The Enzymatic Conversion of Phospholipid Ethanolamine to Phospholipid Choline in Rat Liver

KENNETH D. GIBSON,* JEAN D. WILSON,† AND SIDNEY UDENFRIEND

From the Laboratory of Clinical Biochemistry, National Heart Institute, National Institutes of Health, United States Public Health Service, Bethesda 14, Maryland

It is known from the work of Elwyn et al. (1) that ethanolamine is incorporated as a unit into the choline molecule during the synthesis de novo of this substance. Du Vigneaud et al. (2, 3) showed that at least some of the methyl groups of choline are derived directly from the methyl group of methionine; and in an earlier communication we provided evidence that all the methyl groups originate from this source (4). Recently, as a result of studies with rat liver in vivo (5) and in vitro (6, 7), Bremer and Greenberg proposed that choline is formed by the stepwise transfer of three methyl groups from S-adenosyl methionine to phosphatidyl ethanolamine. The work reported here essentially confirms their findings and offers some further support for the reactions which they formulated.

EXPERIMENTAL PROCEDURE

Preparation of Subcellular Fractions—All operations were performed in a cold room at 3°. Male Sprague-Dawley rats, weighing 100 to 150 g, were killed by decapitation. The livers were excised, cooled in ice, and homogenized with 6 to 9 volumes of 0.25 M sucrose for 2 minutes in a Waring Blender. The homogenate was centrifuged for 10 minutes at 600 X g, and the precipitate was discarded. Mitochondria and microsomes were prepared from the supernatant fluid by differential centrifugation (8). When microsomes alone were required, the crude homogenate was first centrifuged for 10 minutes at 10,000 X g to remove mitochondria and larger fragments; microsomes were then collected from the supernatant fluid either by centrifugation for 1 hour at 75,000 X g, or by centrifugation for 30 minutes at 25,000 X g after the pH had been adjusted to 5.4 to 5.6 by dropwise addition of 1 N acetic acid. Microsomes prepared by either method had about the same activity in the reactions studied here. The particles were suspended in a volume of 0.25 M sucrose equal to the volume of liver from which they were isolated. They could be stored at -20°, at which temperature they were stable for 1 to 2 weeks.

Extraction of Phospholipids—Phospholipids were extracted from incubation mixtures either by Artom's method (9) as described previously (4), or else with chloroform and methanol according to Bligh and Dyer (10). In the latter case, the incubation mixture (1 volume) was shaken vigorously for 2 minutes with 1.25 volumes of chloroform and 2.5 volumes of methanol, and centrifuged. The supernatant fluid was mixed with a further 1.25 volumes of chloroform and 2.5 volumes of 0.5% KCl, and after the pH had been adjusted to 5.4 to 5.6 by dropwise addition of 1 N acetic acid. Microsomes prepared by either method had about the same activity in the reactions studied here. The particles were suspended in a volume of 0.25 M sucrose equal to the volume of liver from which they were isolated. They could be stored at -20°, at which temperature they were stable for 1 to 2 weeks.

Assay for Radioactivity—Unless otherwise stated, choline reineckate was counted in a gas flow counter as described earlier (4). Solutions of phospholipid were evaporated to dryness in a vacuum desiccator; the residues were dissolved in 10 ml of a solution of 0.3% 2,5-diphenyloxazole and 0.05% 1,4-bis-(2-phenyloxazolyl)-benzene in toluene, and counted in a Packard Tri-Carb liquid scintillation counter set at 880 volts. Aliquots (1.0 ml) of aqueous solutions were mixed with 10 ml of a solution of KCl, 0.3% 2,5-diphenyloxazole, 0.02% 1,4-bis-(5-phenyloxazolyl)-benzene, 2% (volume per volume) ethylene glycol, and 10% (volume per volume) methanol in dioxane (20) and counted in a Packard liquid scintillation counter set at 960 volts.

