# THE BIOSYNTHESIS OF CHOLINE AND ITS RELATION TO PHOSPHOLIPID METABOLISM

JON BREMER, PAUL H. FIGARD AND DAVID M. GREENBERG

*Department of Biochemistry, University of California School of Medicine, San Francisco, Calif. (U.S.A.)*

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## SUMMARY

The biosynthesis of choline has been studied in the rat with [3-$^{14}$C]serine, [1,2-$^{14}$C]-aminoethanol and [$^{14}CH_3$]methionine as precursors.

When [$^{14}$C]aminoethanol was given to intact rats, radioactive choline was isolated from the liver phospholipids, but no radioactive phosphocholine was found in an aqueous liver extract. When [$^{14}$C]methionine was given, radioactive mono- and dimethylaminoethanol, as well as radioactive choline, were isolated from the liver phospholipids. The mono- and dimethylaminoethanol had much higher turnover rates than had the choline. When [$^{14}$C]methionine was administered, the incorporation of radioactivity into cytidinediphosphocholine was, in comparison with the incorporation of radioactivity into phospholipid choline, delayed.

*In vitro* experiments showed that choline-labeled lecithin was formed when isolated rat-liver microsomes were incubated with S-adenosylmethionine.

When [$^{14}$C]serine was injected into intact rats, radioactive aminoethanol as well as radioactive choline labeled in both the hydroxyethyl- and in the methyl groups, were isolated. Turnover and specific activity studies showed that phosphoaminoethanol and cytidinediphosphoaminoethanol are unlikely intermediates in the formation of phospholipid aminoethanol from serine.

On the basis of these results it is concluded that phosphatidylserine is decarboxylated to phosphatidylaminoethanol and that the latter compound is methylated to form lecithin. In this reaction all the methyl groups of choline are incorporated by transmethylation from S-adenosylmethionine.

The significance of these results is discussed in relation to fat and phospholipid metabolism.

## INTRODUCTION

The decarboxylation of serine to aminoethanol and the subsequent methylation of aminoethanol to choline are well known reactions[1,2]. The $\beta$-carbon atom of serine is also known to be a precursor of the methyl groups of methionine and choline, presumably through the intermediate formation of a tetrahydrofolic acid–formaldehyde complex. The methyl group of methionine is known to be transferred to choline as an intact unit[3,4]. On the basis of *in vivo* experiments, STEKOL *et al.*[5] have suggested

that the first two methyl groups of choline are synthesized *de novo* and incorporated into choline by a folic acid-dependent mechanism, while the third methyl group is incorporated by transmethylation from adenosylmethionine[4].

We have recently performed extensive *in vitro* experiments to elucidate the mechanism involved in the biosynthesis of choline from free aminoethanol or [$^{14}$C]-phosphoaminoethanol, but no free choline formation could be detected in liver homogenates. These negative results might indicate that a derivative of aminoethanol rather than the free compound is the methyl acceptor in the biosynthesis of choline.

The following reactions are known for the conversion of the above bases to phosphatides[6].

$$\text{Aminoethanol} \longrightarrow \text{phosphoaminoethanol} \longrightarrow \text{cytidinediphosphoaminoethanol} \longrightarrow \text{phosphatidylaminoethanol} \quad (1)$$

$$\text{Choline} \longrightarrow \text{phosphocholine} \longrightarrow \text{cytidinediphosphocholine} \longrightarrow \text{phosphatidylcholine} \quad (2)$$

It is thus evident that there are at least four different possible methyl acceptors in the biosynthesis of choline.

In the conversion of serine to phosphatidylserine, no intermediates are known[7], but phosphoserine and "cytidinediphosphoserine" must be considered as possible substrates for a "serine decarboxylase".

On the basis of these considerations, experiments were designed to identify the immediate precursors in the methylation of aminoethanol and also in the decarboxylation of serine. There are measurable pools in rat liver of phosphoaminoethanol[8], phosphocholine[9], cytidinediphosphoaminoethanol and cytidinediphosphocholine[6]. By injecting the known precursors, [$^{14}$C]serine, [$^{14}$C]aminoethanol, [$^{14}$C]choline and [$^{14}$C]methionine, and following the appearance of label in the phospholipids and in the above mentioned phospholipid precursors, we have found it possible to exclude certain of the possible pathways.

In this paper results are reported which indicate that it is the fully formed phosphatidylserine which is first decarboxylated, and subsequently methylated, to lecithin.

