined

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* A preliminary report of this study has been published (1).
† Research Career Development Award GM-K3-14,018, Department of Health, Education, and Welfare, Bethesda 14, Maryland.
on each fraction by the method of Bartlett (9); nitrogen was analyzed on pooled samples by a micro-Kjeldahl technique; ester equivalents of peak fractions by the procedure of Stern and Shapiro (10); and glycerol by the method of Renkonen (11). The fractions containing ninhydrin-positive material were hydrolyzed in glass-sealed tubes for 48 hours in 2 N HCl at 125°, after which the fatty material was extracted with chloroform and the aqueous phase was concentrated to dryness in a vacuum to remove the HCl. Aliquots of the water-soluble phase were then chromatographed on Whatman No. 1 paper with n-butanol-acetic acid-water (120:30:50), and amino acids were detected by the ninhydrin method.

**Paper Chromatography and Identification of Phospholipids**—Silicic acid-impregnated paper was prepared according to the method of Roos, O’Brien, and Heller (12), and the developing solvents used to separate the phosphatides were chloroform-methanol (4:1, volume for volume) containing 8.0 ml of concentrated aqueous ammonia per liter (13), or the disobutyl ketone-acetic acid system of Marinietti (14). Rhodamine 6G was used to detect the total lipid pattern; ninhydrin in butanol, mixed with 0.2 volume of 2,4-lutidine, for detecting the amino phosphatides; the phosphomolybdate-stannous chloride reagent (15), for demonstrating choline; and the method of Trevelyan, Procter, and Harrison (16), to identify inositol.

**Radioactivity Measurements**—The C14 content of each fraction eluted from the column was immediately determined in a Series 314 E Tri-Carb liquid scintillation counter. A 0.1 to 0.6-ml aliquot of each 7.0-ml fraction was added to 14.0 ml of toluene which contained 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis(2-(5-phenyloxazolyl))benzene. The maximal amount of phospholipid counted contained 150 to 200 μg, and this amount would be present only in the peak tubes. Each sample was counted at least three times, and the recovery of radioactivity in the 150 to 160 tubes (average number of fractions in a run) was excellent. It is important to count the samples as soon as they come off the column, for any lengthy delay causes the development of a yellow color in the lipid sample and lower counting results.

**Gas-Liquid Chromatography**—The fatty acids of each phosphatide, except sphingomyelin, were converted to their methyl esters and analyzed on a Barber-Coleman model 10 apparatus with a diethylene glycol succinate phase coated on acid-washed Chromosorb W (15.4% by weight, 80 to 100 mesh). The column was maintained at 170°, and the flow rate of argon was 2.5 ml per second. Detection of fatty acid esters were made with a Sr90 cell. Two methods of fatty acid esterification were performed: (a) the method of Stoffol, Chu, and Abraham (17) and (b) esterification with 2% H2SO4 in methanol and refluxing for 30 minutes, followed by extraction with n-hexane. Comparable results were obtained with each method; however, the latter procedure was used routinely because of its simplicity. The machine was standardized daily with authentic methyl esters. Only those results which have a direct bearing on the material in this paper are presented.

**RESULTS**

**Separation of Phosphatides from Normal Liver**—The elution pattern of individual phosphatides is shown in the form of a solid line in Fig. 1. The data shown are from one normal animal but have been confirmed on 10 rats. Six clearly defined fractions can be detected.

Fraction I was identified as rat liver cardiolipin because of its

similarities to authentic beef heart cardiolipin kindly given to us by Dr. M. Pangborn of the New York State Health Department. It has a migration rate identical with the standard cardiolipin on silicic acid-impregnated paper and an ester to phosphate ratio of 1.9:2.1. Analyses of its fatty acid content have indicated slight differences from beef heart cardiolipin. Rat liver cardiolipin consistently contains approximately 65% linoleic acid, 17% oleic acid, 7 to 8% palmitic acid, and small but measurable amounts of myristic, palmitoleic, and stearic acids. Cardiolipin from beef heart is very rich in linoleic acid (85 to 90%) and has a comparable amount of oleic acid (10 to 15%), with only trace amounts of palmitic and linoleic acids. The intact phosphatide from rat liver is ninhydrin negative, and chromatography of the water-soluble hydrolysis products of cardiolipin has failed to show the presence of serine, which was previously reported to be present in this fraction (8).