Other Assay Procedures—Total P was determined according to

Vol. 236, No. 3, March 1961
Printed in U.S.A.
TABLE I
Incorporation of C\textsuperscript{14} from \textit{l}-methionine-CH\textsubscript{3}-C\textsuperscript{14} into soluble choline

Incorporation mixtures contained 10 \textmu moles of Tris buffer pH 7.5, 10 \textmu moles of MgCl\textsubscript{2}, 1 \textmu mole of ATP, 1 \textmu mole of TTP, 0 \textmu moles of phosphoethanolamine, 5 \textmu moles of phosphocholine, 5 \textmu moles of sodium, 0.1 \textmu moles of TPN, 0.1 \textmu mole of DPN, 0.1 \textmu mole of tetrahydrofolic acid, 1 \textmu mole of \textit{l}-methionine-CH\textsubscript{3}-C\textsuperscript{14} (1 \muC per \textmu mole), and subcellular fractions of rat liver as shown, in amounts equivalent to 0.5 ml of homogenate, in a volume of 1.0 ml. After 3 hours at 38\textdegree C, the reaction was stopped by heating for 5 minutes at 100\textdegree C, and the tubes were centrifuged. Phosphocholine in the supernatant fluids was hydrolyzed with alkaline phosphatase, and choline was isolated and counted as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Liver fraction</th>
<th>Specific activity c.p.m./\textmu mole choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>180</td>
</tr>
<tr>
<td>Supernatant</td>
<td>70</td>
</tr>
<tr>
<td>Particles (mitochondria + microsomes)</td>
<td>35</td>
</tr>
<tr>
<td>Particles + supernatant</td>
<td>340</td>
</tr>
</tbody>
</table>

TABLE II
Incorporation of C\textsuperscript{14} from \textit{l}-methionine-CH\textsubscript{3}-C\textsuperscript{14} and AMe-CH\textsubscript{3}-C\textsuperscript{14} into phospholipid choline

Incorporation mixtures contained 0.05 M Tris buffer pH 8.0, 0.1 ml of microsomes, and the additions shown in a volume of 1.0 ml. After 1 hour at 38\textdegree C, the reaction was stopped by adding 5 ml of 10% trichloroacetic acid containing 2.5% MgCl\textsubscript{2}. Choline was isolated from the phospholipid and counted as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Additions</th>
<th>Specific activity c.p.m./\textmu mole choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{l}-Methionine-CH\textsubscript{3}-C\textsuperscript{14} (5 \textmu moles, 0.85 \muC)</td>
<td>MgCl\textsubscript{2} (10 \textmu moles), ATP (4 \textmu moles), Liver supernatant (0.5 ml)</td>
<td>220</td>
</tr>
<tr>
<td>AMe-CH\textsubscript{3}-C\textsuperscript{14} (0.55 \textmu mole, 0.005 \muC)</td>
<td>None</td>
<td>370</td>
</tr>
</tbody>
</table>

Bartlett (13), and esters by the method of Rapport and Alonso (14). Aqueous solutions of choline were assayed as follows: the sample (1.0 ml) was mixed with 0.5 ml of a freshly prepared saturated solution of ammonium reineckate in 1 N HCl. After standing for at least 2 hours at 0\textdegree C, the solution was centrifuged in the cold (20 minutes at 3000 x g), and the supernatant fluid was carefully removed by suction through a fine capillary. The precipitate was washed twice with 1.0 ml of ice-cold water and once with 0.5 ml of ice-cold ethanol. It was then dissolved in an isotonic volume (normally 5 to 10 ml) of methyl ethyl ketone, and the optical density was measured at 335 mp.

Sources of Materials—O-Phosphoethanolamine, \textit{l}-homocysteine thiolactone, and AMe\textsuperscript{1} were obtained from the California Foundation for Biochemical Research; ATP, GTP, and CDP-ethanolamine were obtained from the Sigma Chemical Company. \textit{l}-Methionine-CH\textsubscript{3}-C\textsuperscript{14} and ethanolamine-1,2-C\textsuperscript{14} were obtained from Orlando Research, Inc.