Preliminary reports on some of these results have already been published[10,11].

## MATERIALS

The $^{14}$C precursors used were all purchased from Isotopes Specialities Co., Burbank. California. The [3-$^{14}$C]serine had a specific activity of $\sim$ 2.1 mC/mmole, the [1,2-$^{14}$C]-aminoethanol* $\sim$ 1 mC/mmole, the [$^{14}CH_3$]choline $\sim$ 1 mC/mmole and the [$^{14}CH_3$]-methionine $\sim$ 4.5 mC/mmole. When lower specific activities were sufficient, the labeled compounds were diluted with corresponding unlabeled material before use.

The *Clostridium welchii* toxin (200–400 MLD/ml) was kindly supplied by Lederle Laboratories, Pearl River, N.Y.

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* We are indebted to Dr. M. ROTHSTEIN of this laboratory for preparing [1,2-$^{14}$C]phosphoaminoethanol by the following procedure: Aminoethanol hydrochloride (0.3 mmole) in 100% phosphoric acid was kept at 130° for 24 h. After removal of the phosphate with barium hydroxide, the phosphoaminoethanol was eluted from a Dowex 50 ($H^+$) column with 1 *N* acetic acid. The product contained no detectable impurities on paper chromatographs.

## METHODS

Female rats weighing 250–300 g were used for *in vivo* experiments. Both sexes were used for *in vitro* experiments. The animals had free access to food and water until they were sacrificed. All radioactive compounds were administered intraperitoneally in 1–2 ml of 0.9 % NaCl. *In vitro* techniques were as reported elsewhere[11].

*Preparation of aqueous liver extracts and isolation of liver phospholipids*

The method used by DAWSON[12] was modified for the work with whole rat livers. The animals were sacrificed by decapitation and bled as thoroughly as possible. The livers were immediately homogenized in 80–90 ml of 67 % ethanol in a Waring blendor. After centrifugation, the homogenate residues were extracted three more times with 25–30 ml of 67 % ethanol and the combined extracts were concentrated to approximately half the original volume, cleared by centrifugation and then shaken twice with chloroform (50 and 30 ml). The phases were separated by centrifugation and the clear water phase was removed by pipette for column chromatography.

The chloroform–ethanol layers were used to complete the lipid extraction from the homogenate residue. An additional extraction was done with pure chloroform. The chloroform–ethanol was heated to boiling at each extraction.

The combined extracts were evaporated to dryness and the residues extracted with petroleum ether (b.p. 30–60°). The petroleum ether extracts were washed with a little water and concentrated to 3–4 ml and the phospholipids were precipitated by the addition of 8–10 volumes of acetone at —10°. The acetone precipitation was repeated once.

*Hydrolysis of phospholipids*

The isolated phospholipids were hydrolyzed in 1.2 *N* HCl, according to the procedure of PILGERAM *et al.*[2]. After removal of the fatty acids by filtration the mixture was evaporated to dryness and the residue was dissolved in a minimal volume of water for column chromatography.

*Column chromatography*

*The phospholipid bases* were separated chromatographically according to PILGERAM *et al.*[2]. This procedure was also found to give separate peaks for the mono- and dimethylaminoethanol (Fig. 1).

*Phosphoaminoethanol and phosphocholine* were isolated by chromatographing the aqueous liver extracts on the same type of column as the free phospholipid bases. The phosphoderivatives of aminoethanol, mono- and dimethylaminoethanol and choline were eluted in the order mentioned by approx. 7, 10, 13 and 18 resin bed volumes of 2 *N* formic acid.

*Cytidinediphosphoaminoethanol and -choline* were obtained in separate chromatographic peaks by KENNEDY's procedure[13], employing a Dowex-1-formate column when 0.02 *N* formic acid was used in the upper reservoir instead of 0.04 *N* acid. Fractions of 5 ml were collected. Fig. 2 shows two typical runs.

*Separation of phospholipids* was performed according to the procedure of MARINETTI *et al.*[14]. The silicic acid column gave good separation of the "cephalin" and lecithin components of the mixture (Fig. 5A).

*Verification of identity of isolated radioactive compounds*: In addition to the elution points in the column chromatograms, the identity of each of the compounds was verified by paper chromatography.

