BBA 65626

PROPERTIES OF A PHOSPHATIDYLMONOMETHYLETHANOLAMINE *N*-METHYLTRANSFERASE FROM *NEUROSPORA CRASSA*

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(Received March 20th, 1967)

SUMMARY

A soluble preparation was obtained from microsomes of *Neurospora crassa* that catalyzed the two-step methylation of phosphatidylmonomethylethanolamine to phosphatidylcholine with *S*-adenosylmethionine as the methyl donor. Both the phosphatidyl-*N*-monomethylethanolamine and the phosphatidyl-*N*,*N*-dimethylethanolamine *N*-methyltransferase activities had optima near pH 8.0 and showed similar heat denaturation characteristics. The ratio of the two activities remained essentially the same in fractions obtained by precipitation with $(NH_4)_2SO_4$. The results of these studies are consistent with the conjecture based on previous work that one enzyme catalyzes the methylation of both phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine.

INTRODUCTION

Previous studies in this laboratory[1] demonstrated a phosphatide *N*-methyltransferase system in microsomes of *Neurospora crassa* that catalyzed the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine with *S*-adenosylmethionine as the methyl-group donor. This paper describes the properties of a soluble preparation obtained from a normal strain of *N. crassa* which catalyzes the two-step methylation of phosphatidyl-*N*-monomethylethanolamine to phosphatidylcholine. The studies presented here suggest that both of these methyl-group transfers are mediated by a single enzyme.

METHOD

Materials

The 100-mesh silicic acid powder used for the column chromatography of phospholipids was obtained from the Mallinckrodt Chemical Works.

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Protein and phosphate determinations

Protein was determined by the colorimetric procedure of LOWRY *et al.*[2] with citrate replacing tartrate in the reagent[3]. Bovine serum albumin was used as the reference standard for these determinations. The procedure for phospholipid-phosphorus analyses was that used in earlier work[1].

Sonic oscillation treatment of microsomes

The microsomal fraction was prepared from *N. crassa* strain 1A by a procedure described in a previous communication[1]. The microsomes derived from 10 g of moist tissue were suspended in 15 ml 0.05 M Tris–HCl (pH 8.0) which was 0.25 M with respect to sucrose, and disrupted by sonic oscillation for 30 min at 10° in a Raytheon magnetostrictive type instrument (9 kcycles, 50 W). This dispersed preparation was centrifuged for 1 h at 105 000 × *g* in a Spinco Model-L centrifuge to remove the remaining particulate material. The resulting supernatant fraction contained the soluble phosphatidyl-*N*-monomethylethanolamine *N*-methyltransferase activity. This preparation will be referred to in these studies as the initial microsomal dispersion. The microsomal phosphatidylethanolamine *N*-methyltransferase activity[1] was not recovered after disruption by sonic oscillation.

Isolation of the phospholipid substrates

The two substrates for the phosphatidyl-*N*-monomethylethanolamine *N*-methyltransferase, the phosphatides of *N*-monomethylethanolamine and *N,N*-dimethylethanolamine, were obtained from *N. crassa* strain 47904[4]. The total lipids were extracted from the mold[4], and the neutral lipids were removed from the phospholipids by the chromatographic procedure of MEAD AND FILLERUP[5]. The final fractionation of the phospholipids was by chromatography on a dry-packed silicic acid

