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SYNTHESIS OF LECITHIN (PHOSPHATIDYLCHOLINE) FROM PHOSPHATIDYLETHANOLAMINE IN BOVINE BRAIN

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SUMMARY

Choline molecules are needed for the synthesis of acetylcholine and phospholipids in the mammalian brain. An enzymatic activity capable of forming lecithin (phosphatidylcholine) from the step-by-step methylation of phosphatidylethanolamine is identified in the bovine brain. This enzyme(s), phosphatidylethanolamine-N-methyltransferase (EC 2.1.1.17), is localized in the synaptosomal fraction of bovine caudate nucleus, uses S-adenosylmethionine as the methyl donor (apparent K_m = 20 μM), and has a V_{max} of 50–60 pmol/mg protein × h (i.e. about 1 % of that found in rat liver). The brain may be able to meet some of its choline requirements by de novo synthesis.

INTRODUCTION

Lecithin (phosphatidylcholine) is the major phospholipid of the brain, liver, plasma, and most other tissues[27]. It is important not only as a structural component of membranes, but also as a potential source of choline molecules. Choline is required for the synthesis of acetylcholine in neurons: treatments that increase brain choline levels (e.g., administration of choline or lecithin to rats) also increase the synthesis and release of acetylcholine[10,12,16,20,26]. To a great extent, mammals are dependent on exogenous (i.e. dietary) sources of choline[3,5,7,8,11,13,23,24,25]. Of the three enzymatic pathways known to exist in some tissues that catalyze lecithin biosynthesis, only one actually generates new choline molecules (Fig. 1). The cytidine diphosphocholine[21] and base exchange[23] pathways do not cause a net synthesis of choline, but only redistribute preexisting molecules. It is only by the sequential methylation of phosphatidylethanolamine (PE), catalyzed by the enzyme(s) phosphatidylethanolamine-N-methyltransferase (PeMT), that new choline molecules are generated.

Though most of the choline normally present in the body probably derives from dietary lecithins, about 15% is thought to be synthesized de novo, by PeMT in the liver[5,7]. The adrenal medulla[19] and red blood cells[17,18] are also known sites of PeMT activity. In contrast, the brain has been thought to obtain most or all of its choline from the circulation[2,3,15]. Despite evidence from several laboratories that there is PeMT activity in the brain[9,13,22], many authors have discounted the possibility that the brain obtains significant amounts of choline by synthesizing lecithin and liberating choline from that lecithin[1,15].

We present evidence that the brain does possess the ability to synthesize, de novo, the choline moiety of lecithin by the step-by-step methylation of PE. Its lecithin-forming activity per unit weight of tissue (calculated by measuring the [^{3}H]lecithin extracted from the assay mixture when incubated at physiological pH), is comparable to that found in the adrenal medulla[19] and the erythrocyte[18], and about 1% of the activity found in the liver[7]. (This is clearly an underestimate inasmuch as it neglects any radioactivity present as choline or choline products.)

MATERIALS AND METHODS

Special chemicals used in these experiments were purchased from the following sources, except as noted: S-adenosylhomocysteine (98% pure), generously provided by the Bioresearch Corporation, Milan, Italy; S-adenosylmethionine (SAM) from the Sigma Chemical Corporation, St. Louis, Mo.; phospholipid standards from GIBCO, Grand Island, N.Y.; [^{3}H-methyl]phosphatidylcholine, [^{3}H-methyl]phosphorylcholine, [^{3}H-methyl]lysophosphatidylcholine [^{3}H-methyl]choline, and [^{3}H-methyl]S-adenosylmethionine from New England Nuclear Corporation, Boston, Mass.; thin-layer chromatography (TLC) plates (LK5D) from the Whatman Company, Clifton, N.J.; ion exchange resins from the Dow Chemical Corporation, Midland, Mich.; and Scinti-Verse from Fisher Scientific Corporation, Fair Lawn, N.J. All chemicals were reagent grade unless otherwise noted.

Calves were sacrificed by a blow to the head; caudate nuclei were immediately dissected and placed on dry ice. Tissues were stored at —80 °C until used. Pooled specimens from 15 animals were used in assays.

