# Studies in the biosynthesis of hepatic and biliary lecithins

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ABSTRACT Male rats with biliary cannulae were injected with linoleate-1-$^{14}$C, stearate-1-$^{14}$C, palmitate-9-10-$^{3}$H, phosphate-$^{32}$P, L-methionine-methyl-$^{14}$C, and choline-methyl-$^{3}$H in various combinations and the incorporation of these isotopes into the phospholipids of liver, bile, and plasma was determined for 1–4 hr. The results summarized below favor the view (*a*) that exchange of saturated fatty acids plays a role in the formation of lecithins; (*b*) that the unsaturated fatty acids do not undergo significant exchange and determine the pathway of biosynthesis of lecithins; and (*c*) that there is either more than one pool of CDP-choline in liver or a pathway of biosynthesis of lecithin from choline not involving CDP-choline as an intermediate.

Linoleoyl lecithin of liver attained higher specific activity with respect to phosphate-$^{32}$P and choline-methyl-$^{3}$H than did arachidonoyl lecithin. Lecithin in bile attained higher specific activities with respect to phosphate-$^{32}$P, choline-methyl-$^{3}$H, and linoleate-1-$^{14}$C than the corresponding hepatic lecithins. Stearate-1-$^{14}$C and palmitate-9-10-$^{3}$H attained highest specific activities in the hepatic lecithin fraction rich in arachidonic acid. The specific activity of hepatic phosphatidyl ethanolamine was lower with respect to saturated fatty acids, but much higher with respect to $^{32}$P than any lecithin. The ratio of specific activity of $^{3}$H in methyl groups from choline to $^{14}$C in methyl groups from methionine in hepatic sphingomyelin was lower than in hepatic linoleoyl lecithin.

KEY WORDS lecithin · phosphatidyl ethanolamine · liver · bile · plasma · linoleate · palmitate · stearate · phosphate · choline · methionine · CDP-choline · biosynthesis · transacylation · rat

In a previous study (1) it was shown that the fatty acid pattern of the lecithins of bile differed significantly from that of plasma lecithins in man, dogs, rats, and oxen. In all four species bile lecithin was shown to contain predominantly palmitic and linoleic acids, whereas plasma lecithin contained significantly greater amounts of stearic and arachidonic acids. Studies by other investigators have shown that both biliary and plasma lecithins are derived from the liver (2–5). Two preliminary studies in our laboratory showed that both for $^{32}$P-labeled phosphate and for linoleate-1-$^{14}$C bile lecithin attained higher specific activity than any fraction of hepatic lecithin, whether expressed as dpm/$\mu$g-atom of lipid P or as dpm/$\mu$mole of linoleic acid (6, 7). It seems, therefore, that biliary lecithin is secreted from a separate pool within the liver, or is derived by selection from the heterogeneous hepatic pool. The studies reported here were undertaken to investigate interrelationships in the metabolism of various phospholipids in the liver.

## EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats (350–450 g) were provided with biliary cannulae by a modification of the method of Fisher and Vars (8). The cannulae were stitched in a loop under the skin of the abdominal wall and brought out through a subcutaneous tunnel at the point of the right scapula in such a way as to drain into a plastic bottle sutured to the skin of the rat and protected by a wire mesh. Cannulation was performed during sodium amytal anesthesia. The animals were allowed to recover for 24–36 hr before study, and had free access to food (Wayne Lab. Blox, Allied Mills, Inc., Chicago, Ill.) and water containing 0.5% NaCl and 0.2% KCl.

At zero time for each experiment the radioactive tracer dose was injected into the tail vein during light ether anesthesia. Bile was collected hourly and amounted

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; PE, phosphatidyl ethanolamine; SA, specific activity; CDP, cytidine 5′-diphosphate.

to 0.7–1.3 ml/hr per animal, with approximately 1.5 μmoles of lecithin per ml. The total bile from all the animals (three or four rats) in each group was pooled for each time period. Thus for the 1st hr three pools, for the 2nd hr two pools, and for the 3rd and 4th hr single pools were extracted and analyzed. Blood was obtained by cardiac puncture with a heparinized syringe during ether anesthesia at the time of sacrifice, and plasma samples at any given time point were pooled. The livers were rapidly excised and washed in chilled 0.9% NaCl. Equal portions were taken from each animal and pooled for each time point prior to extraction in a Virtis homogenizer in 20 volumes of chloroform–methanol 2:1 as previously described (1).

Liver lipids were fractionated on silicic acid columns as previously described (9). PE was collected as a single fraction. The phosphatidyl choline (lecithin) peak was eluted in 10-ml fractions and alternate fractions were assayed for lipid P and radioactivity. Liver lecithin was divided into two parts: "lecithin A," the portion eluted up to the lipid P peak; and "lecithin B," the portion eluted after the peak. The purity of all fractions was checked by TLC (10), as well as by phosphorus-to-ester ratio (range obtained, 1.75–2.04), as previously described (1). When TLC revealed any trace of contamination, the compounds were purified by TLC prior to analysis. PE and lecithin from bile and plasma were separated by TLC (10); the spots were made visible with 2′,7′-dichlorofluorescein whenever fatty acid analysis was to be done, and with iodine vapor in preparation for determinations of radioactivity.