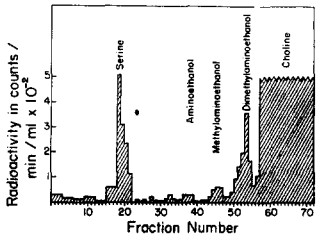


Fig. 1. Radioactivity distribution in column chromatogram of rat-liver phospholipid bases isolated from rat injected with approximately 11.5 $\mu$moles [$^{14}CH_3$]methionine, $3.9 \cdot 10^6$ counts/min. Animal sacrificed 40 min after the injection; 5 ml fractions collected from the column.

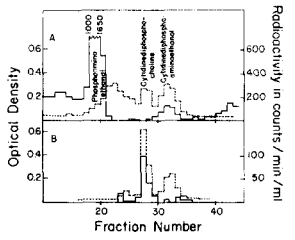


Fig. 2. Radioactivity distribution and u.v. light absorption at 280 m$\mu$ in column chromatogram of rat liver aqueous extract from rats injected with: A: approximately 100 $\mu$moles [1,2-$^{14}$C]-aminoethanol, $6 \cdot 10^6$ counts/min. The animal was sacrificed 75 min after the injection. B: approx. 6 $\mu$moles [$^{14}CH_3$]methionine, $1.9 \cdot 10^6$ counts/min. The animal was sacrificed 300 min after the injection. Fractions of approx. 5 ml collected.

*Phospholipid bases*

The isolated serine gave the same $R_F$ value as the authentic compound on paper chromatograms developed with *n*-butanol–diethyleneglycol–water (4:1:1) (see ref. 15). Whatman No. 4 filter paper was used in this and all other paper chromatography systems.

The identities of aminoethanol, mono- and dimethylaminoethanol and choline were established by the paper chromatography system described below. Fifty g phenol, 50 ml *n*-butanol, 3 ml 80% formic acid and 5 ml water were shaken with solid potassium chloride. The paper was pre-treated with 1 *N* potassium chloride and dried. The salt treatment of the system reduced tailing in the chromatograms. In this system the following $R_F$ values were found: aminoethanol, 0.10; methylaminoethanol, 0.30; dimethylaminoethanol, 0.55 and choline, 0.72. The compounds isolated from rat liver phospholipids gave identical $R_F$ values, estimated from the distribution of radioactivity on the chromatograms. Aminoethanol gave a positive ninhydrin reaction and the choline gave a distinct spot with iodine vapor[16] and with phosphomolybdate[13]. The choline also gave a radioactive Reineckate precipitate, which was recrystallized from acetone–propanol without loss of specific activity.

The identities of mono- and dimethylaminoethanol were further verified by the following tests: The radioactive compounds were eluted from the paper chromatogram with water. Approx. 100 $\mu$moles of the appropriate compounds were added along with 1 ml of 1 *N* sodium hydroxide and 0.8 ml of 1 *M* methyl iodide in ethanol. The mixtures were shaken for 1 h at 45°. The choline formed was precipitated as the Reinecke salt and recrystallized three times from acetone–propanol. The specific activity of the choline Reineckate formed from monomethylaminoethanol was 11, 9 and 10 counts/min/mg after one, two and three recrystallizations respectively and from dimethylaminoethanol 67, 66 and 68 counts/min/mg. In another experiment carrier choline and Reinecke salt were added to samples of isolated radioactive mono-

and dimethylaminoethanol. In this instance, completely inactive choline Reineckates were obtained after one recrystallization from acetone–propanol.

*Phosphoaminoethanol and phosphocholine*

These materials were chromatographed in two different paper systems: Propanol–80% formic acid–water (6:3:1) ($R_F$ values 0.15 and 0.30) and water saturated phenol–80% formic acid (99:1) ($R_F$ values 0.40 and 0.85). The isolated, radioactive compounds moved identically with the synthetic reference compounds in the two systems. Both compounds gave positive spot tests for phosphate[17]. The phosphoaminoethanol gave a positive ninhydrin reaction, the phosphocholine a distinct spot with iodine vapor[16].

*Cytidinediphosphoaminoethanol and -choline*

These compounds were identified by their u.v. absorption spectra and by paper chromatography. The fractions containing cytidinediphosphoaminoethanol gave an absorption spectrum identical with that of cytidine both in acid and alkaline solution. When chromatographed on paper with 0.25 *M* TRIS–HCl buffer, pH 8–ethanol (1:1), and with 0.02 *M* acetic acid–ethanol (4:6), only one u.v. absorbing spot was found with $R_F$ values of 0.71 and 0.50 in the two systems respectively compared to 0.69 and 0.33 reported by KENNEDY AND WEISS[6]. In animals injected with [$^{14}$C]aminoethanol, the compound became strongly radioactive.

