Conversion of ethanolamine to choline (1), with methionine serving as the methyl donor (2), has been established in the intact animal. Stetten (1) observed that the labeled nitrogen of ethanolamine-$N^{15}$ was retained in the choline molecule, and we determined that the carbon chain of ethanolamine is the precursor in vivo of the choline carbon chain (3).

Conversion of ethanolamine to choline by in vitro preparations is in a more doubtful state. The synthesis of choline in vitro from methionine and ethanolamine with rat or guinea pig kidney and liver slices or homogenates (4, 5) has been questioned by Veitch and Zweig (6). These authors have demonstrated that the disappearance of methionine, as judged by the McCarthy-Sullivan method, was not the result of transmethylation but was actually due to the oxidative deamination of the methionine, mainly by $d$-amino acid oxidase present in the tissue preparations. Similarly, the test for choline by precipitating it as the reineckate salt has been claimed to be unreliable, as ethanolamine and other bases that occur give insoluble reineckates (6). However, choline has been reported to be synthesized from $l$-methionine and 2-dimethylaminoethanol by rat liver slices in the presence of 0.001 M KCN, but not by liver homogenates (7).

Since the earlier in vitro experiments did not employ isotopic tracer methods and apparently did not utilize a reliable procedure for the isolation of choline, it appeared that the problem of the synthesis of choline from ethanolamine in vitro warranted reinvestigation. In view of the lack of knowledge of the formation of the phosphatides, the present study of the metabolism of ethanolamine in vitro and the formation of choline was so organized as to permit the investigation to deal with the factors influencing the formation of lecithin or phosphatidylcholine.

**Methods**

Non-fasted rats of the Long-Evans strain, weighing 165 to 175 gm., were killed by a sharp blow with the hand to the back of the head. The liver
or other tissues were immediately removed and placed in ice-cold physiological saline. Several slices totaling 500 mg. of wet weight,1 prepared with a Stadie-Riggs microtome, were incubated for 2 hours in 5 ml. of Krebs-Ringer phosphate solution made up as described by Umbreit et al. (8), with the exception that the CaCl₂ was 0.0016 M. The initial pH of the phosphate solution was 7.4. Incubation was carried out aerobically in 20 ml. beakers at 37° with the Dubnoff apparatus (9). The rate of oscillation was maintained between 90 and 100 cycles per minute.

The radioactive substrates consisted of 3.8 × 10⁻⁴ M ethanolamine-1,2-⁴¹C, prepared as previously reported (3), with 239,500 counts per 100 seconds per beaker, 3.0 × 10⁻⁴ M methionine-⁴¹H₃ with 311,000 counts per 100 seconds per beaker, and 3.0 × 10⁻⁴ M sarcosine-⁴¹H₃ with 185,000 counts per 100 seconds.

The reaction was stopped by the addition of 2 ml. of a 3:1 mixture of ethyl alcohol-ethyl ether. In addition, to facilitate the denaturation of the proteins, the thermostat of the incubator was turned up so as to increase the temperature to 75 to 80° and shaking was continued for another 15 minutes. The denatured tissue was then extracted for phosphatides or stored in the deep freeze until ready for extraction. Extraction for phosphatides, hydrolysis, and the column chromatographic separation of the phosphatide constituents on Dowex 50 resin were carried out as described previously (3), with the exception that approximately 8 mg. of carrier choline were added to the mixture of phosphatides and hydrochloric acid before hydrolysis.

The elution point (Ep) of choline from the Dowex 50 (250 to 500 mesh) resin column may be determined visually by examining the polyethylene collecting planchets for hygroscopic material. The Ep value has been arbitrarily defined in terms of the numbers of the planchets in which choline is found. This value is relatively constant if such conditions as the density and height of the resin bed, rate of flow and normality of the acid eluting liquid, and temperature are held constant.

The radioactivity of choline was determined directly in the planchets containing choline by employing the Tracerlab SC-16 windowless gas flow counter in conjunction with a Nuclear Instrument and Chemical Corporation scaler, model No. 163. In addition, the reineckate salt derivative was prepared by taking up the choline chloride in a minimal volume of water and adding to the aqueous choline 5 times its volume of a 2 per cent Reinecke salt-methanol solution. Choline reineckate was allowed to precipitate quantitatively by being stored in the refrigerator or the deep freeze for 6 hours. The precipitate was then plated on 4.25 cm. Whatman No. 42 filter paper by use of conventional procedures, and counted for radioactivity.

1 The wet weight was taken after gently blotting each tissue slice on filter paper.
It should be pointed out that the resin column chromatography for the separation of choline assures the identity of the compound. No other compound known, which gives the reineckate salt reaction, possesses the same Ep value as choline. This is in contrast to the previous situation for the determination of choline reviewed by Jukes (10).

