

Synthesis of Phosphatidylcholine from Phosphatidylethanolamine by at Least Two Methyltransferases in Rat Pituitary Extracts*

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Rat pituitary extracts contain at least two methyltransferases that methylate phosphatidylethanolamine to phosphatidylcholine using *S*-adenosylmethionine as the methyl donor. The first enzyme methylates phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine and has a high K_m (40–42 μM) for *S*-adenosylmethionine, whereas the second enzyme(s) catalyzes two successive methylations of phosphatidyl-*N*-monomethylethanolamine to phosphatidyl-*N,N*-dimethylethanolamine and then to phosphatidylcholine and has a low K_m (6.7 μM) for *S*-adenosylmethionine. The first enzyme is loosely bound to the membrane fraction; therefore it appears in both particulate (20,000 $\times g$) and supernatant (20,000 $\times g$) fractions, whereas the second enzyme(s) is tightly bound to the membrane and thus appears only in the particulate fraction. Both methyltransferases have two pH optima of 6.5 and 9.5 (9.5 activity > 6.5 activity) and they do not require Mg^{2+} .

Phosphatidylcholine is the major phospholipid of several endocrine tissues, including pituitary gland (1). The enzymatic synthesis of PC¹ from PE via a methylation pathway has been shown to occur in *Neurospora* (2), rat liver (3), bovine adrenal medulla (4), rat brain (5), and erythrocytes (6). This methylation is catalyzed by two enzymes utilizing AdoMet as the methyl donor (4). The first enzyme, methyltransferase I, methylates PE to form PME. The second enzyme, methyltransferase II, catalyzes two successive methylations of PME to PMME and then PC. Studies from several different laboratories have suggested significant biological roles for PC synthesis by methylation pathway in a number of apparently unrelated processes such as maintenance of membrane fluidity (7), regulation of the number of β -adrenergic (8) as well as human growth hormone receptor (9), Ca^{2+} -ATPase activity (10), monocyte chemotaxis (11), lymphocyte mitogenesis (12), and mast cell histamine secretion (13). Since the contribution of phospholipid methylation pathway in total phospholipid synthesis is relatively low when compared with CDP-choline (14, 15) pathway, a recent report has questioned the impor-

tance of methylation pathway in biological functions (16). However, the location and the nature of phospholipids methylated, independent of the concentration of methyltransferase activity, may be critical in eliciting various physiological responses. It has been noted earlier that PC formed by transmethylation contains the major portion of arachidonic acid although transmethylation is only a minor pathway for the PC synthesis compared with the CDP-choline pathway (14, 15).

To explore the possible role of phospholipid methylation in pituitary physiology, we decided to characterize in detail the potential enzymatic methylation of phospholipids by rat pituitary extracts. Our data show the presence in rat pituitary gland of at least two methyltransferases that catalyze the methylation of PE to PC.

EXPERIMENTAL PROCEDURES

Materials—*S*-Adenosyl-L-[methyl-³H]methionine (14 Ci/mmol) was purchased from New England Nuclear. PE, PME, PMME, and PC were obtained from Grand Island Biological Co. (Grand Island, NY), whereas AdoMet was a product of Sigma.

Animals, Preparation of Crude Extract and Its Subcellular Fractionation—Sprague-Dawley rats (female, 150–200 g) were killed by decapitation; then their pituitary glands were removed and rinsed in ice-cold 0.9% saline. For the preparation of crude extract, pituitaries were homogenized in cold TKM buffer (40 mM Tris(hydroxymethyl)amino methane (pH 7.4) + 7.5 mM KCl + 2 mM MgCl_2) (117- μl buffer/pituitary) by means of a small glass homogenizer. For subcellular fractionation, the pituitaries were homogenized in TKM buffer containing 250 mM sucrose. The pellet and supernatant fractions were prepared by centrifuging crude extract at 20,000 $\times g$ for 20 min. The pellet fraction was washed twice with 2 volumes of the same buffer and then resuspended in 1 volume of buffer.