The fractions containing cytidinediphosphocholine gave a somewhat distorted absorption spectrum compared to cytidine, due to some contamination from a neighboring peak in the column chromatogram. When chromatographed on paper with TRIS buffer–ethanol and with acetic acid–ethanol, one major spot was found with $R_F$ values of 0.64 and 0.55 in the two systems respectively, compared to the values 0.59 and 0.36 reported by KENNEDY AND WEISS[6]. When eluted from the paper, the major spot gave the typical u.v. absorption spectrum of cytidine.

The cytidinediphosphocholine was further identified by acid hydrolysis and paper chromatography of the degradation products. The compound was refluxed with 1 *N* HCl for 1 h. By subsequent chromatography on paper with propanol–formic acid–water, the only radioactive spot found had the same $R_F$ value as phosphocholine. It also gave a positive spot reaction for phosphate.

*Degradation of choline*

To determine the distribution of $^{14}$C between the methyl- and the hydroxyethyl groups of choline, the compound was degraded by alkaline permanganate oxidation as described by DU VIGNEAUD *et al.*[3]. Samples of the choline and of the trimethylamine formed during the oxidation were precipitated as chloroplatinates. From the specific activities the distribution of $^{14}$C between choline methyl and hydroxyethyl groups was calculated.

*Determination of specific activities*

The specific activity of choline was determined as explained above. The specific activity of cytidine diphosphocholine was calculated from the radioactivity and the u.v. absorption of the peaks in the column chromatograms. The calculated values must be considered as minimum values, due to the slight contamination of u.v.-absorbing material from a neighboring peak in the chromatograms.

In the experiments with [$^{14}$C]serine, the specific activity of cytidinediphosphoaminoethanol could not be calculated directly from the column chromatograms. The large amount of radioactivity ($\sim$ 100 $\mu$C) injected into the animals led to a relatively high "background" radioactivity in all the column fractions, and no distinct radioactive peak corresponding to cytidinediphosphoaminoethanol was detectable. A rough estimation of the specific activity was obtained by chromatographing the isolated cytidinediphosphoaminoethanol on paper with acetic acid–ethanol (see above), eluting the u.v.-absorbing spot from the paper and measuring the absorption and the radioactivity in the eluate. Only 4–6 counts/min on the planchets were obtained. The calculated values therefore must be considered as only rough approximations.

The specific activities of aminoethanol, phosphoaminoethanol and of serine were calculated on the basis of quantitative determinations of these compounds with ninhydrin according to MOORE AND STEIN[18].

## RESULTS

Table I shows the results of an experiment in which the total radioactivity incorporated into phosphoaminoethanol, phosphocholine, phospholipid aminoethanol and phospholipid choline was measured after the injection of [1,2-$^{14}$C]aminoethanol, [1,2-$^{14}$C]phosphoaminoethanol and [$^{14}CH_3$]choline. It is seen that injection of both choline and aminoethanol (and phosphoaminoethanol) led to the appearance of radioactive choline in the phospholipids, whereas only choline gave measurable activity in the water soluble phosphocholine.

These results indicated that phosphocholine is not an intermediate in the biosynthesis of lecithin from aminoethanol, and we therefore had to consider cytidinediphosphoaminoethanol or phosphatidylaminoethanol as the possible methyl acceptors.

In the experiments shown in Figs. 3 and 4, [$^{14}CH_3$]methionine was chosen as the labeled precursor, because the cytidinediphosphoaminoethanol and -choline peaks appear very close together in the column chromatogram. As a consequence it was

TABLE I

TOTAL RADIOACTIVITIES OF COMPOUNDS ISOLATED FROM RAT LIVER AFTER THE INTRAPERITONEAL INJECTION OF LABELED AMINOETHANOL, PHOSPHOAMINOETHANOL, OR CHOLINE

The animals were sacrificed 2 h after the injections.

| *Compound injected* | *Radioactivity of compounds isolated from liver in counts/min* | | | |
|---|---|---|---|---|
| | *Phosphoaminoethanol* | *Phosphocholine* | *Phospholipid aminoethanol* | *Phospholipid choline* |
| Approximately 10 $\mu$moles [1,2-$^{14}$C]aminoethanol, $2.5 \cdot 10^6$ counts/min | 13,500 | Not detectable | 280,000 | 65,000 |
| Approximately 10 $\mu$moles [1,2-$^{14}$C]phosphoaminoethanol, $3 \cdot 10^6$ counts/min | 25,000 | Not detectable | 370,000 | 58,000 |
| Approximately 10 $\mu$moles [$^{14}CH_3$]choline, $2.5 \cdot 10^6$ counts/min | — | 130,000 | — | 215,000 |

difficult to secure reliable measurements of the cytidinediphosphocholine radioactivity in the vicinity of a highly radioactive cytidinediphosphoaminoethanol peak.