RESULTS AND DISCUSSION

The incubation of radioactive ethanolamine with rat liver slices in the absence of any cofactors resulted in a very small yield of phosphatidyl-

**Table I**

Effect of Factors on Formation of Phosphatidylcholine

<table>
<thead>
<tr>
<th>No. of experimental results</th>
<th>Ethanolamine</th>
<th>Methionine</th>
<th>Homocysteine</th>
<th>Betaine</th>
<th>Sarcosine</th>
<th>Cysteine</th>
<th>Total counts in phosphatidylcholine per 100 sec.</th>
</tr>
</thead>
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<tr>
<td>6</td>
<td>+*</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>7</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1124</td>
</tr>
<tr>
<td>3</td>
<td>+*</td>
<td>+†</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1928</td>
</tr>
<tr>
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<td>99</td>
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<tr>
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<td>+</td>
<td>0</td>
<td>+*</td>
<td>0</td>
<td>170</td>
</tr>
</tbody>
</table>

* Radioactive.
† 7.5 \( \times 10^{-4} \) M methionine.

choline, representing about 0.04 per cent conversion (Table I). This was in contrast to the yield of phosphatidylcholine, representing 2.14 per cent conversion, obtained in vivo (3). Three possible explanations for the low yield were considered: (a) the 3 hour period of incubation was too long and most of the choline formed had been oxidized, (b) the radioactive choline found in the liver tissue of the intact animal may have originated in some other tissue, and (c) there was a relative absence of necessary cofactors or methylating substances. However, shorter periods of incubation of 30,

2 The term phosphatidylcholine is used to describe the source of the radioactive choline since free choline is considered to be practically insoluble in petroleum ether (11).
110 PHOSPHATIDYLCHOLINE FORMATION

60, and 90 minutes gave similar low values, as did incubations of tissue
derived from spleen, kidney, intestine, and the pancreas.

Effect of Methionine and Methionine-C\textsuperscript{14}H\textsubscript{3} on Phosphatidylcholine For-
mation—The addition of non-radioactive methionine, as a possible methyl-
ating substance, to the liver slices caused a maximal yield, under optimal
conditions, of phosphatidylcholine, of about 19 times the basal level (Table
I). The conversion under these conditions is approximately 0.8 per cent,
which represents a considerable increase above the level of 0.04 per cent
conversion, in the presence of ethanolamine alone, but still falls short of the
2.1 to 2.2 per cent conversion obtained \textit{in vivo}.

The optimal concentration of methionine for the maximal yield of phos-
phatidylcholine under our conditions was $7.5 \times 10^{-4} \text{ M}$; the use of higher
concentrations of methionine either decreased the yield or exerted no effect.
The ethanolamine substrate concentration apparently did not represent a
limiting factor, as 65 to 70 per cent of the ethanolamine substrate always
remained after the reaction was stopped.

As indicated in Table I, the transfer of the C\textsuperscript{14} methyl group of methio-
nine to phosphatidylcholine is greater than the conversion of the C\textsuperscript{14}-la-
beled chain of ethanolamine on an equimolar basis.\textsuperscript{3} This increased yield
of radioactive phosphatidylcholine, in the presence of labeled methionine,
may of course be accounted for by the fact that, on an equimolar and ran-
dom basis, for every labeled ethanolamine molecule there will be three
labeled methyl groups entering into the reaction.

It will be noted that the presence of methionine is required for appreci-
able formation of radioactive phosphatidylcholine from labeled ethanol-
amine, whereas ethanolamine is not required for appreciable formation of
radioactive phosphatidylcholine from labeled methionine.\textsuperscript{3} The observa-
tions of the low yield of phosphatidylcholine when labeled ethanolamine
alone was incubated with liver slices and the necessity of adding methionine
to the incubation of ethanolamine-1,2-C\textsuperscript{14} to achieve maximal phosphatidyl-
choline production indicate that the concentration of methionine available
for methylation purposes in the rat liver slice is limited and that the liver
slice contains a considerable metabolic pool of ethanolamine. This obser-

\textsuperscript{3} The results given in Table I indicate a slightly greater formation of choline from
methionine in the absence of ethanolamine. In a more recent experiment performed
by Mr. Akira Nakao, a 15 per cent greater increase in the label of choline (counted as
the reineckate) was obtained in the presence of $2 \times 10^{-3} \text{ M}$ ethanolamine in the
incubation medium than in its absence. In this same experiment it was determined
that the methyl groups of choline contained over 90 per cent of the radioactivity when
the radioactive substrate was methionine-C\textsuperscript{14}H\textsubscript{3}. In earlier experiments with etha-
nolamine-1,2-C\textsuperscript{14}, it was found that about 20 per cent of the label appeared in the
methyl groups of the choline. The methyl groups were cleaved as trimethylamine
and measured as tetramethylammonium iodide.
vation is supported by the widespread occurrence of ethanolamine phosphate in tissues (12) and the regular excretion of ethanolamine in the urine (13).