For experiment 1, the animals were given linoleate-1-$^{14}$C (4 μc) and $^{32}PO_4$ (310 μc) simultaneously by intravenous injection. In experiment 2, the rats were injected with 4.0 μc (0.1 μmole) of stearate-1-$^{14}$C, 16.0 μc (0.5 μmole) of palmitate-9,10-$^{3}$H, and 280 μc of $^{32}PO_4$ by the same route. In experiment 3, each rat was given 12.5 μc of L-methionine-methyl-$^{14}$C, 100 μc of choline-methyl-$^{3}$H, and 280 μc of $^{32}PO^4$ by intravenous injection. Radioactive phosphate was obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn. All other radioactive materials were purchased from Nuclear-Chicago Corporation, Des Plaines, Ill. The purity of the linoleate-1-$^{14}$C, palmitate-9,10-$^{3}$H, and stearate-1-$^{14}$C was checked by TLC and GLC. More than 98% of the radioactivity was recovered as linoleic, palmitic, and stearic acids, respectively. The radiochemical purity of the choline-methyl-$^{3}$H was reported by the manufacturer as >98% by paper chromatography in three different systems, while that of the L-methionine-methyl-$^{14}$C was >98% by dilution analysis and paper chromatography. These compounds and the phosphate-$^{32}$P were not checked further before use, and were used within 1 month of receipt.

In experiment 1, radioactivity was determined in a Nuclear-Chicago series 720 liquid scintillation spectrometer using Bray's solution (11). Quenching was corrected for by internal standardization. For experiments involving $^{3}$H, $^{14}$C, and $^{32}$P simultaneously (experiments 2 and 3), a Nuclear-Chicago Mk. 1 liquid scintillation spectrometer was set up in the following manner. For each isotope, a solution of a labeled compound was dissolved in toluene solution containing 0.5% diphenyloxazole and 0.03% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 20-ml aliquots were dispensed into glass counting vials. Reference standard *n*-hexadecane was used for $^{3}$H and $^{14}$C. Crude phospholipid prepared from the liver of a rat injected with $^{32}PO_4$ was used as a source of lipid-soluble $^{32}$P and the least quenched sample was arbitrarily assigned an efficiency of 100%. A channel attenuator was adjusted for maximal counts at wide discriminator settings for each isotope. The upper discriminator of the tritium channel was lowered until the count rate of an unquenched tritium sample was reduced by 50%. The lower discriminator on the $^{14}$C channel was raised until the count rate of a 0.1 μc tritium standard was less than 2 cpm. Similarly, the lower discriminator of the $^{32}$P channel was raised to exclude $^{14}$C. Attenuator settings were found which gave maximal count rates for the $^{133}$Ba external standard and an unquenched sample. External standardization was carried out by counting in two channels at this attenuator setting, with discriminator settings at 0.0–3.3 in one channel and 3.3–9.9 in the second. The external standard was printed out as a ratio of these two channels. This was constant for vials of similar content, whereas the total count varied considerably. An increasing quantity of chloroform (25 to 500 μl) was added to a series of 20 vials for each isotope. Each vial was counted at the isotope settings and again at external standard settings, and a set of graphs was prepared giving counting efficiencies for $^{3}$H, $^{14}$C, and $^{32}$P, per cent $^{14}$C counts in the $^{3}$H channel, and per cent $^{32}$P counts in the $^{3}$H and $^{14}$C channels, each plotted against external standard count ratio. By the use of these graphs, the amount of each isotope in a sample could be calculated. We assumed that quenching materials found in these experiments would behave as chloroform, but recognized that quench correction and isotope separation in this manner are valid only when all the isotopes are fully dissolved. Random checking of samples by emptying the contents of sample vials into fresh vials and recounting indicated that all of the materials counted in the experiments reported in this paper were fully dissolved.

The fatty acid methyl esters of the liver, bile, and plasma phosphatidyl choline and of the liver PE were prepared by a modification of the boron trifluoride procedure (12). The lipid was refluxed for 10 min with

14% $BF_3$ in methanol in a boiling water bath and the reaction mixture was extracted with hexane. The 10 min refluxing time yielded 95% esterification when checked by TLC and gave results similar to those obtained by refluxing with 2% $H_2SO_4$ in methanol for 4 hr. Refluxing times of 3, 5, and 10 min with $BF_3$ gave identical fatty acid patterns on subsequent GLC. The methyl esters were analyzed in a F & M model 400 gas chromatograph equipped with a flame ionization detector and a 180 cm glass column (I.D. 0.2 cm) containing 5% diethylene glycol succinate on Diatoport S, at a flow rate of 60 ml of helium per min (inlet pressure 40 psi) at 165° C. The gas chromatograph was originally calibrated and subsequently checked daily with mixture H 105 from The Hormel Institute, Austin, Minn. and agreed with the stated percentages of components to within 3%. Fatty acids were identified by their retention times relative to stearic acid in standard runs, and were quantified by triangulation and calculated as moles per cent.

Aliquots of the rat livers at each time period in experiment 3 were homogenized in 0.24 M sucrose in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 800 *g* for 15 min and the debris discarded. The supernatant fraction was centrifuged at 9000 *g* for 20 min and the mitochondrial fraction was washed with 0.24 M sucrose and recentrifuged at 9000 *g* for 20 min. The supernatant fraction from the first centrifugation at 9000 *g* was centrifuged at 82,500 *g* for 90 min and the new supernatant solution was designated the soluble fraction. The microsomal residue was resuspended once in 0.24 M sucrose and recentrifuged at 82,500 *g* for an additional 90 min. Lipids were extracted as described above, and the lecithin and PE were separated by TLC (10).

Lecithins from bile and whole liver at the various time periods were further fractionated by TLC on 25% silver nitrate-impregnated Adsorbosil (Applied Science Laboratories Inc.) without binder by a modification of the method of Arvidson (13), with methanol–chloroform–water 65:25:2 as solvent. The three detectable bands were eluted and assayed for radioactivity, lipid P, and fatty acid composition. These bands were found to be predominantly linoleoyl, arachidonoyl, and probably docosahexaenoyl lecithins.