\textsuperscript{1}The following abbreviation is used: AMe, \textit{s}-adenosylhomocysteine.

AMe-CH\textsubscript{3}-C\textsuperscript{14} was made enzymatically from ATP and \textit{l}-methionine-CH\textsubscript{3}-C\textsuperscript{14} according to Cantoni (15). It was isolated and stored as the reineckate, and aqueous solutions were prepared by Cantoni’s method as required. The preparation used in these studies was about 65% pure when first isolated. The purity slowly deteriorated to about 40% during prolonged storage at -20\textdegree C. However, the bulk of the impurities were removed when the AMe was extracted into aqueous solution. After storage for several months, an aqueous extract was chromatographed on paper in ethanol-water-acetic acid (65:34:1, volume per volume) (16). A small spot was observed in the position of methylthio adenosine; this contained about 0.6% of the radioactivity found in the AMe. No other radioactive or ultraviolet-absorbing area could be detected. Crystalline \textit{s}-adenosylhomocysteine was prepared enzymatically from adenosine and \textit{l}-homocysteine according to de la Haba and Cantoni (17).

Phosphatidylethanolamine and phosphatidylserine were isolated from calf brain according to Lee (18). Phosphatidylethanolamine was also prepared from rat liver, by passing a chloroform-methanol extract (10) through a column of silica gel (19:1), and eluting the phosphatidylethanolamine with chloroform-methanol (4:1). Although by no means pure, these preparations were adequate for the present work.

RESULTS

When homogenates of rat liver were incubated with methionine-CH\textsubscript{3}-C\textsuperscript{14}, phosphocholine, and various cofactors, a significant incorporation of C\textsuperscript{14} into the soluble choline was observed. Fractionation of the homogenate revealed that the particulate and supernatant fractions each had low enzyme activity individually, although together they were more active than the original homogenate (Table I). In subsequent experiments it was found that the microsomal fraction was more active than the mitochondria. It was also found that the incorporation was absolutely dependent on the presence of ATP and Mg\textsuperscript{2+}, but that none of the other cofactors listed in Table I had any effect on it.

In the majority of these experiments, choline was isolated from the phospholipid as well as from soluble choline, and it was found in every case that phospholipid choline was much more highly labeled than phosphocholine.\textsuperscript{2} The conditions for incorporation from methionine-CH\textsubscript{3}-C\textsuperscript{14} into lipid choline were similar to the conditions for incorporation into soluble choline; however, when AMe-CH\textsubscript{3}-C\textsuperscript{14} was substituted as radioactive precursor, the requirement for ATP, Mg\textsuperscript{2+} and the supernatant fraction was abolished, and the incorporation proceeded in the presence of microsomes alone (Table II). The addition of ethanolamine, phosphoethanolamine, or CDP-ethanolamine did not enhance or dilute the incorporation from AMe-CH\textsubscript{3}-C\textsuperscript{14} into lipid choline, nor did the addition of phosphatidylethanolamine or phosphatidylserine from calf brain (Table III). In other experiments these phosphatides were tested at various pH values between 7.0 and 10.0, and in no case was any stimulation observed. These results suggest that the major route of choline synthesis in rat

\textsuperscript{2}Similar results have been obtained in vivo (J. D. Wilson, K. D. Gibbon, and S. Udenfriend, unpublished observations). Methionine-CH\textsubscript{3}-C\textsuperscript{14} (1.0 \muC) was injected intraperitoneally into a rat, and the animal was killed after 15 minutes; choline isolated from the liver phospholipid contained 90% c.p.m. per \textmu mole, as compared with 25 c.p.m. per \textmu mole in phosphocholine and 60 c.p.m. per \textmu mole in free choline. After 30 minutes, the specific activities were 3500, 260, and 65 c.p.m. per \textmu mole, respectively.
liver occurs by transfer of methyl groups from AMe to an acceptor which may be phosphatidylethanolamine, and that microsomes contain both the acceptor and the enzymes responsible for the transfer. Accordingly, the transfer of methyl groups from AMe to microsomal lipid was studied further.