Preparation of tissue fractions

Tissues were prepared by a modification of the method of Fuxe et al.[11]. One gram of caudate was homogenized in 10 ml of 0.32 M sucrose. The homogenate was centrifuged at 1000 × *g* for 10 min to remove tissue debris and nuclei. The supernatant fluid was centrifuged at 20,000 × *g* for 10 min to obtain a crude mitochondrial pellet fraction. This pellet was diluted to a final volume of 1 ml with 0.32 M sucrose, and applied to a discontinuous density gradient containing 9 ml of 1.2 M sucrose and 3 ml of 0.8 M sucrose; this was then centrifuged at 100,000 × *g* for 60 min at 4 °C. The fraction at the 0.8–1.2 M sucrose interface was collected; we call this the synaptosomal fraction. In some cases, the postmitochondrial supernatant fluid was centrifuged at 100,000 × *g* for 60 min at 4 °C, to obtain a microsomal fraction.

PeMT(s) assay

PeMT activity was assayed using the method of Hirata et al.[19]. Product formation was linear for up to 30 min. The assay medium contained 25 μl of a solution comprised of 50 mM Tris·HCl buffer (pH 7.5), 15 mM $MgCl_2$, and 0.2 mM EDTA, to which was added 20 μl tissue preparation (80 μg protein). The reaction was started by the addition of 5 μl radioactive SAM. The amount and specific activities of the [^{3}H]SAM used in each experiment are described in table and figure legends. As the figure legends indicate, the media also contained 100 μg of a particular phospholipid substrate. The reaction continued for 30 min at 37 °C in a Dubnoff shaker, and was stopped by the addition of 3 ml chloroform/methanol/HCl (100:50:1, v/v). These extracts were washed twice with 2 ml 0.1 M KCl in 50% methanol. Individual tritiated phospholipids were purified by thin-layer chromatography, as described below. A heated preparation of enzyme (15 min at 80 °C) was used as a blank.

Identification of reaction products

Reaction products were identified essentially as described by Hirata et al.[19]. A portion (0.8 ml) of the chloroform phase was concentrated to a volume of 20 μl, using a Savant Speed-Vac lyophilizing centrifuge. This was applied to a silica gel TLC plate along with internal standards (PE, phosphatidyl-N-monomethyl-ethanolamine (P1), phosphatidyl-N-dimethylethanolamine (P2), and lecithin (phosphatidylcholine; PC)), and developed using chloroform/propionic acid/n-propyl alcohol/water (2:2:3:1, v/v) to 12 cm; the segments were visualized by exposure to iodine vapor, and scraped into scintillation vials. Phospholipids were eluted with 0.5 ml methanol, and then 15 ml Scinti-Verse was added and the samples were counted.

To establish that the radioactivity found in the newly formed lecithin actually was present as [^{3}H]choline, 0.8 ml of the chloroform phase was chromatographed and eluted as described above, evaporated to dryness in a Savant Speed-Vac lyophylizing centrifuge, resuspended in 100 μl of 6 N HCl, and heated in a stoppered tube at 100 °C for 1 h to hydrolyze the phosphorylcholine bond. A known lecithin standard, labeled with tritium on the methyl carbon of choline, was treated identically. The heated mixtures were evaporated to dryness and resuspended in 100 μl water. These mixtures were then applied to a 3 cm column of Dowex 50W-X8 (200-400 mesh) hydrogen-form cation exchange resin. The columns were washed once with 5 ml water, 5 times with 5 ml 0.2 N HCl, and 4 times with 5 ml 2.0 N HCl. Each 5 ml fraction was collected in a scintillation vial and counted in 15 ml Scinti-Verse. The Dowex was then removed from the column and counted in the same manner. Identical chromatography was performed on authentic tritium-labeled choline, phosphorylcholine, PC, and lyso-PC.

pH and magnesium dependence

The PeMT assay was performed as described above. P2 was added as exogenous substrate. Sodium acetate (50 mM) buffer was used to prepare reaction mixtures of pH 6.0 and 6.5. Tris·HCl buffer (50 mM) was used to obtain pH 7.0, 7.5, 8.0 and 8.5, and glycine NaOH (50 mM) was used to obtain pH 9 and higher. pH was verified in each sample during incubation. Magnesium chloride was added at a concentration of 15 mM. Protein was assayed by the method of Bradford[4].