Effect of Homocysteine, Cysteine, and Betaine on Phosphatidylcholine Formation—Homocysteine, as a precursor of methionine, would be expected to play an important role in the formation of phosphatidylcholine. The per cent conversion attained was 0.25, which is approximately 52 per cent of the yield procured in the presence of an equimolar concentration of unlabeled methionine (Table I). The action of homocysteine is intrinsic and not due to its SH group alone, since the presence of cysteine did not increase the average activity of choline above that of experiments in which ethanolamine-1,2-C^{14} was incubated alone.

Betaine was relatively ineffective as a precursor of methyl groups for phosphatidylcholine. The mean activity in phosphatidylcholine resulting from betaine was 34.5 per cent of the yield obtained from homocysteine. However, when homocysteine and betaine were added simultaneously to the substrate, the conversion of ethanolamine to phosphatidylcholine was as much as 4 times that obtained with betaine alone and nearly 2 times the yield obtained in the presence of homocysteine (Table I). The small effect of betaine on the formation of phosphatidylcholine in vitro and the synergistic action of betaine and homocysteine suggest that such a pool does not exist. At least it is not a pool in the sense that there is a transfer directly between these two particular members, i.e. choline and betaine, each participating equally. The transfer of deuterio methyl groups from betaine to choline (14, 15) may be explained by homocysteine serving as the carrier (16) for the betaine methyl groups. However, the function of the betaine methyl groups appears to require further investigation.

Effect of Homogenization on Formation of Phosphatidylcholine—Attempts to secure the methylation of ethanolamine to choline and phosphatidylcholine with rat liver homogenates in the absence and presence of adenosine triphosphate and folic acid were negative. The results were also negative when the homogenization was performed for periods as short as 30 seconds in a container surrounded by an ice bath.

Conversion in Vivo of Ethanolamine-1,2-C^{14} to Phosphatidyl Fatty Acids—After 4 hours, the magnitude of the conversion of ethanolamine to phosphatidylcholine with rat liver homogenates in the phosphatidyl fatty acids, isolated from the whole liver of rat experiments in vivo, amounted to 0.015 per cent of the total injected activity (3 μc).

The fatty acids contained in the residue from the filtration of the hydrochloric acid hydrolysate of the phospholipide were isolated and counted by redissolving in acetone and transferring the solution to flat metal cups. Upon evaporation of the acetone the cups were assayed for radioactivity.

Other Observations—Sarcosine-C^{14}H_3 showed a very small conversion of
0.05 per cent to phosphatidylcholine. The incubation of homocysteine with labeled sarcosine increased the yield of phosphatidylcholine about 70 per cent in two experiments. The interpretation of the increase in the yield of phosphatidylcholine in the presence of homocysteine is the same as with betaine, namely that homocysteine acted as a carrier of the sarcosine methyl group or more probably of the one carbon fragment derived from the methyl group of sarcosine.

The per cent conversion in vitro of ethanolamine-1,2-C\textsuperscript{14} (3.8 \times 10^{-4} \, \text{M}, 239,500 counts per 100 seconds) to glycine was found to be 0.27 per cent, thus confirming the in vivo results reported by Weissbach and Sprinson (17). Glycine was isolated by adding 300 mg. of carrier glycine to the in vitro mixture upon termination of the incubation. The preparation was then homogenized. After homogenization, ethyl ether was used to extract the 1 ml. of 30 per cent trichloroacetic acid used to denature the incubation mixture. Following the extraction, absolute ethyl alcohol was added to precipitate the glycine. Glycine was crystallized twice from aqueous alcohol. The acetyl derivative of the recrystallized glycine was prepared, isolated, and degraded, and the glycine resulting from the degradation was then crystallized twice more before counting.

SUMMARY

In the absence of methionine, ethanolamine-1,2-C\textsuperscript{14} was not appreciably converted to phosphatidylcholine by rat liver slices. The addition of methionine increased the yield about nineteen times. The addition of methionine-C\textsuperscript{14}H\textsubscript{3} increased the yield of phosphatidylcholine about 3-fold above that obtained with unlabeled methionine. Evidence is presented which indicates that the methionine pool is much smaller than the ethanolamine pool. Homocysteine possesses 52 per cent of the activity of methionine, on an equimolar basis, in stimulating phosphatidylcholine formation, whereas betaine possesses only 34.5 per cent of the activity of homocysteine. Homocysteine and betaine together increased the yield of phosphatidylcholine considerably above the sum of the individual effects of each alone. The effect of homocysteine is intrinsic and is not due to the SH grouping alone.

Homogenization of the liver tissue resulted in complete loss of the ability to form choline. The conversion in vivo of ethanolamine-1,2-C\textsuperscript{14} to rat liver phosphatidyl fatty acids amounted to 0.015 per cent of the total injected activity. Sarcosine-C\textsuperscript{14}H\textsubscript{3} showed a small conversion of 0.05 per cent to phosphatidylcholine. The presence of homocysteine increased the yield about 70 per cent. The conversion in vitro of ethanolamine-1,2-C\textsuperscript{14} to glycine was 0.27 per cent.
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