Transfer of Methyl Groups to Microsomal Lipid—The variation with pH of the rate of incorporation of radioactivity from AMe-CH₂C⁴ into microsomal lipid is shown in Fig. 1. The system is much more active in the alkaline region than at neutral pH values, the optimum being near pH 10.0. The effect of AMe concentration on incubation has not been investigated thoroughly, because of the complexity of the system; however, the rate is approximately constant at concentrations of AMe above 10⁻⁴ M. The reaction is not stimulated by addition of Mg++, Mn++, ATP, CTP, cysteine, or glutathione. The system is relatively stable to acetone drying and freeze-drying. It is also remarkably stable to alkali; exposure to pH values up to 12.0 for 1 hour at 0°C does not affect the activity of the microsomes.

The experiment in Table IV shows that the incorporation of C⁴ into lipid is in fact due to transfer of methyl groups to choline. In this experiment the lipid extract was hydrolyzed and choline reprecipitated from a portion of the hydrolysate. The reprecipitated was dissolved in methyl ethyl ketone, and choline was

**TABLE III**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Specific activity c.p.m./pmole phospholipid choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1120</td>
</tr>
<tr>
<td>Ethanalamine (5 pmoles)</td>
<td>1170</td>
</tr>
<tr>
<td>Phosphoethanolamine (6 pmoles)</td>
<td>940</td>
</tr>
<tr>
<td>CDP-ethanolamine (2 pmoles)</td>
<td>1250</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (6 mg)</td>
<td>800</td>
</tr>
<tr>
<td>Phosphatidylserine (6 mg)</td>
<td>900</td>
</tr>
</tbody>
</table>

**FIG. 1.** pH and incorporation of C⁴ into microsomal lipid. Incubation mixtures contained buffer as shown, 0.3 ml of microsomes, and 0.28 pmole of AMe-CH₂C⁴ (0.2 µc per pmole) in a volume of 0.8 ml. After 90 minutes at 37° the reaction was stopped by addition of chloroform and methanol, and the lipid was assayed for radioactivity and P as described under "Experimental Procedure." ○—○, 0.06 M Tris buffer; O—O, 0.12 M NaHCO₃-Na₂CO₃ buffer.

**TABLE IV**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Radioactivity c.p.m./ml</th>
<th>Choline µmole/ml</th>
<th>Specific activity c.p.m./µmole choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole lipid hydrolysate</td>
<td>4900</td>
<td>2.35</td>
<td>1920</td>
</tr>
<tr>
<td>Isolated lipid choline</td>
<td>1090</td>
<td>0.48</td>
<td>2270</td>
</tr>
</tbody>
</table>

**TABLE V**

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>µmole lipid P c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>720</td>
</tr>
<tr>
<td>0.1</td>
<td>800</td>
</tr>
<tr>
<td>0.2</td>
<td>770</td>
</tr>
<tr>
<td>0.3</td>
<td>770</td>
</tr>
<tr>
<td>0.4</td>
<td>600</td>
</tr>
<tr>
<td>0.5</td>
<td>480</td>
</tr>
</tbody>
</table>

The time course of incorporation is shown in Fig. 2; in this experiment the rate of the reaction was almost constant for 80 minutes. In other experiments the initial rate was not maintained for such a long period, the reaction frequently terminating completely after 30 to 60 minutes. An explanation for this became apparent when it was found that S-adenosylhomocysteine is a potent inhibitor of the incorporation (Table VI). Inhibition by this compound may also account for the observation in Table V that the incorporation does not remain proportional to enzyme concentration when large amounts of microsomes are present. Attempts have been made to demonstrate reversal of the reaction...
0.5 ml of microsomes, and 0.12 pmole of AMe-CH$_3$ (0.2 µc per pmole) in a volume of 0.8 ml. After incubation at 38° for the period shown, the reaction was stopped by addition of chloroform and methanol, and the lipid was assayed for radioactivity and P