Assay of Phospholipid Methylation—The methylation of phospholipids was measured by incorporation of [³H]methyl group from *S*-adenosyl-L-[methyl-³H]methionine into phospholipids (4). The reaction mixture (50 μl) contained buffer (2.5 μmol , pH 6.0–10.0), MgCl_2 (0.5 μmol), sodium EDTA (5 nmol), *S*-adenosyl-L-[methyl-³H]methionine (2 μCi , 10 nmol) and tissue extract (10–50 μg of protein). The reaction was initiated by the addition of tissue extract and run in a 12-ml stoppered glass tube at 37 °C for 5 min. To stop the reaction, 3 ml of chloroform/methanol/hydrochloric acid (2/1/0.02, v/v/v) containing 50 $\mu\text{g}/\text{ml}$ of butylated hydroxytoluene followed by 2 ml of 0.1 M KCl in 50% methanol was added. The mixture was vigorously vortexed twice and then centrifuged at 200 $\times g$ for 10 min. The aqueous phase was aspirated, the chloroform phase was rewashed with 2 ml of 0.1 M KCl in 50% methanol, and 1 ml of the chloroform phase was transferred to a miniscintillation vial. After the solvent was evaporated to dryness at 80–85 °C, 4 ml of scintillation fluid (Formula-963, New England Nuclear) was added and the radioactivity was measured in a Packard Liquid Scintillation Counter.

Identification of Reaction Products—To identify the products of phospholipid methylation, the chloroform phase was evaporated to dryness under nitrogen gas at 23 °C and the residue was dissolved in a small volume to chloroform. The sample was applied on a Silica Gel G plate and the chromatogram developed in solvent 32 (chloroform/propionic acid/*n*-propyl alcohol/water (3/2/6/1, v/v/v/v)) at 23 °C

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¹ The abbreviations used are: PC, phosphatidylcholine; AdoMet, *S*-adenosyl-L-methionine; PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-monomethylethanolamine; and PMME, phosphatidyl-*N,N*-dimethylethanolamine.

in ascending mode. The phospholipid standards were chromatographed simultaneously and their positions were visualized by spraying a saturated solution of iodine in chloroform. The areas corresponding to standard phospholipids (PC, PMME, and PME) were scrapped separately and then the radioactivated was extracted with chloroform/methanol (2:1).

The chemical identity of the individual methylated phospholipids was further established by mild alkaline hydrolysis (17) followed by paper chromatography of their deacylated products. Briefly, samples containing individual [*methyl-³H*]phospholipids were transferred into a 50-ml round-bottomed flask. The solvent was removed at 45 °C *in vacuo* with constant swirling to avoid splashing. The residue was redissolved in 8.3 ml of absolute ethanol. Water (0.65 ml) was then added, followed by 0.25 ml of 1 N NaOH. The mixture was incubated at 37 °C for 20 min. Ethyl formate (0.4 ml) was then added and, after mixing, the solution was incubated for a further 5-min period at 37 °C. This neutralized excess of alkali by the reaction ethyl formate + NaOH = sodium formate + ethanol. The hydrolysate was then taken to dryness *in vacuo* at 50 °C. One volume of water was equilibrated with 2 volumes of isobutanol, CHCl₃ mixture (1:2, v/v), by shaking for a few minutes and allowing the phase separation. Samples from 1 ml of the upper aqueous phase and 2 ml of the lower organic phase were added to the dry residue of the hydrolysate, and the flask was shaken and warmed to 37 °C to ensure the quantitative solubilization of the hydrolysis products. The emulsion was then transferred to a centrifuge tube. After centrifugation (1000 × *g* for 15 min), the upper aqueous phase was collected with a Pasteur pipette and then lyophilized. The residue was dissolved in 50 μl of water immediately before paper chromatography. The samples were applied on a Whatman No. 3mm paper and the chromatogram developed in solvent 38 (isopropanol, water, 28% ammonia (7/2/1, v/v/v)) at 23 °C in ascending mode. The phospholipid standards (PC, PMME, and PME) and the products of their alkaline hydrolysis (glycerylphosphorylcholine, *N,N*-