## RESULTS

### *Fatty Acid Patterns*

The fatty acid composition of lecithin and PE remained essentially unchanged from one experiment to another (Table 1), so that the interpretation of the studies is not complicated by alterations in the fatty acid compositions. No antioxidants were used in these studies and some loss

TABLE 1 FATTY ACID COMPOSITION OF VARIOUS PHOSPHOLIPIDS IN THE THREE EXPERIMENTS

| | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 20:4 | Other |
|---|---|---|---|---|---|---|---|
| | | | | *mole % of total fatty acids* | | | |
| Liver lecithin A | 21.5 (19.7–22.4) | 0.9 (0.0–2.6) | 30.1 (28.5–32.1) | 5.3 (5.0–5.5) | 11.7 (9.1–14.0) | 28.6 (27.3–29.7) | 0.5 |
| Liver lecithin B | 29.9 (27.9–31.4) | 0.4 (0.0–1.2) | 23.8 (22.6–25.2) | 6.9 (6.1–7.7) | 18.9 (17.3–21.0) | 20.9 (19.9–22.4) | 0.5 |
| Bile lecithin | 43.0 (39.5–45.6) | 1.2 (0.0–3.5) | 8.3 (6.6–11.8) | 6.3 (5.9–6.9) | 30.8 (28.9–31.7) | 9.7 (8.8–10.6) | 3.1 |
| Plasma lecithin | 27.8 (27.3–28.3) | 0.9 (0.0–1.9) | 25.6 (23.4–26.7) | 8.3 (6.4–10.2) | 19.8 (19.6–20.1) | 18.0 (16.1–19.9) | 0 |
| Liver PE | 23.4 (22.7–23.7) | 0.9 (0.0–2.5) | 32.6 (30.5–34.6) | 6.0 (5.6–6.6) | 10.9 (10.1–11.4) | 25.8 (24.9–27.4) | 1.4 |
| *Silver nitrate fractions (experiment 3 only)* | | | | | | | |
| Liver lecithins | | | | | | | |
| Dienoic | 33.9 (33.4–34.4) | 0 | 21.9 (20.3–23.3) | 8.2 (7.5–9.0) | 33.8 (34.3–37.0) | 0 | 0 |
| Tetraenoic | 25.4 (24.6–26.3) | 0 | 29.5 (25.9–34.7) | 4.2 (3.7–4.7) | 5.1 (4.6–5.7) | 37.1 (36.6–37.6) | tr. |
| >Tetraenoic | 33.2 (29.4–37.0) | 0 | 29.2 (26.6–31.8) | 7.5 (6.3–8.6) | 6.1 (5.9–6.3) | 13.9 (10.9–17.8) | 10.4 (8.9–11.2) |
| Bile lecithins | | | | | | | |
| Dienoic | 41.3 (36.2–46.4) | 0 | 7.1 (6.6–7.7) | 6.7 (6.5–9.0) | 37.3 (34.3–40.3) | 1.3 (0.0–2.6) | 0 |
| Tetraenoic | 39.0 (32.4–45.0) | 0 | 9.5 (7.9–11.1) | 5.4 (4.1–5.8) | 13.3 (11.3–15.2) | 28.6 (26.4–30.8) | 0 |

The figures represent the mean and range of a minimum of six determinations, at least two from each of the three experiments. Silver nitrate fractionation was done in experiment 3 only; the data here represent the means and ranges of at least six determinations.

Fatty acids are designated by number of carbon atoms: number of double bonds. Lecithin A is that portion of total liver lecithin eluted from a silicic acid column up to the lipid P peak; lecithin B is the portion eluted thereafter.

TABLE 2 SPECIFIC ACTIVITIES OF PHOSPHOLIPIDS AFTER I.V. INJECTION OF LINOLEATE-1-$^{14}$C (4.0 $\mu$C) AND PHOSPHATE-$^{32}$P (310 $\mu$C)

| Time | Liver Lecithin A | | Liver Lecithin B | | Bile Lecithin | | Plasma Lecithin | | Liver PE | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $^{14}$C | $^{32}$P | $^{14}$C | $^{32}$P | $^{14}$C | $^{32}$P | $^{14}$C | $^{32}$P | $^{14}$C | $^{32}$P |
| *min* | | | | | | | | | | |
| 60 | 995 | 2,490 | 1,520 | 3,370 | 2,230 (3) | 320 | 980 | 510 | 790 | 25,530 |
| | 4,250 | — | 4,020 | — | 3,600 (3) | — | 2,500 | — | 3,600 | — |
| 120 | 1,060 | 4,240 | 2,110 | 5,760 | 4,760 (2) | 4,890 | 1,390 | 1,580 | 1,560 | 35,350 |
| | 4,520 | — | 5,590 | — | 7,750 (2) | — | 3,550 | — | 7,140 | — |
| 180 | — | — | — | — | 4,050 | 9,560 | — | — | — | — |
| | — | — | — | — | 6,590 | — | — | — | — | — |
| 240 | 1,010 | 11,070 | 1,490 | 14,120 | 3,650 | 16,190 | 2,130 | 10,200 | 430 | 43,140 |
| | 4,310 | — | 3,940 | — | 5,940 | — | 5,430 | — | 1,970 | — |

Results are from samples pooled from a minimum of three rats. Where more than one pool was used the number of pools is shown in parentheses. For each time period, the upper line of numbers is dpm per $\mu$g-atom of lipid P; the lower, dpm per $\mu$mole of linoleate. Liver lecithin A and B are as defined in Table 1.

of polyunsaturated fatty acids may therefore have occurred. However, the resultant error, if present, was apparently constant throughout the studies, and the data obtained are in good agreement with the results of McFarlane, Gray, and Wheeldon (14) and of Glenn, Opalka, and Tischer (15).