After 30 minutes at 38°, the reaction was stopped and lipid was extracted with chloroform and methanol, and assayed for radioactivity by coupling the transfer of methyl groups from choline to S-adenosylhomocysteine with the methylation of nicotinamide, methylethanolamine; De, dimethylethanolamine; Ch., choline. In these experiments the peaks responding in RF values to monomethylethanolamine, dimethylaminoethanolamine, and choline were identified by chromatography on paper as described previously (4). When the paper was cut into strips and scanned in a paper strip scanner, only three radioactive peaks were found, corresponding to R$_f$ values to monomethylaminoethanolamine, dimethylaminoethanolamine, and choline.

**Table VI**

<table>
<thead>
<tr>
<th>S-Adenosylhomocysteine µmole</th>
<th>Specific activity c.p.m./µmole lipid P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>480</td>
</tr>
<tr>
<td>0.25</td>
<td>450</td>
</tr>
<tr>
<td>0.51</td>
<td>290</td>
</tr>
<tr>
<td>0.85</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>

![Graph](https://via.placeholder.com/150)

**Fig. 2.** Time course of incorporation of C$^{14}$ into microsomal lipid. Incubation mixtures contained 0.06 M Tris buffer pH 9.0, 0.5 ml of microsomes, and 0.12 µmole of AMe-CH$_3$-$^1$-$^4$ (0.2 µc per µmole) in a volume of 0.8 ml. After incubation at 38° for the period shown, the reaction was stopped by addition of chloroform and methanol, and the lipid was assayed for radioactivity and P as described under "Experimental Procedure."

![Graph](https://via.placeholder.com/150)

**Fig. 3.** Incorporation of C$^{14}$ into bases of microsomal phospholipid. The conditions of incubation and the methods of extraction and hydrolysis of the phospholipid and chromatography of the bases are described in the text. Incubation times: A, 45 seconds; B, 2 minutes; C, 5 minutes; D, 10 minutes. Me., mono-methylethanolamine; De, dimethylethanolamine; Ch., choline. Experiments C and D were performed with a different batch of microsomes from those used in experiments A and B.

by coupling the transfer of methyl groups from choline to S-adenosylhomocysteine with the methylation of nicotinamide, with the use of partially purified nicotinamide methyltransferase (10). However, no formation of N-methyl nicotinamide could be detected with the sensitive fluorometric assay of Huff and Perlzweig (20).

**Incorporation of C$^{14}$ into Phospholipid Monomethylethanolamine and Dimethylethanolamine—Duplicate incubation mixtures were set up containing 0.045 M Tris buffer pH 9.0, 8.4 ml of microsomes, and 5.2 µmoles of AMe-CH$_3$-$^1$-$^4$ (1 µc per µmole) in a volume of 11.4 ml. After incubation for various periods, the reaction was stopped by addition of 20 ml of 10% trichloroacetic acid containing 2.5% MgCl$_2$. Phospholipid was extracted from the washed precipitates and hydrolyzed according to Artom (9) as described under "Experimental Procedure." The hydrolysates were evaporated to dryness; the residues were suspended in water and washed with chloroform; and the aqueous layers were applied to columns (50 by 1 cm) of Dowex 50-X8 in the hydrogen form. The columns were washed with 100 ml of water and eluted with 1.5 M HCl (21). Fractions (4 ml) were collected with an automatic fraction collector and 1.0-ml aliquots were assayed for radioactivity as described under "Experimental Procedure." The distribution of radioactivity in the phospholipid bases is shown in Fig. 3. After incubation for 5 or 10 minutes, the elution pattern (Fig. 3, C and D) was similar to the pattern found in the phospholipid bases of whole rat liver after injection of methionine-CH$_3$-$^1$-$^4$ (4, 5). At earlier times there was more radioactivity in dimethylethanolamine than in choline (Fig. 3, A and B). The fact that dimethylethanolamine had more radioactivity than monomethylethanolamine even as early as 45 seconds after addition of AMe-CH$_3$-$^1$-$^4$ (Fig. 3A) suggests that the transfer of the second methyl group, to monomethylethanolamine, is considerably more rapid than the transfer of the first methyl group to ethanolamine. In these experiments the peaks were identified by chromatography on paper as described previously (4). When the paper was cut into strips and scanned in a paper strip scanner, only three radioactive peaks were found, corresponding in R$_f$ values to monomethylaminoethanolamine, dimethylaminoethanolamine, and choline.