Fraction II was identified as phosphatidylethanolamine by chromatographic studies on silicic acid paper, hydrolysis of the intact phosphatide, and identification of ethanolamine in the water-soluble products. There was no evidence of phosphatidylserine in this fraction, and the material was always eluted from the column with a sharp symmetrical peak. Phosphatidylethanolamine had an ester to phosphate ratio of 1.9:2.1 and a nitrogen to phosphate ratio of approximately 1. Analyses of the fatty acid content of phosphatidylethanolamine from 10 different animals have yielded the following pattern: stearic, 32 to 39%; arachidonic, 25 to 31%; palmitic, 16 to 20%; docosahexenoic, 12 to 14%; linoleic, 4 to 7%; and oleic, 4 to 7%. The amount of docosahexenoic acid in this fraction is greater than it is in phosphatidylserine and phosphatidylethanolamine, and it is of further interest since this acid is absent in cardiolipin and phosphatidylserine.

Fraction III was identified as phosphatidylinositol by chromatography on silicic acid-impregnated paper with two different solvent systems and hydrolysis of the intact phospholipid, which showed the presence of inositol by the method of Trevelyan, Procter, and Harrison (16). It was free of phosphatidylethanolamine.

1 The acid was identified by comparison with methyl-4,7,10,13,16,19-docosahexenoate which was obtained from the National Institutes of Health.

**FIG. 1** Incorporation of palmitic acid-1-C14 into phosphatides of normal rat liver. Fractions have been identified as follows: I, cardiolipin; II, phosphatidylethanolamine; III, phosphatidylinositol; IV, phosphatidylserine; V, phosphatidylcholine; VI, sphingomyelin.
but the water-soluble hydrolytic products contained small
amounts of serine and only trace amounts of ethanolamine. This
fraction had an ester to phosphate ratio of 1:8:2.1, and the peak
tube containing this component has shown a nitrogen to phos-
phate ratio as low as 0.085. Phosphatidylcholine (43 to 53%), arachidonic acid (35 to 45%), and palmitic acid (4 to 6%), but also contained definite measurable amounts of oleic and linoleic acids. It was void of docosahexaenoic acid.

Fraction IV was identified as phosphatidylserine after exten-
sive modification of the original column chromatographic separa-
tions. The original loading ratio (milligrams of lipid phosphorus
per g of silicic acid) was about 1.0 and did not provide a clean
separation between phosphatidylcholine and Fraction V (phos-
atidylcholine). The original separations also showed the
presence of a ninhydrin-positive material in the latter half of the
phosphatidylcholine fraction. By decreasing the loading ratio
to 0.2 or 0.3, it was possible to resolve Fraction IV. This com-
ponent, phosphatidylserine, had a migration rate on silicic acid
paper identical with that of phosphatidylcholine when the system
of Rouser, Bauman, and Kritchevsky (13) was employed. How-
ever, phosphatidylserine was ninhydrin-positive whereas phos-
atidylcholine was negative. Separation of the two can be
obtained by employing the solvent system of Marinetti (14),
as is shown in a later section. Our location of phosphatidy-
serine between phosphatidylcholine and phosphatidylethanolamine
agreed with the results of Karnovsky and Wallach (18) on leu-
ocyte phospholipids. Hydrolysis of this component and subse-
quent chromatography of the water-soluble products showed
that serine was the major amino acid present, but trace amounts
of ethanolamine could also be detected. Chromatography of
large amounts (100 μg) of the intact phosphatidase did not show
the presence of phosphatidylethanolamine, so that the possibility
existed that the detection of ethanolamine in the hydrolysis
products of this fraction was due either to conversion of serine
to ethanolamine during hydrolysis or to the presence of free
ethanolamine. We are currently studying this component (phosphatidylserine) by the incorporation of L-serine-1-C14, which
labels only this fraction of the phospholipids. The nitrogen
to phosphate ratio of phosphatidylserine was slightly greater than
the expected one (1.3:1.5), but the ester to phosphate ratio was
approximately 2. Analysis of the fatty acid content of phos-
atidylserine gave the following pattern: stearic, 50 to 58%;
linolenic, 4 to 6%; and palmitic, 2 to 3%.

Fraction V was phosphatidylethanolamine (PC), and it was studied
as one major combined fraction as well as in two subdivisions,
which were obtained as follows. The material eluted off the
column after the last tube from Fraction IV, up to and including
the tube at the phosphate peak in Fraction V, was pooled and
designated as PC-1. The remaining material in Fraction V was
then pooled and was referred to as PC-2. The fraction was
divided because of the skewed nature of the phosphate curve
itself as well as the asymmetry of the palmitic acid-1-C14 incor-
poration. This latter point is discussed below. As shown in Table I, there is a definite difference in the fatty acid pattern
of the two subdivisions of Fraction V. Thus there are greater
amounts of palmitic acid in the PC-2 component. The results
indicate that Fraction V contains at least two phosphatidy-
cholines which differ in their acyl groupings.