*Experiment 1.* Because of the different time course of incorporation of linoleic acid and phosphate into bile and liver lecithin observed in two preliminary experiments, linoleate-1-$^{14}$C and phosphate-$^{32}$P were injected simultaneously (Table 2). Bile lecithin SA (as dpm/$\mu$g atom lipid P) for linoleate-1-$^{14}$C rose to a peak at 2 hr and then declined. The SA of bile lecithin-$^{14}$C reached a value approximately twice that seen in any liver lecithin fraction. Calculation of the SA of linoleate-1-$^{14}$C based on the linoleic acid content of bile lecithin and liver lecithin B at peak time showed the former to be 7750 while the latter was 5590 dpm/$\mu$mole of linoleic acid, a ratio of 1:4:1. The SA of plasma lecithin linoleate-1-$^{14}$C was still rising slowly at 4 hr, when it was appreciably lower than that of bile lecithin but similar to that of liver lecithin B. In similar preliminary experiments linoleate-1-$^{14}$C incorporation into hepatic lecithin was measured at 15 and 30 min as well as at the time intervals reported here, and the SA at 15 and 30 min was shown to be lower than at 1 and 2 hr.

The SA of $^{32}$P followed a different time course from that of linoleate-1-$^{14}$C: it rose more slowly and was still increasing at 4 hr in all fractions. The SA of liver lecithin-$^{32}$P exceeded that of bile lecithin at 1 and 2 hr, but by 4 hr, bile lecithin was 1.1 times as active as liver lecithin B (whose SA exceeded that of liver lecithin A). Liver PE showed much greater incorporation of $^{32}$P than any lecithin fraction. At no time was sufficient PE found in bile for significant counts to be obtained for either $^{32}$P or $^{14}$C.

Bile neutral lipids were separated on TLC and the areas of adsorbent corresponding to partial glycerides and free fatty acids were taken for counting. Only trace amounts of material were obtained and these did not contain significant radioactivity (<twice background).

*Experiment 2.* The data presented in Table 3 show that after injection of labeled stearate and palmitate, the highest SA of bile lecithin, whether calculated as dpm/$\mu$g-atom of lipid-P or dpm/$\mu$mole of palmitic or stearic acid, was lower than that of hepatic lecithin A or B. This is in sharp contrast to the pattern of incorporation of linoleate in experiment 1.

It is also of note that the ratio of SA of either fatty acid (dpm/$\mu$mole of fatty acid) to that of $^{32}$P (dpm/$\mu$g-atom lipid P) in liver PE was lower than the same ratio in bile or plasma lecithin throughout the period of study. If incorporation of $^{32}$P is a measure of de novo phospholipid synthesis, then the ratio of fatty acid SA to $^{32}$P SA should be the same in all phospholipids unless fatty acids are incorporated into lecithin by a pathway other than by way of phosphatidic acid, e.g., by transacylation. Furthermore, in liver lecithin A and B and in bile lecithin the SA (dpm/$\mu$mole) of stearic acid was higher than that of palmitic acid, whereas the reverse was true in the liver PE fraction. Such inequalities of distribution of fatty acid radioactivity are consistent with the view that transacylation is important in determining the final fatty acid composition of phospholipids. That this difference was not due to variation in groups of animals is shown by the fact that in this experiment, as in the previous one (Table 2), the SA of bile lecithin in terms of $^{32}$P was greater than that of liver lecithin B by a factor of 1.1 at 4 hr, and the SA of liver lecithin B at 2 and 4 hr was greater than that of liver lecithin A. Liver PE again showed far higher SA for $^{32}$P than any lecithin fraction studied.

Liver lecithin and triglyceride fatty acids were each fractionated by GLC. Less than 1% of $^{3}$H was found in fatty acids other than palmitic acid. Less than 2% of $^{14}$C activity was present in fatty acids other than stearic acid.

TABLE 3 SPECIFIC ACTIVITIES OF VARIOUS PHOSPHOLIPIDS AFTER SIMULTANEOUS I.V. INJECTION OF PALMITATE-9,10-$^{3}$H (16 μc), STEARATE-1-$^{14}$C (4.0 μc), AND PHOSPHATE-$^{32}$P (280 μc)

| | | Time in Min | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 60 | | 120 | | 180 | | 240 | |
| Liver lecithin A | $^{3}$H | 8,520 | 19,800 | 3,300 | 7,650 | — | — | 3,370 | 7,850 |
| | $^{14}$C | 17,190 | 28,560 | 6,900 | 11,460 | — | — | 6,790 | 11,280 |
| | $^{32}$P | 4,070 | — | 5,270 | — | — | — | 14,030 | — |
| Liver lecithin B | $^{3}$H | 8,960 | 14,950 | 4,370 | 7,310 | — | — | 4,860 | 8,130 |
| | $^{14}$C | 10,320 | 21,680 | 5,700 | 11,980 | — | — | 5,040 | 10,590 |
| | $^{32}$P | 3,600 | — | 6,300 | — | — | — | 17,130 | — |
| Bile lecithin | $^{3}$H | 4,650 (3) | 5,420 | 9,010 (2) | 10,450 | 9,300 | 10,800 | 8,530 | 9,930 |
| | $^{14}$C | 1,640 (3) | 9,880 | 3,040 (2) | 18,310 | 3,140 | 18,920 | 1,680 | 10,120 |
| | $^{32}$P | 1,330 (3) | — | 5,800 (2) | — | 10,080 | — | 18,680 | — |
| Plasma lecithin | $^{3}$H | 2,370 | 4,260 | 3,670 | 6,640 | — | — | 4,390 | 7,950 |
| | $^{14}$C | 2,380 | 4,650 | 3,760 | 7,340 | — | — | 5,380 | 10,510 |
| | $^{32}$P | 370 | — | 1,510 | — | — | — | 3,700 | — |
| Liver PE | $^{3}$H | 5,630 | 12,100 | 5,060 | 10,850 | — | — | 4,640 | 9,940 |
| | $^{14}$C | 7,110 | 10,910 | 4,980 | 7,640 | — | — | 4,820 | 7,390 |
| | $^{32}$P | 22,840 | — | 31,970 | — | — | — | 38,370 | — |

Results are from samples pooled from a minimum of three rats. Where more than one pool was used, the number of pools is shown in parentheses. For each time period, the first column of data is expressed in dpm per μg-atom of lipid P; the second, dpm $^{3}$H per μmole of palmitate or dpm $^{14}$C per μmole of stearate. Liver lecithin A and B are as defined in Table 1.