**Product of the Reaction—**Rat liver microsomes (10.0 ml) were mixed with 4.0 ml of 0.5 M Tris buffer pH 9.0, and 4.0 ml of a solution containing 0.4 µmoles of AMe-CH$_3$-$^1$-$^4$ (1.0 µc per µmole), and the mixture was incubated at 38° for 5 minutes. At the end of this time, 40 ml of methanol and 30 ml of chloroform were added; after vigorous shaking, the mixture was centrifuged. The supernatant fluid was shaken with a mixture of 20 ml of chloroform and 20 ml of 1% NaCl, and centrifuged. The lower layer was dried over anhydrous Na$_2$SO$_4$ and the solvent removed under reduced pressure. The residue was dissolved as far as possible in 1.0 ml of anhydrous ether and centrifuged. Acetone (5 ml) was added to the filtrate to precipitate the phospholipid. After standing overnight at 0°, the precipitate was collected by centrifugation, washed with acetone twice by suspension and centrifugation, and dried in a vacuum. This material (60 mg) was dissolved in 5 ml of chloroform and applied to a column of 15 g of silicic acid (35 by 1 cm). The column was eluted with a gradient of methanol and chloroform as described by Wren (22). Initially the mixing chamber (diameter, 7.6 cm) contained 200 ml of chloroform, and the reservoir, which was a 250-ml Erlenmeyer flask, contained 250 ml of methanol. Fractions of approximately 5 ml were collected with an automatic fraction collector. Aliquots of 0.5 ml from each fraction were evaporated to dryness and counted as described under "Experimental Procedure." Total P and ester groups were determined on further aliquots from each fraction. The results are shown in Fig. 4.
The radioactivity was eluted in three major peaks, A, B, and C; several minor phospholipid peaks which were also eluted contained no radioactivity. The fractions were combined as shown in Fig. 4 and evaporated to dryness; the residues were hydrolyzed by refluxing for 3 hours in 6 N methanolic HCl, and again evaporated to dryness. A portion of the residue from Peak C was found to contain 2.56 μmoles of P per ml and 2.40 μmoles of choline per ml. This gives a ratio of 1:0.94:1.99 for P-choline, in good agreement with the theoretical value for lecithin. When the hydrolysate was chromatographed on paper by the solvent system described previously (4) and the paper was scanned in a paper strip scanner, radioactivity could be detected only in the position corresponding to choline (Rf 0.95). In another experiment in which radioactive phospholipid obtained in this manner was chromatographed on silicic acid, the lecithin peak was hydrolyzed and choline was isolated as the reineckate. This was then dissolved in methyl ethyl ketone, and choline was extracted into dilute HCl as in the experiment of Table III. This choline and the hydrolyzed lecithin from which it was isolated were both found to have 84 c.p.m. per μmole of choline, showing that all the radioactivity in the lecithin was in the choline residue. When the residues from the hydrolysis of Peaks A and B were chromographed on paper and scanned as before, two radioactive areas were obtained for each peak. In Peak B, the positions corresponding to dimethylthanolamine (Rf 0.85) and choline contained approximately equal amounts of radioactivity. In Peak A, the radioactive areas were in the positions of monomethylethanolamine (Rf 0.73) and dimethylthanolamine, the former having about one-fifth the radioactivity of the latter. Ethanolamine was also identified in both Peaks A and B; no radioactivity could be detected in this area in either case.