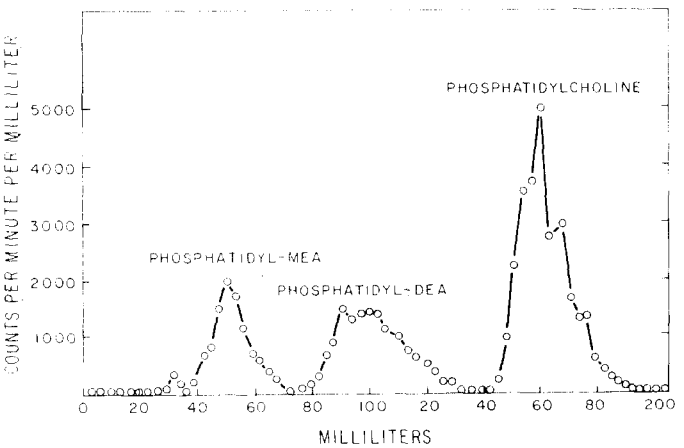

Fig. 1. A typical elution pattern for phosphatides of *N*-monomethylethanolamine (MEA), *N,N*-dimethylethanolamine (DEA) and choline from the silicic acid column described in the text. Trace amounts of the three enzyme-prepared phosphatides[1], labeled with ^{14}C in the *N*-methyl groups, were added as markers to the column sample consisting of 300 mg of the total phospholipids from *N. crassa* strain 47904. Eluate fractions were assayed by conventional counting procedures.

column, developed for this purpose in this laboratory. This procedure effected the separation of the three *N*-methylated derivatives of phosphatidylethanolamine. A typical elution pattern for these phospholipids is shown in Fig. 1. The silicic acid used as the stationary phase in this procedure was heated for 1 h at 110° and immediately packed into a glass chromatographic column (28 mm in diameter) to a height of 17.5 cm. The column packing was protected from moisture while it cooled, and all subsequent procedures were carried out at room temperature. About 300 mg of the crude phospholipid fraction from *N. crassa* strain 47904 were dissolved in 2 ml of chloroform–methanol (70:25, v/v) and applied to the column. After the sample entered the upper region of the dry column, it was followed by three 1 ml portions of the eluting solvent, chloroform–methanol–water (70:25:2.7, v/v/v). A thick filter-paper disc which fits snugly inside the column was then tamped onto the surface of the silicic acid column. This prevented disfiguration of the first few cm of the silicic acid as it became wetted by further addition of the eluting solvent. The dry column was then developed further with the eluting solvent descending under a solvent head of about 100 cm. The presence of the desired phospholipids in the eluate fractions could be determined with bioassays[4,6] or by isotope measurements when labeled samples were employed. The individual phospholipid substrates in pooled column fractions were determined quantitatively by assay with *N. crassa* strain 34486 (refs. 4, 6).

Dispersion of phospholipid substrates

The phosphatide substrates which were to be added to the enzyme incubations were dispersed immediately before use by cavitation for 5 min with a Branson Sonifier. The dispersion medium was an aqueous solution containing 0.25 M sucrose, 0.5 mM EDTA, and 0.01 M Tris–HCl (pH 8.0). The resulting substrate dispersions were 1.3 mM for phosphatidyl-*N*-monomethylethanolamine and 1.0 mM for phosphatidyl-*N*,*N*-dimethylethanolamine.

Enzyme incubations

In the standard procedure for determining the *N*-methyltransferase activity, the soluble enzyme derived from the microsomes of *N. crassa* was incubated at 37° for 10 or 15 min with *S*-adenosyl-L-[*Me*-^{14}C]-methionine and a phospholipid substrate. The final vol. of the assays was 0.7 ml, and Tris–HCl was used to maintain the mixture at an appropriate pH. The samples were heated to stop the reaction and assayed for the labeled products formed by a procedure described in a previous communication[1]. In this procedure the labeled phospholipid bases from assay samples were released by acid hydrolysis, chromatographed on paper, and quantitatively determined in a strip counter. Within each experiment the enzyme activities were comparable since a fresh sample of dispersed phospholipid substrate was prepared each time. The difficulty inherent in getting reproducible phospholipid dispersions caused some daily variations in the measured activity of the *N*-methyltransferase.