TABLE 4 SPECIFIC ACTIVITIES OF VARIOUS PHOSPHOLIPIDS AFTER THE SIMULTANEOUS I.V. INJECTION OF CHOLINE-METHYL-$^{3}$H (100 μc), L-METHIONINE-METHYL-$^{14}$C (12.5 μc), AND PHOSPHATE-$^{32}$P (280 μc)

| | | Time in Min | | | | |
|---|---|---|---|---|---|---|
| | | 15 | 60 | 120 | 180 | 240 |
| | | *dpm per μg-atom of lipid P* | | | | |
| Liver lecithin A | $^{3}$H | 2,340 | 19,100 | 30,540 | — | 54,680 |
| | $^{14}$C | 16,630 | 26,400 | 24,280 | — | 24,670 |
| | $^{32}$P | 180 | 1,580 | 4,330 | — | 11,640 |
| Liver lecithin B | $^{3}$H | 3,010 | 22,430 | 38,010 | — | 62,180 |
| | $^{14}$C | 14,475 | 18,880 | 21,450 | — | 20,620 |
| | $^{32}$P | 145 | 1,580 | 5,700 | — | 13,390 |
| Bile lecithin | $^{3}$H | — | 15,320 (3) | 57,500 (2) | 98,160 | 102,680 |
| | $^{14}$C | — | 11,440 (3) | 17,110 (2) | 16,870 | 17,780 |
| | $^{32}$P | — | 710 (3) | 5,970 (2) | 14,010 | 19,320 |
| Plasma lecithin | $^{3}$H | 430 | 5,870 | 21,890 | — | 47,800 |
| | $^{14}$C | 390 | 12,140 | 19,040 | — | 22,170 |
| | $^{32}$P | 80 | 290 | 2,100 | — | 7,650 |
| Liver PE | $^{3}$H | 0 | 0 | 0 | — | 0 |
| | $^{14}$C | 0 | 0 | 0 | — | 0 |
| | $^{32}$P | 6,640 | 19,840 | 29,640 | — | 37,240 |
| Liver sphingomyelin | $^{3}$H | 280 | 2,000 | 4,490 | — | 14,940 |
| | $^{14}$C | 370 | 1,590 | 2,870 | — | 6,540 |
| | $^{32}$P | 50 | 130 | 330 | — | 1,580 |

Results are from samples pooled from a minimum of three rats. Where more than one pool was used, the number of pools is shown in parentheses. Liver lecithin A and B are as defined in Table 1.

*Experiment 3.* The results of this experiment are presented in Tables 4–6. As in previous experiments, the SA of phosphate-$^{32}$P in liver PE rose more rapidly than in any lecithin fraction. Bile lecithin SA for $^{32}$P exceeded liver lecithin B SA by a factor of 1.4 at 4 hr. Liver lecithin A again had a lower SA for $^{32}$P than did liver lecithin B at 2 and 4 hr (Table 4).

Methyl-$^{14}$C derived from methionine attained its highest SA (dpm/μg-atom lipid P) in liver lecithin A, with somewhat lower values in liver lecithin B and in plasma and bile lecithin. Significant incorporation of this label into liver sphingomyelin occurred. By contrast, choline-methyl-$^{3}$H was incorporated preferentially into bile lecithin; indeed after the 1st hr, bile lecithin tritium SA exceeded that of liver lecithin B by a factor of 1.5–1.65. As expected, choline-methyl-$^{3}$H was incorporated readily into sphingomyelin, but neither $^{14}$C nor $^{3}$H was found in hepatic PE.

Table 5 gives the corresponding data for the lecithin and PE of the subcellular fractions of the liver. The lecithin and PE of the microsomal fraction attained higher SA than those of the same phospholipids in the soluble

TABLE 5 SPECIFIC ACTIVITIES OF PHOSPHATIDYL CHOLINE AND PHOSPHATIDYL ETHANOLAMINE FROM SUBCELLULAR FRACTIONS OF LIVER

| | | Time in Min | | | |
|---|---|---|---|---|---|
| | | 15 | 60 | 120 | 240 |
| | | *dpm per μg-atom lipid P* | | | |
| Phosphatidyl choline | | | | | |
| Soluble fraction | $^{3}H$ | 3,140 | 20,300 | 22,540 | 21,730 |
| | $^{14}C$ | 10,910 | 20,300 | 16,540 | 8,900 |
| | $^{32}P$ | 265 | 1,270 | 5,200 | 6,000 |
| Mitochondrial | $^{3}H$ | 4,650 | 17,540 | 27,170 | 47,730 |
| | $^{14}C$ | 7,740 | 18,370 | 18,500 | 19,470 |
| | $^{32}P$ | 70 | 1,300 | 5,020 | 11,770 |
| Microsomal | $^{3}H$ | 3,330 | 23,050 | 33,240 | 57,870 |
| | $^{14}C$ | 17,130 | 25,620 | 23,000 | 21,960 |
| | $^{32}P$ | 640 | 2,630 | 6,260 | 14,270 |
| Phosphatidyl ethanolamine | | | | | |
| Soluble fraction | $^{32}P$ | 2,170 | 6,350 | 9,260 | 11,820 |
| Mitochondrial | $^{32}P$ | 3,190 | 10,440 | 21,920 | 29,640 |
| Microsomal | $^{32}P$ | 11,340 | 29,030 | 42,040 | 54,790 |

Rats were given simultaneous I.V. injections of choline-methyl-$^{3}H$ (100 μc), L-methionine-methyl-$^{14}C$ (12.5 μc), and phosphate-$^{32}P$ (280 μc).