TABLE I

Subcellular distribution of PeMT activity in calf caudate

Fractions were prepared as described in text. SAM (4.5 μM; specific activity = 21,000 dpm/pmol) was added. After incubation with 100 μg exogenous P2 the radioactivity in the TLC segment corresponding to authentic lecithin, P2, or P1 was counted. Results are expressed as means ± S.E.M. for 3 experiments.

Fraction	*mg protein/ fraction*	*C(^{3}H)$_3$ incorporation into product pmol/mg × 30′*	
Homogenate	211	Lecithin	0.87 ± 0.07
		P2	0.63 ± 0.04
		P1	0.31 ± 0.03
Mitochondria	39	Lecithin	1.31 ± 0.05
		P2	0.88 ± 0.03
		P1	0.55 ± 0.09
Synaptosomes	3	Lecithin	3.25 ± 0.18
		P2	2.35 ± 0.04
		P1	1.26 ± 0.11
Microsomes	8	Lecithin	0.81 ± 0.06
		P2	0.45 ± 0.03
		P1	0.25 ± 0.03

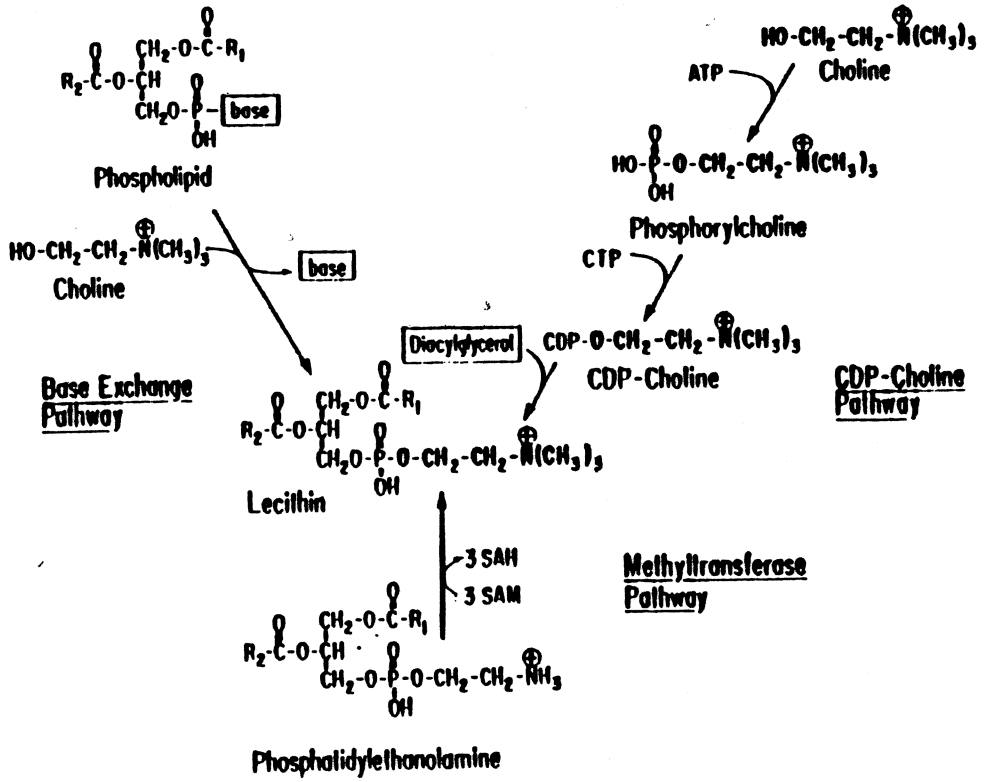

Fig. 1. Pathways of lecithin (phosphatidylcholine) synthesis.