The subfractions of phosphatidylethanolamine were inseparable on

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>PC-1</th>
<th>PC-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic</td>
<td>6-9</td>
<td>Trace</td>
</tr>
<tr>
<td>Palmitic</td>
<td>49-40</td>
<td>6-8</td>
</tr>
<tr>
<td>Palmitolei</td>
<td>35-53</td>
<td>20-26</td>
</tr>
<tr>
<td>Stearic</td>
<td>4-7</td>
<td>14-17</td>
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<tr>
<td>Oleic</td>
<td>3-5</td>
<td>6-8</td>
</tr>
<tr>
<td>Linolenic</td>
<td>6-8</td>
<td>19-26</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>32-38</td>
<td>7-9</td>
</tr>
<tr>
<td>Docosahexenoic</td>
<td>7-9</td>
<td>1-3</td>
</tr>
</tbody>
</table>

* PC-1 and PC-2 represent subfractions of phosphatidylethanolamine as explained in text.

Fraction VI was tentatively identified as sphingomyelin but
was not confirmed as rigorously as the others. It has a migration
rate on silicic acid paper similar to a sphingomyelin standard
obtained from Nutritional Biochemicals Corporation, and its
chromatographic location corresponded to that reported by other
investigators. It contained choline as a major nitrogen component
and also traces of free ethanolamine and free serine.

Paper Chromatographic Studies of Phosphatides of Normal Liver—The phospholipid material contained in the peak tubes of the various fractions was generally used for these studies, although combined tubes in each fraction gave similar results. The solvent systems of Rouser et al. (13) and Marinetti (14) were used, since a combination of both provided good separation and identification, whereas either one alone was inconclusive. Reproductions of chromatograms obtained with the two systems are shown in Fig. 2, A and B. The chloroform-methanol system
do not hallucinate.

![Fig. 2. Separation of phosphatides on silicic acid-impregnated paper. A, descending chromatography in chloroform-methanol-
ammonium hydroxide (80:20:0.8). Paper, 140 mm X 150 mm;
time, 4 hours at 27°C. B, ascending chromatography in diisobutyl-
ketone-acetic acid-water (40:20:3). Paper, 140 mm X 300 mm;
time, 17 hours at 2°. Black spots indicate ninhydrin-positive;
other spots were obtained with rhodamine 6G stain with ultra-
violet observation. CL, cardiolipin; PE, phosphatidylethanol-
amine; PI, phosphatidylaginositol; PS, phosphatidylserine; PC,
phosphatidylethanolamine.](http://www.jbc.org/Downloaded from http://www.jbc.org/ by guest on August 28, 2017)
The study of Marinetti (Fig. 2B) gave better separation of the phosphatides with the exception of phosphatidylserine, which migrated just behind phosphatidylethanolamine. A combination of the two systems thus allowed for identification of phosphatidylserine and phosphatidylethanolamine.

Separation of Phosphatides from Fatty Liver of Cerium-treated Rats—The elution of individual phosphatides from the hepatic tissue of an animal that had received cerium 36 hours previously is shown in Fig. 3. Incorporation of palmitate-1-Cl4 into those fractions is also shown here. Since this type of fatty liver is due to an increase in triglycerides, there is little change in the level of total liver phospholipids and no consistent change in the level of individual phosphatides. Six clearly defined fractions, corresponding to the phosphatides from normal liver, were observed at 24, 30, and 36 hours after injection of cerium. There was, however, a sharp decrease in phospholipid content of liver in animals 40 hours or more after injection of cerium. This was at a time when massive necrosis of hepatic tissue was taking place and when the mortality rate was high.

Chemical studies on phosphatides from such fatty livers have given comparable results to those obtained on normal livers with the exception of the cardiolipin fraction. This fraction had an

![Graph depicting the incorporation of palmitic acid-1-C14 into phosphatides of fatty liver after cerium injection 36 hours previously.](http://www.jbc.org/)
ester to phosphate ratio of 2 when obtained from normal rats, but the ratio is elevated to 3 to 4 in fatty livers. There is no explanation for this at the present time.