The data from chromatograms of the phospholipid bases, isolated after the injection of radiomethionine, favored phosphatidylaminoethanol as the methyl acceptor. Fig. 1 shows that radioactive mono- and dimethylaminoethanol, the presumed intermediates in choline biosynthesis, were found in the phospholipids. Fig. 3 further shows that these compounds have a high turnover rate compared to what is known of other phospholipid bases. The methylation of phosphatidylaminoethanol is further supported by the finding that the specific radioactivity of cytidinediphosphocholine seemed to reach its maximum later than did the phospholipid choline (Fig. 4). Admittedly, the accuracy of the determination of cytidinediphosphocholine's specific activity was poor. This was calculated from counting rates of 20–100 counts/min on

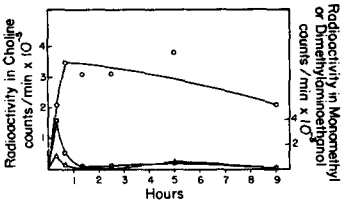


Fig. 3. Time curves for the appearance of radioactivity in phospholipid monomethylaminoethanol, dimethylaminoethanol and choline after the intraperitoneal injection of approx. 6 $\mu$moles [$^{14}CH_3$]methionine, $1.9 \cdot 10^6$ counts/min. △, monomethylaminoethanol; □, dimethylaminoethanol; ○, choline.

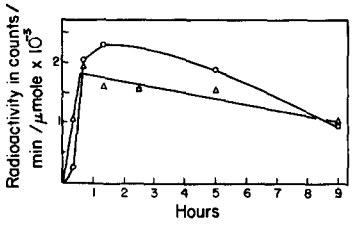


Fig. 4. Time curves for specific radioactivity of cytidinediphosphocholine and phospholipid choline after the intraperitoneal injection of approx. 6 $\mu$moles [$^{14}CH_3$]methionine, $1.9 \cdot 10^6$ counts/min. ○, cytidinediphosphocholine; △, choline.

the planchets. On the other hand, considering the relative pool sizes of cytidinediphosphocholine and of phosphatidylcholine in rat liver, the former evidently would have a specific activity many times that of phospholipid choline, if it were an intermediate.

Our conclusions from these *in vivo* experiments have been verified by *in vitro* experiments. Fig. 5 shows that incubating isolated microsomes with [$^{14}CH_3$]adenosylmethionine led to the formation of radioactive phospholipidcholine (Fig. 5B), and that this choline was bound to lecithin (Fig. 5A).

In another *in vitro* experiment, the extracted radioactive phospholipids were treated with *Clostridium welchii* toxin in ether–alcoholic solution according to the procedure of HANAHAN AND VERCAMER[19]. From the reaction mixture, radioactive phosphocholine was isolated by paper chromatography, in the two systems used for this compound (see above) confirming the identity of the radioactive lipid as lecithin.

The "cephalin" peak in the phospholipid chromatogram from this *in vitro* experiment also contained some radioactivity. This activity was shown to be due to dimethylaminoethanol after hydrolyzing a sample of the isolated "cephalin" with hydrochloric acid and chromatographing the hydrolyzate on paper in the phenol–butanol–formic acid system (see above). The small amount of radioactive monomethylaminoethanol present in the phospholipid hydrolyzate, could not be localized in the phospholipid chromatogram.

Fig. 6 shows the labeling of liver phospholipid bases after the injection of [3-$^{14}$C]-

serine. Only a very small fraction (< 0.5 %) of the injected radioactivity was incorporated, compared to the degree of incorporation of radioactive aminoethanol, choline or methionine-methyl (Table I, Fig. 3). The turnover time of phosphatidylserine in rat liver is known to be of the same order as that of the other phosphatides[20]. This low incorporation is probably due to extensive dilution of the injected serine by internal, inactive serine.