Nature of the Methyl Acceptor—So far, all attempts to obtain a preparation which could be stimulated by adding exogenous phosphatidylethanolamine have been unsuccessful. When acetone-dried microsomes were extracted with ethanol and petroleum ether at 0°, their activity was partially or totally lost; in no case could it be restored by the addition of phosphatidylethanolamine. The incorporation of C14 from AMe-CH1-2-C14 was at least partially destroyed by deoxycholate, Tween 20, Tween 80, Triton X-100 (Rohm and Haas Company) and Tergitol TMN in concentrations which solubilized the microsomes to some extent; again there was no restoration of activity by phosphatidylethanolamine from calf brain or rat liver. Digitonin and Pluracol E-600 had little or no effect on the activity. Attempts were made to fractionate the particles with digitonin, deoxycholate, or alkaline pH; none of these experiments resulted in a reversible separation of the activity into more than one component.

Since this approach failed, the following experiment was performed. Microsomes (10 ml) were mixed with a solution containing 1500 μmoles of Tris buffer pH 8.0, 100 μmoles of MgCl2, 500 μmoles of ATP, 2 μmoles of CTP, and 5 μmoles of ethanolamine-1,2-C14 (10 μc) in 5 ml. The mixture was incubated at 35° for 20 minutes, after which it was cooled in ice, and the microsomes were recovered by centrifugation for 1 hour at 105,000 × g. They were suspended in 15 ml of 0.25 m sucrose and dialyzed for 16 hours against 1 liter of 0.01 m Tris buffer pH 8.0, with several changes of the buffer. Ethanolamine, isolated from the phospholipid of these microsomes, as described elsewhere (23), was found to contain 1800 c.p.m. per μmole. When the microsomes were incubated with nonradioactive AMe, there was a significant increase of radioactivity in the lipid choline which did not occur when AMe was omitted or when the particles were heated at 100° before incubation (Table VII, Experiment 1). In a similar experiment, in which the prior incubation period with ethanolamine-1,2-C14 (125 μmoles, 1.0 μc) was extended to 40 minutes, and a larger concentration of AMe was then used, there was a considerably greater transfer of radioactivity to choline in the presence of AMe, and again, none when it was omitted (Table VII, Experiment 2). The results of all these studies are consistent with the hypothesis that the substrate for methylation is a bound form of phosphatidyl ethanolamine, possibly a lipoprotein.

**DISCUSSION**

There are five known derivatives of ethanolamine, each of which might possibly serve as a substrate for the synthesis of new choline. These are ethanolamine itself, phosphoethanolamine, glyceryl phosphorylethanolamine, CDP-ethanolamine, and...
phospholipid ethanolamine. Dawson (24) has produced evidence that glycercyl phosphorylethanolamine is in fact a breakdown product of phospholipid, but no studies of this sort have been reported for the other derivatives. The observation of Bremer and Greenberg (5) that when methionine-CH\textsubscript{3}-C\textsuperscript{14} was injected into rats radioactivity reached a maximum in CDP-choline more than 1 hour after it did in lecithin suggests that CDP-choline is not a precursor of lecithin in this reaction, even though it is an intermediate in the synthesis of lecithin from free choline (25, 26). In the present work it has been found that radioactivity from AMe-CH\textsubscript{3}-C\textsuperscript{14} is incorporated into lecithin in rat liver preparations far more readily than it is into the choline-phosphoethanolamine fraction, and a similar finding has been made in vivo, with methionine-CH\textsubscript{3}-C\textsuperscript{14} as precursor. The simplest explanation for all these results is that the methyl acceptor in the synthesis de novo of choline is phospholipid ethanolamine, and in fact there is now considerable evidence for this hypothesis.