Incorporation of Palmitate-l-14C into Phosphatides of Fatty Liver—The distribution of radioactive palmitate in the phosphatides from a fatty liver is shown as a dotted line in Fig. 3. The pattern of incorporation is dissimilar from that of control animals in a number of respects. The biggest change is the marked reduction in the amount of isotope found in phosphatidylethanolamine. The analytical data for this animal are shown in the lower half of Table II. The decrease in total radioactivity in phosphatidylethanolamine is not due entirely to a lowered uptake by the fatty liver, since the incorporation into PC-2, although decreased, is not as pronounced. It is the relative distribution of the isotope that changed. This is further shown by the increase in total activity in phosphatidylinositol and the sharp rise in its specific activity, whereas the reverse is taking place in the phosphatidylethanolamine fraction. Seven experiments with cerium-treated animals at different time intervals after injection have consistently shown a sharp decline in incorporation into phosphatidylethanolamine. The sharp increase in specific activity in phosphatidylinositol is not as pronounced at 24 hours after cerium injection, and sometimes we find the incorporation of palmitate into cardiolipin greater than into phosphatidylethanolamine in the fatty livers at this time interval. The increased specific activity of phosphatidylserine is believed to be due to contamination with phosphatidylcholine, and the monitoring of radioactivity in this region (Fig. 3) demonstrates a decrease in isotopic content while the level of phosphatidylserine is increasing. The asymmetrical labeling of the phosphatidylcholine region is again due to higher palmitate concentration in the latter half of this fraction.

DISCUSSION

The deposition and removal of triglycerides from liver may be directly related to phospholipid metabolism, and even more specifically to the metabolism of an individual phosphatide. In this study, we have followed the incorporation of palmitic acid into the individual phosphatides under normal conditions and in a state in which the liver is retaining neutral lipid. A short time period (10 minutes) was allowed for the incorporation in order to decrease the conversion of palmitate to other fatty acids. A sharp decrease in the incorporation of palmitic acid into phosphatidylethanolamine of fatty livers was observed at a time when this acid was being incorporated into cardiolipin and phosphatidylcholine at a greater rate than in normal animals. This finding is significant since it illustrates that the metabolism of one phosphatide may be affected adversely while other phosphatides are unaffected or are stimulated to greater metabolic activity. Thus, investigators studying phospholipid metabolism, without analyzing the effects on individual phosphatides, could draw erroneous conclusions. It might also explain the discrepancy that the majority of workers have found decreased phospholipid metabolism in fatty liver (5) whereas Christie and Judah (6), working with isolated mitochondria, reported an increase in phosphatide metabolism. The possibility should also be considered that the metabolism of a given phosphatide in one cell fraction, e.g. mitochondria, might be affected differently than in another part of the cell, e.g. microsomes.

The biosynthetic pathway for cardiolipin and phosphatidyl-ethanolamine involves phosphatidic acid as a direct precursor, whereas phosphatidylcholine is synthesized from a diglyceride and CDP-ethanolamine (24). The greater incorporation of palmitate into the two former phosphatides in the cerium-induced type of fatty liver may indicate that the conversion of phosphatidic acid to the appropriate diglyceride needed for phosphatidyl-ethanolamine synthesis is decreased, or that the synthesis and breakdown of phosphatidylcholine is depressed.

The results with normal animals show that palmitate is rapidly incorporated into all phosphatides with the exception of phosphatidylserine. Phosphatidylethanolamine is rich in palmitic acid, and it rapidly incorporated palmitate. Therefore, it is puzzling that phosphatidylserine lacks radioactive palmitate, whereas phosphatidylcholine is synthesized from phosphatidyl-ethanolamine by the exchange of serine for ethanolamine (19). The difference in radioactive palmitate content may mean that free serine exchanges with a moiety different from the main phosphatidylcholine fraction, despite the fact that a heterogeneity similar to that observed in phosphatidylethanolamine could not be demonstrated for phosphatidylethanolamine. Another possibility is that the phosphatidylserine formed from phosphatidylethanolamine rapidly loses its palmitate in exchange for another fatty acyl group. It should be stressed that the reaction mechanisms by which a phosphatide renews its intramolecular components are not fully known, and the possibility remains that acyl groups may be renewed without the net synthesis of the entire molecule (25).

An interesting finding with regard to the fatty acid composition of the individual phosphatides was the absence of docosahexanoic acid in cardiolipin and phosphatidylcholine. As stated above, these two phosphatides have different biosynthetic pathways from the other phosphatides studied, and the presence of this acid in a phosphatidic acid pool may allow for the selectivity that must be operative in order to differentiate phosphatidic acid molecules differing only in their acyl groups.

SUMMARY

1. Palmitic acid-1-14C was rapidly incorporated into normal rat liver phosphatides, with the exception of phosphatidylserine.
2. Phosphatidylserine was resolved and identified as a separate fraction by silicic acid column chromatography.
3. A pronounced decrease in palmitate incorporation into phosphatidylethanolamine occurred in fatty liver after cerium administration. This decrease occurred at a time when greater palmitate incorporation was observed into phosphatidylcholine and cardiolipin.
Acknowledgment—The authors are grateful to Dr. John A. Muntz for his critical reading of the manuscript.

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Joseph L. Glenn, Edward Opalka and Konrad Tischer


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