The striking feature in this experiment is that the phospholipid serine reached its maximum activity within 40 min, whereas the phospholipid aminoethanol activity kept on increasing for at least 5 h after the serine injection. The same gradual increase was found for the choline hydroxyethyl group. The same feature is evident in the methionine experiment, where the aminoethanol activity rose linearly through all the 9 h of the experiment (Fig. 7).

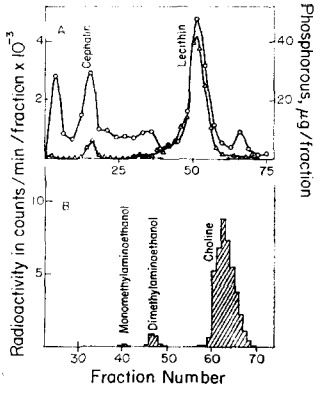


Fig. 5. Radioactivity distribution in column chromatograms of phospholipids isolated from rat-liver microsomes incubated with [$^{14}CH_3$]adenosylmethionine. Microsomes from approximately one rat liver were incubated with [$^{14}CH_3$]adenosylmethionine, 3.5 µmoles = $1 \cdot 10^6$ counts/min; reduced glutathione, 17 µmoles; tris(hydroxymethyl)aminomethane buffer, pH 7.25, 250 µmoles. Total volume: 6 ml, incubation time 2 h, temperature, 37°; atmosphere, air. The phospholipids were isolated from the incubation mixture as reported elsewhere[11]. Approx. 10% of the added radioactivity was recovered in the phospholipids, of which 0.4 were chromatographed on a silicic acid column according to MARINETTI *et al.*[14] (A). O, phosphorus; △, radioactivity. Approximately 0.6 of the isolated phospholipids were hydrolyzed and chromatographed on a Dowex 50 (strong anionic resin) according to PILGERAM *et al.*[2] (B).

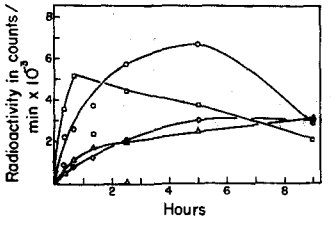


Fig. 6. Time curves for the appearance of radioactivity in phospholipid serine, aminoethanol and choline hydroxyethyl- and choline methyl groups after the intraperitoneal injection of 6 µmoles [3-$^{14}$C]serine, $3.8 \cdot 10^6$ counts/min. □, serine; ◇, aminoethanol; △, choline-hydroxyethyl; O, choline-methyl.

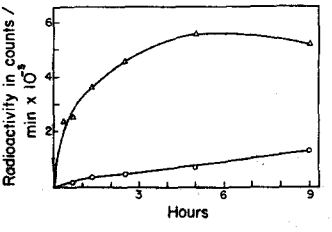


Fig. 7. Time curves for the appearance of radioactivity in liver phospholipid serine and -aminoethanol after the intraperitoneal injection of approximately 6 µmoles [$^{14}CH_3$]methionine, $1.9 \cdot 10^6$ counts/min. △, serine; O, aminoethanol.

TABLE II

TOTAL RADIOACTIVITIES AND SPECIFIC RADIOACTIVITIES OF COMPOUNDS ISOLATED FROM LIVER OF RATS INJECTED INTRAPERITONEALLY WITH APPROXIMATELY 49 μmoles [3 $^{14}$C]SERINE*

| | *Total radioactivity in counts/min in liver phospholipid bases* | | | *Total counts/min in aqueous liver extract* | *Specific radioactivity of phospholipid bases (counts/min/μmole)* | | *Specific radioactivity of compounds in aqueous liver extract (counts/min/μmole)* | |
|---|---|---|---|---|---|---|---|---|
| | *Serine* | *Amino-ethanol* | *Choline*** | *Phospho-amino-ethanol* | *Serine* | *Amino-ethanol* | *Phospho-amino-ethanol* | *Cytidine-diphospho-amino-ethanol**** |
| Animal sacrificed after 2 h | 35,700 | 8,500 | 39,800 | 1,850 | 3,650 | 140 | — | ~ 340 |
| Animal sacrificed after 4 h | 34,700 | 16,600 | 57,450 | 2,500 | 3,750 | 290 | 320 | ~ 380 |

* $31 \cdot 10^6$ counts/min.
** Determination of intramolecular distribution of radioactivity not performed.
*** Only approximate values based on counting rates of 4–6 counts/min. Not corrected for possible radioactivity in the cytidine portion of the molecule.