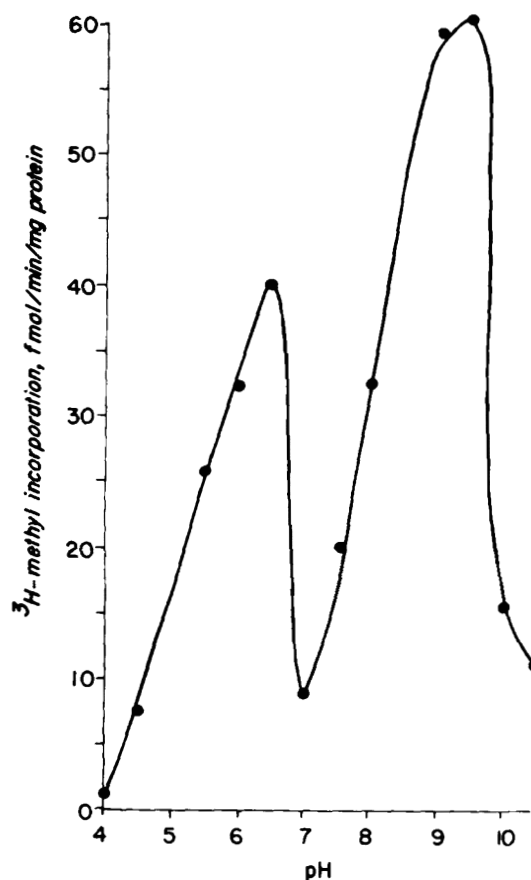


FIG. 1. Effect of pH on [³H]methyl incorporation into phospholipids. Crude pituitary extracts were used for methylation, as described under "Experimental Procedures," except that buffers at various pH values were used. The buffers used were 50 mM sodium acetate between pH 4.0 and 6.5, Tris-acetate between pH 7.0 and 9.5, and sodium borate between pH 10.0 and 10.5.

TABLE I

Subcellular distribution of phospholipid methylating activity at pH 6.5 and 9.5

Pituitary glands were homogenized in TKM buffer containing 250 mM sucrose and then fractionated into supernatant and particulate fractions as described under "Experimental Procedures." The enzyme activity presented below is expressed as mean ± S.E. using 5 independent determinations.

Fraction	pH 6.5	pH 9.5	Ratio (9.5/6.5)
	pmol [³ H]methyl incorporated/min/mg protein		
Crude extract	60.2 ± 16.5	111.3 ± 13.2	2.5 ± 0.8
Particulate (20,000 × <i>g</i>)	32.8 ± 12.0	152.0 ± 28.6	6.1 ± 1.5
Supernatant (20,000 × <i>g</i>)	136.5 ± 14.7	320.4 ± 42.5	2.3 ± 0.1

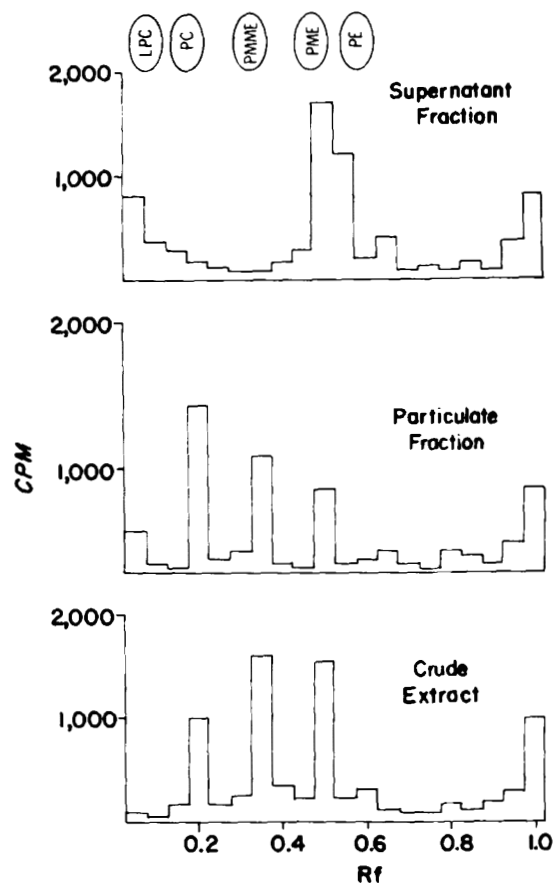


FIG. 2. Chromatographic pattern of the ³H-methylated phospholipids after incubation of rat pituitary crude extracts, particulate (20,000 × *g*) and supernatant (20,000 × *g*) fractions. The reaction products were isolated and chromatographed as described under "Experimental Procedures."

dimethylglycerylphosphorylethanolamine, and *N*-monomethylglycerylphosphorylethanolamine) were chromatographed simultaneously. The position of the hydrolysis product of different methylated phospholipids was visualized by spraying the chromatogram with acid molybdate reagent of Hanes and Isherwood (18) as modified by Dawson (17) followed by irradiation with UV light. The modified acid molybdate reagent contained 10 ml of 72% (w/v) perchloric acid, 20 ml of 5 N HCl, 40 ml of 5% ammonium molybdate, and 130 ml of water.