Subcellular distribution of PeMT(s)

PeMT activity was found to be concentrated within the synaptosomal fraction (prepared from the crude mitochondrial fraction); its specific activities in crude mitochondrial membranes and in microsomes were less than half as great as in synaptosomes (see Table I). (In contrast, microsomes of liver and adrenal medulla[24,25] have relatively high PeMT activities.) The postmicrosomal fraction was not assayed. Because of its high specific activity, the synaptosomal fraction was used in subsequent experiments. The total radioactivity present as PC or its precursors was about 60% of the total count in the chloroform phase. The step-by-step conversion of PE to lecithin was demonstrated by adding PE, PI, or P2 (Fig. 2A–C) to the reaction mixture. PE had no discernible effect on the levels of [^{3}H]PI or [^{3}H]P2 observed at the end of the incubation (compare Fig. 2A with 2B and 2C); addition of PI or P2 caused 3.5- or 3.0-fold enhancements in the accumulations of tritiated P2 and lecithin, respectively. The addition of more than 100 μg of PI or P2 to the reaction mixtures did not increase the yields of radioactive products. The identities of the radioactive compounds present at the solvent front were not determined.

Identification of label in the choline moiety

Samples of reaction mixture, containing added PE, were chromatographed on silica gel TLC plates. The lecithin segments were eluted, and the eluates were hydrolyzed and chromatographed on an ion exchange resin. Most of the label incorporated into the material chromatographing with lecithin was found to migrate with authentic [^{3}H]choline (Fig. 3). Authentic choline-labeled [^{3}H]lecithin subjected to similar hydrolysis and ion exchange chromatography yielded a similar [^{3}H]choline peak.

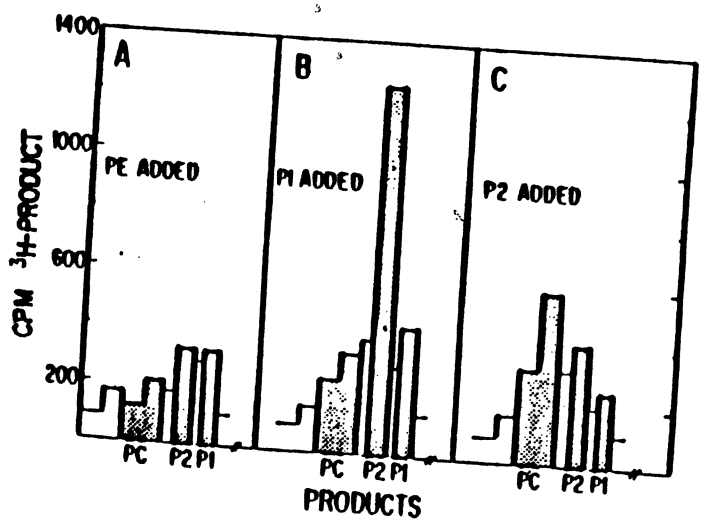

Fig. 2. Thin-layer chromatography of PeMT products. Synaptosomes were incubated with SAM (4.5 μM; 10,000 dpm/pmol). In A, PE was added; in B, PI was added; and in C, P2 was added (all at 100 μg/tube) to the reaction mixture. Results represent radioactivity on TLC plate scraped in sequence. Stippled areas indicate segments to which authentic standard migrated.