These observations indicate that there must be an intermediate with a long turnover time between serine and phosphatidylaminoethanol.

To elucidate further the mechanism of this conversion, another experiment was undertaken, in which a larger amount of [3-$^{14}$C]serine was injected to overcome the internal dilution (Table II). In this experiment the aqueous extracts of the livers were chromatographed to isolate the phosphoaminoethanol and the cytidinediphosphoaminoethanol. A significant radioactive peak was found corresponding to cytidinediphosphocholine, but no distinct peak corresponding to cytidinediphosphoaminoethanol. There was also a small peak corresponding to phosphoaminoethanol. As mentioned in METHODS, the cytidinediphosphoaminoethanol isolated by paper chromatography was also found to be slightly radioactive. The small amount of the compound present permitted only a rough calculation of its specific activity. The specific activity of phosphoaminoethanol could be calculated more accurately, because a larger amount of this compound was present.

As seen from Table II, the water soluble derivatives of aminoethanol were found to have a somewhat higher specific activity than the phospholipid aminoethanol. At first sight, therefore, this experiment does not seem to exclude these compounds as being intermediates in the formation of phosphatidylaminoethanol from serine. We consider it unlikely that these compounds are intermediates for the following reasons: From our experiments it is evident that the specific activity of phosphatidylaminoethanol keeps on rising for at least 5 h after the injection of radioserine. During this time the immediate precursor must have a higher specific activity. Considering then the sizes of the metabolic pools in rat liver of phosphoaminoethanol (approx. 5 μmoles), cytidinediphosphoaminoethanol ($<$ 1 μmoles) and of phosphatidylaminoethanol ($>$ 50 μmoles), and the turnover time of many hours of the latter compound, one would expect the immediate precursor to have a specific activity many times greater than that of the phosphatidylaminoethanol. This is evidently not so. A more reasonable explanation, therefore, of our findings, is that the turnover of the phosphatidylaminoethanol is not uniform, but that the breakdown of a more active

phospholipid fraction gives rise to the phosphoaminoethanol and cytidine-diphospho-aminoethanol with a specific activity somewhat higher than the average aminoethanol specific activity.

## DISCUSSION

The results of the *in vivo* experiments indicate that the decarboxylation of serine and the methylation of aminoethanol take place after these compounds have been incorporated into the phospholipids. The methylation of phosphatidylaminoethanol is further supported by *in vitro* experiments. We have also shown that S-adenosyl-methionine is the donor of all the methyl groups in choline[11]. On this basis therefore, we suggest the metabolic cycle shown in Fig. 8, showing the interrelationship between phosphatide- and methyl group metabolism. The reactions on the right side of the

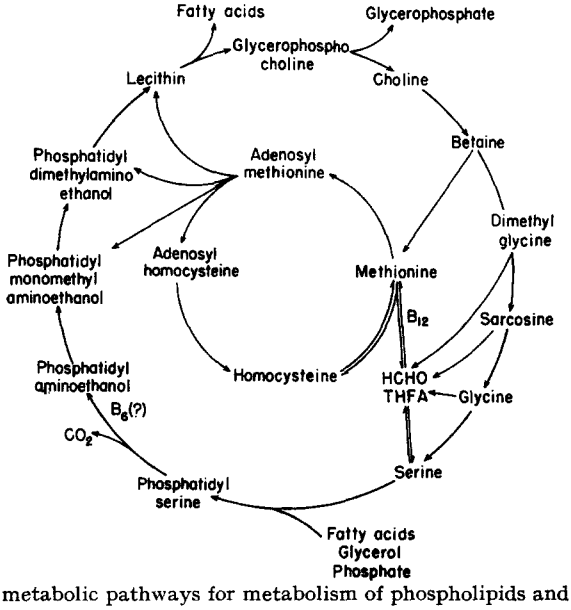


Fig. 8. Proposed metabolic pathways for metabolism of phospholipids and methyl groups.

diagram, involving compounds freely soluble in water, are well known, and all of them have been shown to take place *in vitro*[21]. This is also true of the synthesis of methionine from formaldehyde or serine[22–24], although the exact mechanism in this conversion is not known. Numerous *in vivo* experiments[25,26], and recently also *in vitro* experiments with enzymes from microorganisms[27,28], strongly suggest that vitamin $B_{12}$ is a cofactor in the *de novo* synthesis of methionine methyl groups. There is also evidence for an oxidative demethylation of methionine[29].