RESULTS

Properties of Phospholipid Methyltransferase(s) from Rat Pituitary Extracts—To determine the optimal pH for phospholipid methylation, rat pituitary extracts were incubated with *S*-adenosyl-L-[*methyl-³H*]methionine at various pH val-

ues between 4 and 10 and then the amount of [^3H]methyl radioactivity incorporated into phospholipids was determined, as described under "Experimental Procedures." The data presented in Fig. 1 show the presence of two pH optima (6.5 and 9–9.5) for phospholipid methylation by pituitary extracts. The incorporation of [^3H]methyl radioactivity into organic (chloroform) phase after extraction of the reaction mixture with chloroform, methanol, HCl (2/1/0.02, v/v/v) did not require the presence of Mg^{2+} , and the incorporation increased with increasing concentration of enzyme protein and the time of incubation. The radioactivity extracted into chloroform phase was not volatile after heating at 80–85 °C to dryness.

Subcellular Distribution of Phospholipid Methyltransferase(s) Activity—A subcellular distribution of pH 6.5 and 9.5 activities is presented in Table I. The activity at pH 9.5 was always greater than the activity at 6.5 for all three fractions. The highest specific activity was found in the supernatant fraction at both pH values. Thus, it was not clear whether the activities at pH 6.5 and 9.5 were due to the same or different enzymes. Therefore, we decided to determine the nature of the product(s) of phospholipid methylation by various fractions at pH 6.5 and 9.5.

Identification of the Products of Phospholipid Methylation—To identify the products, the standard reaction mixture (50 μl) was scaled up to 250 μl . The [^3H -methyl]phospholipids were extracted into chloroform phase, evaporated to dryness under nitrogen gas at 23 °C, and then various products were identified as described under "Experimental Procedures." Fig. 2 shows the TLC analysis of the products of phospholipid methylation catalyzed by crude extract, supernatant, and particulate fractions at pH 9.5. In the case of crude extract (*bottom*) and particulate fraction (*middle*), three major radioactive peaks with R_F values corresponding to PME, PMME, and PC appeared. However, the products of reaction catalyzed by supernatant fraction (*top*) had only one radioactive peak corresponding to the same R_F value as PME. The analysis of the products formed by various fractions at pH 6.5 exhibited the same results.

The radioactive products co-migrating with standard methylated phospholipids (PC, PME, and PMME) were recovered from the TLC plate and then deacylated by mild alkaline hydrolysis as described under "Experimental Procedures." The deacylation of PC, PMME, and PME leads to the formation of glycerolphosphoryl derivatives of choline (GP-choline), *N,N*-dimethylethanolamine (GP-MME) and *N*-mono-

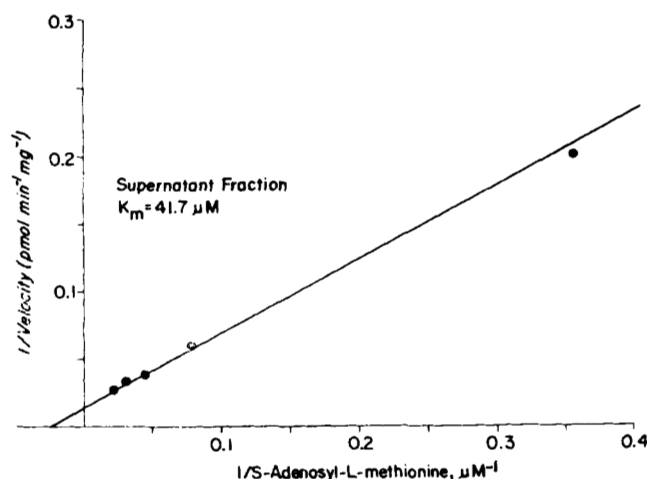


FIG. 3. Effect of S-adenosyl-L-methionine concentration on phospholipid methylation by supernatant (20,000 \times g) fraction at pH 9.5. The data show a double reciprocal plot of velocity versus AdoMet concentration (1–50 μM).