TABLE 6 SPECIFIC ACTIVITIES OF THE LECITHINS OF LIVER AND BILE AFTER THE SIMULTANEOUS I.V. INJECTION OF CHOLINE-METHYL-$^{3}H$ (100 μc), L-METHIONINE-METHYL-$^{14}C$ (12.5 μc) AND PHOSPHATE-$^{32}P$ (280 μc)

| | Time in Min | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 15 | | | 60 | | | 120 | | | 240 | | |
| | $^{3}H$ | $^{14}C$ | $^{32}P$ | $^{3}H$ | $^{14}C$ | $^{32}P$ | $^{3}H$ | $^{14}C$ | $^{32}P$ | $^{3}H$ | $^{14}C$ | $^{32}P$ |
| | *dpm per μg-atom lipid P* | | | | | | | | | | | |
| Liver lecithins | | | | | | | | | | | | |
| Total | 3,010 | 14,475 | 145 | 22,430 | 18,880 | 1,580 | 30,540 | 24,280 | 4,330 | 62,180 | 20,620 | 13,390 |
| Dienoic | 5,100 | 8,720 | 290 | 30,820 | 18,540 | 2,280 | 42,130 | 16,560 | 6,710 | 73,190 | 15,870 | 15,690 |
| Tetraenoic | 2,910 | 11,460 | 130 | 14,550 | 22,010 | 760 | 24,000 | 22,880 | 2,560 | 39,400 | 18,350 | 6,400 |
| >Tetraenoic | — | — | — | 25,530 | 36,000 | 2,610 | 41,590 | 41,990 | 6,740 | 56,230 | 30,530 | 17,790 |
| Bile lecithins | | | | | | | | | | | | |
| Total | — | — | — | 17,980 | 12,860 | 680 | 47,750 | 20,740 | 5,600 | 98,180 | 19,970 | 16,130 |
| Dienoic | — | — | — | 17,900 | 9,940 | 670 | 41,520 | 13,590 | 5,100 | 107,940 | 14,930 | 18,140 |
| Tetraenoic | — | — | — | 12,630 | 17,920 | 650 | 21,700 | 8,800 | 2,200 | 51,860 | 19,540 | 7,750 |

The lecithins were fractionated by TLC using silver nitrate impregnated Silica Gel G. The fatty acid composition of the various fractions is shown in Table 1. For these studies all samples of bile at 60 min and both samples at 120 min were pooled prior to analysis. The data for bile at "240" min are based on the pooled samples obtained at 180 and 240 min.

Liver lecithin values refer to liver lecithin B at 15, 60, and 240 min and to liver lecithin A at 120 min. Liver lecithin A and B are as defined in Table 1.

and mitochondrial fractions. Even though the SA of the microsomal lecithin was higher for all three isotopes than that of the other subcellular fractions, it did not approach that of bile lecithin in respect of $^{32}P$ and choline-methyl-$^{3}H$.

The results of silver nitrate fractionation of the lecithins of bile and liver are shown in Table 6. In both tissues the SA of $^{32}P$ in linoleoyl lecithin was higher than that in arachidonoyl lecithin at all times, and significantly so after the 1st hr. In liver lecithin, the linoleoyl type is predominantly labeled with choline-methyl-$^{3}H$ at all times except at 15 min, whereas the arachidonoyl type has a relatively higher SA in respect of methyl-$^{14}C$ derived from methionine. However, by 120 min choline-methyl-$^{3}H$ label exceeded the methyl-$^{14}C$ derived from methionine, even in this fraction. This contrast between the linoleoyl and arachidonoyl types of lecithin is even more marked in bile lecithin. The highly unsaturated fraction (>tetraene) was not detectable in bile lecithin, but was found as a minor component in liver lecithin. This fraction had an SA for $^{32}P$ similar to that of the linoleoyl type.

## DISCUSSION

Investigations by previous workers using radioactive-tracer techniques have demonstrated that plasma lecithins are derived from hepatic lecithins (2–4). The evidence for the hepatic origin of bile lecithins is, however, less direct (5). Nevertheless, on a priori grounds, biliary lecithin is likely to be derived from the heterogeneous hepatic pool (5, 15–17). The possibility that biliary lecithins are secreted by the bile duct epithelium

cannot be ruled out, but seems very unlikely in view of the observations of Bouchier and Cooperband (18) in two patients with biliary obstruction, which showed that the resultant "white bile," which contained no bilirubin, was also totally devoid of phospholipids.