The exact mechanism for serine incorporation into phosphatides is not known, but a recent report by DILS AND HÜBSCHER[7] indicates a mechanism different from that reported by KENNEDY AND WEISS for aminoethanol and choline[6]. DILS AND HÜBSCHER found "subcellular" particles to incorporate serine into phospholipids in the presence of calcium ions and phosphate. They also found that choline is incorporated under the same conditions, a result which indicates the existence of a third pathway for lecithin biosynthesis in addition to the pathway reported by KENNEDY AND WEISS, and the one reported in this paper.

It has been shown that serine is converted to aminoethanol[1], and also that aminoethanol[2], mono- and dimethylaminoethanol[30] can serve as precursors for choline. It is, however, a striking fact that, except for recent work by STEKOL *et al.*[4], the reaction products have always been isolated from the phospholipids, a fact which to a certain extent supports our conclusion that these reactions represent interconversion of phospholipids. The accumulation of mono- and dimethylaminoethanol in the phospholipids of choline-requiring mutant strains of *Neurospora crassa*[31], also fits in with the proposed metabolic scheme.

STEKOL *et al.*[4] have reported the isolation of free choline after incubating dimethylaminoethanol and adenosylmethionine with rat-liver homogenate. At present, it is not possible to explain with certainty the differences between their and our results. One possibility is that the free choline isolated in their experiments, represents a hydrolytic product of lecithin, which therefore might be the primary product. The low yield and the use of relatively large amounts of liver homogenates may indicate such an explanation. Their report also contains no experimental data showing the requirement for free dimethylaminoethanol in the formation of the choline isolated. In *in vivo* experiments with folic acid-deficient rats[5], these workers obtained results indicating that the first two methyl groups of choline are formed by a folic acid-dependent mechanism and that the third methyl group is transferred from adenosylmethionine. Our *in vitro* experiments do not support this theory.

In turnover studies it has been found that glycerophosphoaminoethanol and glycerophosphocholine behave as breakdown products from the phospholipids[12], and further that glycerophosphocholine is broken down to glycerophosphate and choline[32]. FAIRBAIRN[33] reported that free fatty acids are probably the primary breakdown products of lecithin. Thus there is good experimental support for all the steps in the proposed metabolic cycle.

The cycle represents a new pathway for lecithin biosynthesis. Experiments by GROTH *et al.*[34] suggest that this pathway is quantitatively more important in the rat than the pathway(s) involving utilization of free choline. These workers compared the incorporation of [$^{14}CH_3$]choline and [1,2-$^{14}$C]choline into phospholipids in rats. They found that the methyl-labeled choline is twice as efficient as the hydroxyethyl-labeled choline as a lecithin precursor both in liver and in brain. Considering the fact that only one of the three methyl groups of choline is reutilized by direct transmethylation by way of betaine to methionine, while the two other methyl groups are oxidized and probably are subject to a high dilution in the "formaldehyde–formate pool", and are therefore poorly reutilized for methyl synthesis (*cf.* the low conversion to choline methyl of the serine-3-carbon compared to that of methionine methyl in our experiments), it can be said almost with certainty that this new pathway is very important in the normal rat.

GROTH *et al.*[34] also found different half-lives for the lecithins formed from the two choline precursors, indicating different turnover rates for the lecithins formed by the two pathways. In our experiments, the specific activities of cytidinediphosphoaminoethanol and -choline were found to rise above the average specific activities of phospholipid aminoethanol and -choline. This may also indicate a non uniform turnover of the phosphatides.

Further, the two pathways seem to have different intracellular localizations. The cytidine-dependent incorporation of choline into phospholipid is reported to take

place in the mitochondria[6]. We have found that the methylation of phosphatidylaminoethanol takes place in the microsome fraction[11]. In this connection it is worth noticing that the acylation of glycerophosphate to phosphatidic acid[35] and the synthesis of neutral fat[36] also occur in the microsome fraction of the cell. Consequently the methylation of the phosphatidylaminoethanol in the microsomes may prove to be a finding of considerable importance, and the possibility should be given consideration that the proposed "phospholipid cycle" represents a mechanism by which neutral fat and long chain fatty acids are transported into the mitochondria for utilization (see also ARTOM[37]).

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