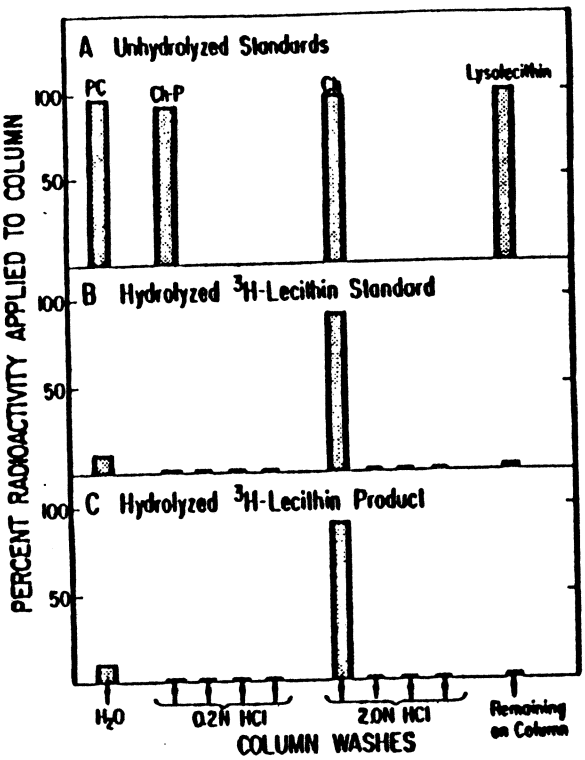

Fig. 3. Identification of base moiety in lecithin, using ion exchange chromatography. Samples were prepared as described in text. In A, authentic lecithin (phosphatidylcholine [PC]), lysophosphatidylcholine (lysoPC), phosphorylcholine (Ch-P), and choline (Ch) were applied. In B, PC tritiated on the choline moiety was hydrolyzed and applied. In C, lecithin produced by the PeMT reaction (performed with added P2) and identified with TLC was hydrolyzed and applied. Columns were washed with water, and eluted with 0.2 N HCl and 2.0 N HCl as noted.

PeMT(s) enzyme kinetics

The relation between reaction velocity and SAM concentration yielded a typical Michaelis-Menten curve, with an apparent K_m = 20 μM, V_{max} = 50-60 pmol/mg protein × h (Fig. 4). S-Adenosylhomocysteine, a known inhibitor of SAM-dependent reactions, inhibited the methylation of PE in a mixed competitive/non-competitive manner, with a K_i = 35-60 μM.

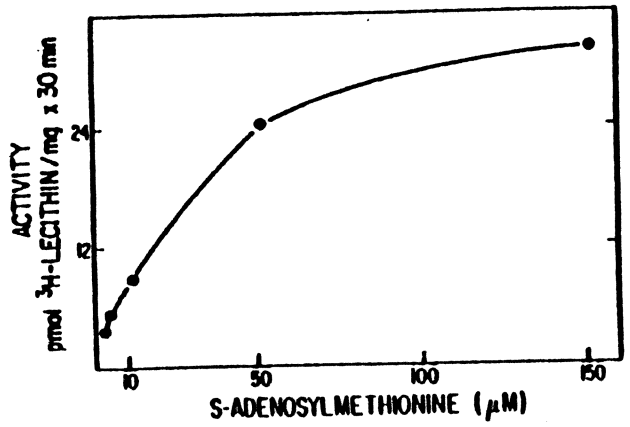

Fig. 4. Kinetics of PeMT reaction (methylation of P2). Concentrations of SAM tested were 3.2 μM, 4.5 μM, 12.2 μM, 52 μM, and 152 μM (Michaelis–Menten plot).