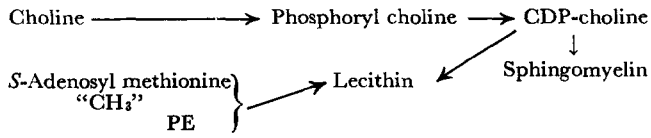
The studies reported here confirm the heterogeneity of hepatic lecithins reported by others (5, 15–17, 19). Structural heterogeneity is demonstrated by the results of silver nitrate TLC (Table 1) and metabolic heterogeneity by the greater incorporation of phosphate-$^{32}$P and choline-methyl-$^{3}$H by the linoleoyl lecithin than by the arachidonoyl type (Table 6). Collins (17) reported a similarly greater incorporation of radioactive phosphate into the fraction corresponding to our lecithin B. Isozaki, Yamamoto, Amako, Sakai, and Okita (19) showed that the fraction corresponding to our liver lecithin A was labeled to a greater extent than that corresponding to our lecithin B after the intramuscular injections of L-methionine-methyl-$^{14}$C, whereas $^{32}PO_4$ labeling was greater in the fraction corresponding to our lecithin B. The data obtained in experiment 3 confirm these findings and extend them in that they show that choline is preferentially incorporated into linoleoyl lecithin, whereas methylation of PE preferentially leads to arachidonoyl lecithin. This latter observation is not surprising in view of the high content of arachidonic acid in PE. Our results differ from those of Isozaki et al. (19) in that these authors found relatively low incorporation of $^{32}PO_4$ into PE as compared to lecithin; in the present studies (Tables 2–4) and those of Ulsamer and Glenn (20), the reverse was true.

Linoleate-1-$^{14}$C attained a higher SA in bile lecithin than in any liver phospholipid (Table 2). The linoleoyl lecithin of bile also had higher SA for $^{32}PO_4$ and choline-methyl-$^{3}$H than the corresponding liver lecithin (Table 6). This indicates that the linoleoyl lecithin destined for bile is not in equilibrium with liver linoleoyl lecithin; therefore, either it is derived from a separate pool or the preexisting hepatic linoleoyl lecithin does not mix rapidly with freshly synthesized lecithin.

Van den Bosch and Van Deenen (21) demonstrated the presence of an enzyme in rat liver homogenates which cleaves the fatty acid in the 1-position of lecithin. Lands (22, 23) has shown that microsomal preparations from rat, guinea pig, bovine, and pig liver contain enzymes capable of acylating monoacyl glycerophosphoryl choline. The 1-position was preferentially acylated with saturated fatty acids and the 2-position with unsaturated fatty acids. If diglyceride or phosphatidic acid were the sole precursor of phospholipid fatty acids, the ratio of SA of fatty acids in bile lecithin to that in hepatic lecithin would not vary from one type of fatty acid to another. However, the peak SA of bile lecithin with respect to linoleic acid was higher (Table 2), while those for palmitic and stearic acids were lower (Table 3) than the corresponding highest values for hepatic lecithins. Therefore, the incorporation of fatty acids into lecithins occurs by additional routes. The low SA of fatty acids in PE, especially in relation to the $^{32}$P, indicates that methylation of PE does not make a significant contribution of radioactive fatty acids to the lecithins. Accordingly, it is thought that the inequalities in distribution of radioactive fatty acids between the various lecithins was brought about by exchange of the fatty acids. However, it is not possible, from experiments 1 and 2, to say whether this occurs at position 1 or at position 2. The great difference between arachidonoyl lecithin and linoleoyl lecithin in incorporation of methyl groups from methionine and from choline (Table 6) suggests that linoleic acid is not exchanged for arachidonic acid or vice versa. Collins (17) considered that it was the saturated fatty acids that determined the rate of incorporation of phosphate-$^{32}$P—that is, that palmitoyl lecithin turned over faster than the stearoyl type. Our data on the other hand indicate that the unsaturated fatty acid at the 2-position (24) determines the pathway of biosynthesis of lecithins and that the saturated fatty acids at position 1 undergo acyl exchange. This view is consistent with the data of Collins (17), since linoleoyl lecithin is largely also palmitoyl lecithin and arachidonoyl lecithin is largely also stearoyl lecithin.

The experiments using $^{32}PO_4$ have consistently shown a markedly higher SA in PE than in any lecithin. This, together with the higher SA of stearate in lecithin A than in PE, can be explained either by the rapid transacylation of the saturated fatty acid coincident with the conversion of PE to lecithin, or on the assumption that the conversion of PE to lecithin makes a minor contribution to the formation of arachidonoyl lecithin. Our data do not allow us to differentiate between these two alternatives, but favor the former.

SCHEME I




Scheme I represents the currently accepted pathways of biosynthesis of lecithin and sphingomyelin (25). Bjørnstad and Bremer (26) interpreted their results in the light of scheme I as indicating a rapid equilibrium between CDP-choline and lecithin, irrespective of whether the latter is derived via CDP-choline or PE. However, such an equilibrium is not consistent with the data obtained in this study or those of others (17, 19), which show marked differences in the relative rates of incorporation of phosphate, choline, and methyl groups from methionine into linoleoyl and arachidonoyl lecithins, as such an equilibrium would eliminate these differences. Our

TABLE 7 RATIOS OF SPECIFIC ACTIVITIES OF CHOLINE-METHYL-$^{3}H$ AND L-METHIONINE-METHYL-$^{14}C$ INCORPORATED INTO CHOLINE CONTAINING PHOSPHOLIPIDS OF LIVER AND BILE AT VARIOUS TIME INTERVALS