In the first place, radioactive monomethyllethanolamine, dimethyllethanolamine, and choline have been identified in rat liver phospholipid after the injection of methionine-CH\textsubscript{3}-C\textsuperscript{14} into rats (4, 5), and also in the phospholipid of rat liver microsomes after these were incubated with AMe-CH\textsubscript{3}-C\textsuperscript{14} (6) and Fig. 3). The time course of incorporation, at least into phosphatidyl(dimethyl)ethanolamine, is consistent with the view that it is a precursor of phosphatidylethanolamine. Monomethyllethanolamine and dimethyllethanolamine have also been identified as constituents of the phospholipid of a choline-lacking mutant of Neurospora crassa (27), and recently phosphatidyl(dimethyl)ethanolamine has been shown to be present and metabolically active in normal rat liver (28). Secondly, there was no stimulation of incorporation from AMe-CH\textsubscript{3}-C\textsuperscript{14} into choline by ethanolamine, phosphoethanolamine, or CDP-ethanolamine (Table III). Thirdly, radioactive lecithin, in which only the choline residue was labeled, has been isolated from microsomes incubated with AMe-CH\textsubscript{3}-C\textsuperscript{14} (7) and Fig. 4). Fourthly, synthetic phosphatidyldimethylethanolamine was found to stimulate the incorporation of C\textsuperscript{14} into microsomal lipid (29). Finally, although it has not been possible to demonstrate a similar stimulation by exogenous phosphatidylethanolamine, a direct conversion to lipid choline of ethanolamine-C\textsuperscript{14} previously incorporated into the microsomes, either as such or as a partially methylated product, was observed when these were incubated with AMe (Table VII). We have recently obtained evidence which suggests that in liver, serine is decarboxylated to form ethanolamine only after incorporation into phospholipid (22), so that reactions in the series which leads from serine to choline all appear to involve the corresponding phosphatides as substrates.

At present it is not possible to assign a physiological function to this sequence of reactions. It seems unlikely that they serve primarily as a mechanism for the synthesis de novo of choline. By the introduction of quaternary ammonium groups, the conversion of cephalin to lecithin could alter profoundly the charge on a lipid membrane, and thereby change its structure or permeability. Such an alteration may play a part in determining the lamellar structure of the endoplasmic reticulum (30), or in some of the functions of intracellular transport and conduction which have been suggested for this component of the cytoplasm (31). It is significant in this connection that in rat liver both the methylation of phosphatidylethanolamine and the decarboxylation of phosphatidylserine occur primarily in microsomes, which recent evidence has shown to be identical with the granular components of the endoplasmic reticulum (30).

**SUMMARY**

1. Homogenates of rat liver incorporate C\textsuperscript{14} from S-adenosylmethionine-CH\textsubscript{3}-C\textsuperscript{14} into phospholipid choline and to a much lesser extent into phosphocholine. The activity is entirely associated with subcellular particles, and incorporation proceeds with no other additions. The optimal pH is near 10.0. S-Adenosylhomocysteine is a potent inhibitor, but no evidence could be obtained for reversal of the reaction.

2. Radioactive monomethyllethanolamine and dimethyllethanolamine have been identified as constituents of the phospholipid of microsomes incubated with S-adenosylmethionine-C\textsuperscript{14}. The incorporation into dimethyllethanolamine is consistent with its being a precursor of choline. Lecithin containing radioactive choline has been isolated from a similar incubation mixture.

3. When microsomes were incubated with ethanolamine-C\textsuperscript{14}, and adenosine and cytidine triphosphates, radioactivity was incorporated into the lipid ethanolamine. When microsomes labeled with C\textsuperscript{14} in this manner were incubated with S-adenosylmethionine, a significant increase was observed in the radioactivity of the lipid choline.

4. These results support the hypothesis that lecithin can be synthesized by the transfer of methyl groups to phosphatidylethanolamine. A possible physiological implication of this reaction is discussed.

**REFERENCES**

18. Lees, M. B., in S. P. Colowick and N. O. Kaplan (Editors),


The Enzymatic Conversion of Phospholipid Ethanolamine to Phospholipid Choline in Rat Liver
Kenneth D. Gibson, Jean D. Wilson and Sidney Udenfriend