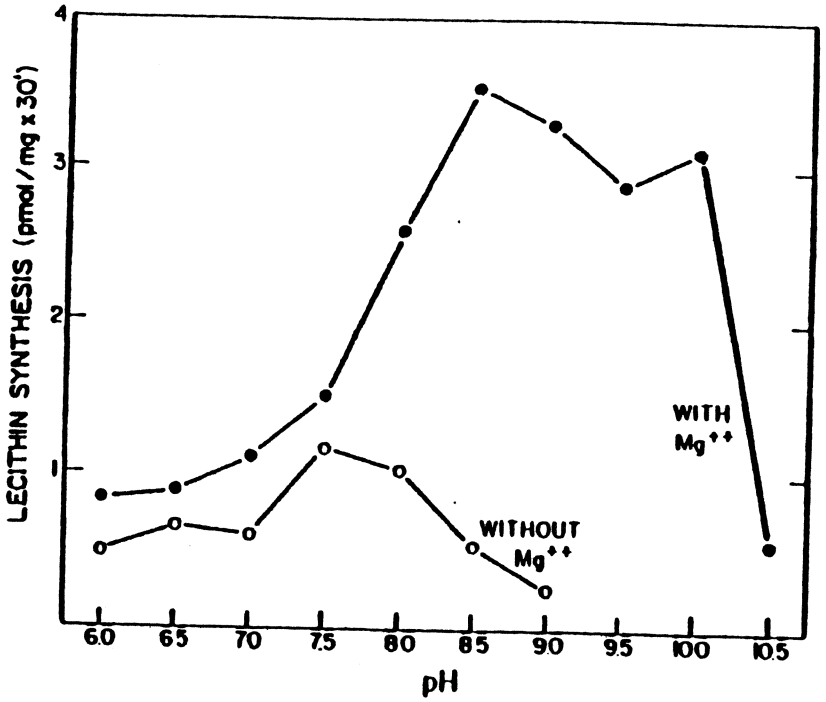

Fig. 5. pH and magnesium dependence of enzyme that converts P2 to lecithin. Sodium acetate buffer (50 mM) was used to generate media pH 6.0 and 6.5; Tris·HCl (50 mM) was used for pH 7.0 8.5, and NaOH-glycine (50 mM) for pH ≥ 9.0. Magnesium chloride was added at a concentration of 15 mM; SAM concentration was 4.5 mM. 100 μg P2 added as substrate.

pH and magnesium dependence

When incubated with magnesium, the enzyme producing lecithin exhibited a pH maximum of 8.5. In the absence of magnesium, lecithin producing enzyme activity was significantly lower, and exhibited its pH maximum at 7.5 (Fig. 5). In contrast, the formation of P2 and P1 exhibited little pH dependence in the range tested (7.0–10.5).

DISCUSSION

These data show that calf caudate contains an enzyme (or enzymes) that sequentially methylates PE, P1 and P2 to form lecithin, and that utilizes either exogenous or endogenous phosphatides as substrates. (That more than a single enzyme may be involved in the overall process is suggested by the observation that the methylation of P2, but not of PE, exhibits marked pH dependence, peaking at pH 8.5.) The enzyme(s) is concentrated within the synaptosomal fraction, uses SAM as its methyl donor, and is inhibited by S-adenosylhomocysteine; it is stimulated by magnesium and exhibits pH maxima of 7.5 without magnesium and 8.5 (or higher) in the presence of magnesium. Crews, Hirata and Axelrod (personal communication) have independently found evidence of enzymes in rat brain synaptosomes that methylate PE to form lecithin.

There has been a great deal of controversy as to the existence of PeMT activity in the brain, despite evidence indicating in vivo and in vitro activity[9,13,22]. Some authors have concluded[1,15] that the brain is unable to synthesize choline de novo on the basis of evidence that it fails to convert intraventricularly administered [^{14}C]ethanolamine

to choline, and that the fatty acid composition of rat brain PE differs significantly from that of lecithin. Since no data are available concerning the metabolic heterogeneity of brain PE, it is possible that a small pool exists which is not formed from ethanolamine, and which, in spite of such observations, does constitute a substrate for PeMT. Also, young rats may have greater PeMT activity than older rats[22] (hence we chose to use brain samples from calves for our preparation).

It remains to be proven that the brain can liberate free choline from the lecithin it forms, or that such choline is converted to acetylcholine. Because the brain contains alkaline phosphatases, glycerophosphocholine diesterase[1,2] and the base-exchange pathways[23], it should be able to cleave choline from lecithin. At certain times, a net efflux of choline from the brain can be detected (by arteriovenous differences)[1]. Moreover, brain choline levels rise sharply after decapitation[4], probably reflecting in part the hydrolysis of brain lecithin.

In preliminary experiments using synaptosomes from rat brain, we observed that free [^{3}H]choline is formed from [^{3}H]SAM in the presence of added P2. The quantities of [^{3}H]choline in the assay mixture are considerably greater than those of [^{3}H]lecithin. The characterization of synaptosomal [^{3}H]choline synthesis will be the subject of a future report.

We find that the specific activity of PeMT in the calf caudate is highest in the synaptosomal fraction (Table I). However, since synaptosomes constitute less than 2% of total tissue protein, *total* PeMT activity is considerably greater in other subcellular fractions. Since lecithin is used generally as a membrane constituent as well as in cholinergic nerve terminals as a potential source of choline for acetylcholine synthesis, it is not surprising that PeMT activity should be ubiquitous.

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