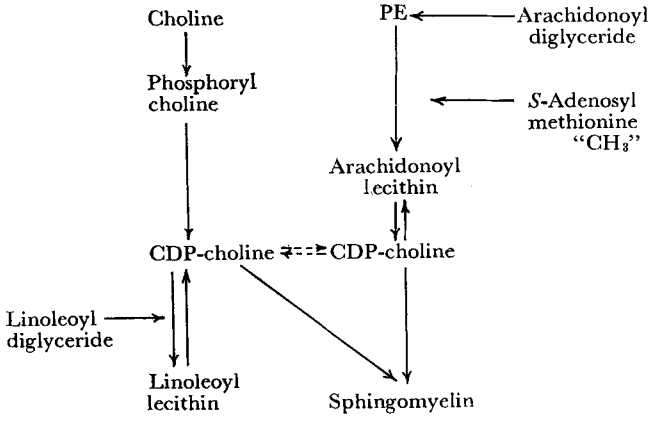
| | Time in Min | | | |
|---|---|---|---|---|
| | 15 | 60 | 120 | 240 |
| | $^{3}H/^{14}C$ | | | |
| Liver lecithins | | | | |
| Dienoic | 0.59 | 1.66 | 2.54 | 4.61 |
| Tetraenoic | 0.25 | 0.66 | 1.05 | 2.15 |
| >Tetraenoic | — | 0.71 | 0.99 | 1.84 |
| Liver sphingomyelin | 0.76 | 1.26 | 1.56 | 2.28 |
| Bile lecithins | | | | |
| Dienoic | — | 1.80 | 3.05 | 7.23 |
| Tetraenoic | — | 0.70 | 2.47 | 2.65 |

The lecithins were separated by TLC using silver nitrate impregnated Silica Gel G.

data in fact show a progressive divergence with time between the ratio of SA of choline-methyl-$^{3}H$ to methyl-$^{14}C$ derived from methionine in linoleoyl lecithin, which rose from 0.59 at 15 min to 4.61 at 4 hr, and the corresponding ratio in arachidonoyl lecithin, where it rose from 0.25 to 2.15 in the same period (Table 7). Therefore, there cannot be a rapid equilibrium between one CDP-choline pool and the hepatic lecithins.

On the basis of scheme I, the ratio of SA of methyl groups from choline to those from methionine in hepatic sphingomyelin should never be lower than in any hepatic lecithin fraction. In fact the results of experiment 3 (Table 7) show that, except at 15 min, this ratio in linoleoyl lecithin is increasingly greater than in sphingomyelin. These observations indicate that choline is either incorporated into lecithin by a pathway independent of CDP-choline, or that there may be more than one CDP-choline pool in the liver. These and the preceding observations would be consistent with the following scheme. The results obtained for bile lecithin, showing higher SA for linoleate (Table 2), choline (Table 4), and phosphate (Tables 2–4) than in hepatic lecithins suggest, however, that the situation may be even more complex. Reasons for believing that linoleoyl lecithin destined for bile may be formed in a separate pool have already been discussed.

SCHEME II




The inclusion of phosphate-$^{32}P$ in each experiment served to demonstrate the reproducibility of patterns of incorporation from experiment to experiment. Since each lipid sample was derived from a group of three or four rats, effects of animal to animal variation were reduced. Therefore, it is felt that the observations recorded are significant.

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## REFERENCES

1. Balint, J. A., E. C. Kyriakides, H. L. Spitzer, and E. S. Morrison. 1965. *J. Lipid Res.* **6:** 96.
2. Fischler, M. D., C. Entenman, M. L. Montgomery, and I. L. Chaikoff. 1943. *J. Biol. Chem.* **150:** 47.
3. Popják, G., and H. Muir. 1950. *Biochem. J.* **46:** 103.
4. Borgström, B., and T. Olivecrona. 1961. *J. Lipid Res.* **2:** 263.
5. Zilversmit, D. B., and E. Van Handel. 1958. *Arch. Biochem. Biophys.* **73:** 224.
6. Balint, J. A., M. Savage, H. L. Spitzer, and E. C. Kyriakides. 1965. *Federation Proc.* **24:** 343.
7. Balint, J. A., D. A. Beeler, and H. L. Spitzer. 1966. *Federation Proc.* **25:** 210.
8. Fisher, B., and H. M. Vars. 1951. *Am. J. Med.* **222:** 116.
9. Balint, J. A., W. L. Nyhan, P. Lietman, and D. A. Turner. 1961. *J. Lab. Clin. Med.* **58:** 548.
10. Skipski, V. P., R. F. Peterson, J. Sanders, and M. Barclay. 1963. *J. Lipid Res.* **4:** 227.
11. Bray, G. A. 1960. *Anal. Biochem.* **1:** 279.
12. Metcalf, L. D., and A. Schmitz. 1961. *Anal. Chem.* **33:** 363.
13. Arvidson, G. A. E. 1965. *J. Lipid Res.* **6:** 574.
14. McFarlane, M. G., G. M. Gray, and L. W. Wheeldon. 1960. *Biochem. J.* **77:** 626.
15. Glenn, J. L., E. Opalka, and K. J. Tischer. 1963. *J. Biol. Chem.* **238:** 1249.
16. Harris, P. M., D. S. Robinson, and G. Getz. 1960. *Nature.* **138:** 742.
17. Collins, F. D. 1963. *Biochem. J.* **88:** 319.
18. Bouchier, I. A. D., and S. R. Cooperband. 1965. *Gastroenterology.* **49:** 354.
19. Isozaki, M., A. Yamamoto, T. Amako, Y. Sakai, and H. Okita. 1962. *Med. J. Osaka Univ.* **12:** 285.
20. Ulsamer, A. G., and J. L. Glenn. 1966. *Biochim. Biophys. Acta.* **125:** 525.
21. Van den Bosch, H., and L. L. M. Van Deenen. 1965. *Biochim. Biophys. Acta.* **106:** 326.
22. Lands, W. E. M., and P. Hart. 1965. *J. Biol. Chem.* **240:** 1905.
23. Lands, W. E. M. 1965. *J. Am. Oil Chemists' Soc.* **42:** 465.
24. Menzel, D. B., and M. S. Olcott. 1964. *Biochim. Biophys. Acta.* **84:** 133.
25. Kennedy, E. P. 1961. *Federation Proc.* **20:** 934.
26. Bjørnstad, P., and J. Bremer. 1966. *J. Lipid Res.* **7:** 38.