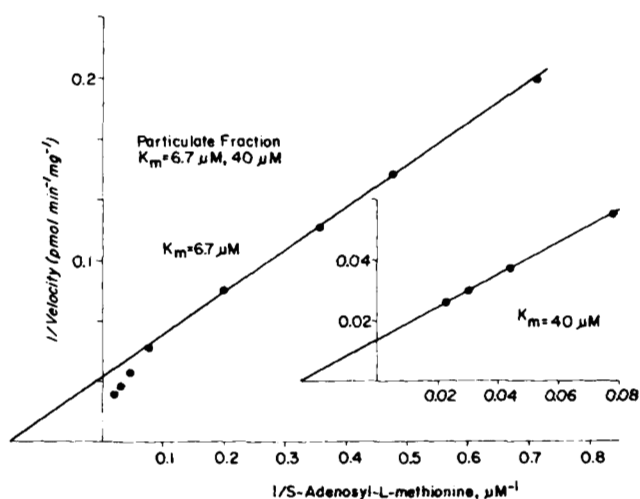


FIG. 4. Double reciprocal plot of velocity of phospholipid methylation at various S-adenosyl-L-methionine concentrations. Large plot shows AdoMet concentrations between 1.4 and 43.5 μM , whereas inset shows concentrations of AdoMet between 12.8 and 43.5 μM .

methylethanolamine (GP-ME), respectively. On paper chromatography, the deacylated derivatives of PC (0.45), PMME (0.68), and PME (0.56) showed R_F values similar to standard GP-choline (0.42), GP-MME (0.63), and GP-ME (0.60), respectively.

Possible Presence of Multiple Methyltransferases in Pituitary Extracts—The above observations suggest the presence of multiple methyltransferases in pituitary extracts. Phospholipid methylation by particulate and supernatant fractions at pH 9.5 was measured using various concentrations of [^3H]AdoMet (1–50 μM). A Lineweaver-Burk plot of the resulting data shows an apparent K_m for AdoMet of 41.7 μM for the supernatant fraction (Fig. 3). However, such a plot of the data for the particulate fraction showed the presence of two separate components. One component had an apparent K_m for AdoMet of 6.7 μM , and the second component had an apparent K_m of 40 μM (Fig. 4). The measurement of K_m values at pH 6.5 gave similar results. Thus, the second component apparently is methyltransferase I (methylating PE to PME, see *top* of Fig. 2), whereas the first component is methyltransferase II (methylating PME to PC). From the data presented here it is not obvious whether the methylation of PME to PC is catalyzed by one or two enzymes.

DISCUSSION

Phosphatidylcholine can be synthesized by two alternative pathways, the incorporation of CDP-choline to α,β -diacylglycerate or the stepwise methylation of PE (3, 19). This report provides evidence that the synthesis of PC can be carried out by two phospholipid methyltransferases in pituitary extracts. The first enzyme (methyltransferase I), which catalyzes the formation of PME from PE, has a high apparent K_m for AdoMet (41 μM) and no requirement for Mg^{2+} . The second enzyme (methyltransferase II), which catalyzes two successive methylations of PME to form PC, has a low apparent K_m for AdoMet (6.7 μM) and no requirement for Mg^{2+} . Both methyltransferases have two pH optima (pH 6.5 and 9.5). The activity at pH 9.5 is always greater than that at pH 6.5. The ratio of activities at these two pH values is 6.1 for the particulate fraction but 2.3 for the soluble fraction.

The phospholipid methyltransferase activity of the pituitary is much higher than that reported for brain (5), erythrocytes (6), and bovine adrenal medulla (4), but not liver (20).

Also the properties of two methyltransferases from pituitary differ from those of bovine adrenal medulla and brain (4, 5). Pituitary methyltransferase I has high K_m for AdoMet (40–42 μM), two pH optima, and no Mg^{2+} requirement, whereas the adrenal and brain enzymes have low K_m for AdoMet (2–4 μM), a single pH optimum between 7.0 and 7.5, and require Mg^{2+} for full enzymatic activity. In contrast, the methyltransferase II from pituitary has low K_m for AdoMet (6.7 μM), two pH optima, and no Mg^{2+} requirement, whereas the adrenal and brain enzymes have high K_m for AdoMet (100–110 μM), a single pH optimum (10.0–10.5), and also no Mg^{2+} requirement.

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