RESULTS

Dependence of N-methyltransferase activity on pH

An experiment was carried out to determine the pH dependence of each of the

two reactions catalyzed by the phosphatidyl-*N*-monomethylethanolamine *N*-methyltransferase recovered in the microsomal dispersions. The data in Table I show that both reactions have optimal rates near pH 8.0. When phosphatidyl-*N*-monomethylethanolamine is used as the substrate at pH 7.2, only a small amount of the phos-

TABLE I

THE EFFECT OF pH ON THE PHOSPHATIDYL-*N*-MONOMETHYLETHANOLAMINE *N*-METHYLTRANSFERASE ACTIVITY IN THE SOLUBLE MICROSOMAL DISPERSION

Assay mixtures contained enzyme (0.71 mg protein), 80 μmoles Tris–HCl (pH 7.2, 8.0, or 9.0), 25.5 mμmoles *S*-adenosyl-L-[*Me*-^{14}C]methionine (4.9 μC/μmole) and either 260 mμmoles phosphatidyl-*N*-monomethylethanolamine or 200 mμmoles phosphatidyl-*N*,*N*-dimethylethanolamine in a total vol. of 0.7 ml. The samples were incubated for 10 min at 37°. The products formed are expressed on the basis of the protein content of the enzyme preparations.

Substrate	*Sample*	*pH*	*mμmoles produced per mg protein/10 min*	
			Phosphatides of N,N-dimethylethanolamine	*Phosphatides of choline*
Phosphatidyl-*N*-monomethylethanolamine	1	7.2	2.46	0.09
	2		2.53	0.10
	1	8.0	2.85	0.31
	2		2.57	0.33
	1	9.0	0.95	0.44
	2		0.91	0.48
Phosphatidyl-*N*,*N*-dimethylethanolamine	1	7.2		0.88
	2			0.83
	1	8.0		0.95
	2			0.92
	1	9.0		0.23
	2			0.13

phatidyl-*N*,*N*-dimethylethanolamine that is initially formed is converted further to phosphatidylcholine. This fact made it convenient to do *N*-methyltransferase studies at pH 7.2 when it was desirable to measure the initial methylation of phosphatidyl-*N*-monomethylethanolamine with a minimal amount of correction for the product lost due to further methylation.

Time course of the N-methyltransferase reactions

The methylation of the phosphatides of *N*-monomethylethanolamine and *N*,*N*-dimethylethanolamine was studied as a function of time at pH 7.2 and 8.0. Incubations similar to those described in Table I were analyzed after 5-, 10- and 15-min intervals to determine if the enzyme activities were altered during this time. These studies showed that the enzyme retained its original activity for at least 15 min at both pH 7.2 and pH 8.0.

TABLE II

HEAT DENATURATION OF PHOSPHATIDYL-*N*-MONOMETHYLETHANOLAMINE *N*-METHYLTRANSFERASE ACTIVITIES IN MICROSOMAL DISPERSIONS

Samples were heated at 45° or 50° for 10 min and the relative *N*-methyltransferase activities with phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine as substrates were determined in the usual manner. Assay incubations of 0.7-ml volume contained enzyme (0.71 mg protein in Expt. 1 and 0.92 mg in Expt. 2, 100 μmoles Tris–HCl (pH 7.2), 50 mμmoles *S*-adenosyl-L-[*Me*-^{14}C]methionine, and either 260 mμmoles of phosphatidyl-*N*-monomethylethanolamine or 400 mμmoles of phosphatidyl-*N*,*N*-dimethylethanolamine. The samples were incubated for 10 min at 37°.

Expt.	*Treatment*	*Activity remaining* (%)*	*Ratio***
1	None	100	1.02
	45° for 10 min	44	0.90
	50° for 10 min	36	0.89
2	None	100	1.30
	45° for 10 min	63	1.35
	50° for 10 min	49	1.39

* Based on phosphatidyl-*N*-monomethylethanolamine as substrate.

** Ratio of phosphatidyl-*N*-monomethylethanolamine to phosphatidyl-*N*,*N*-dimethylethanolamine *N*-methyltransferase activities expressed in terms of molar ratios of products formed.

Michaelis constants

Attempts to determine Michaelis constants for phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine were not feasible be-

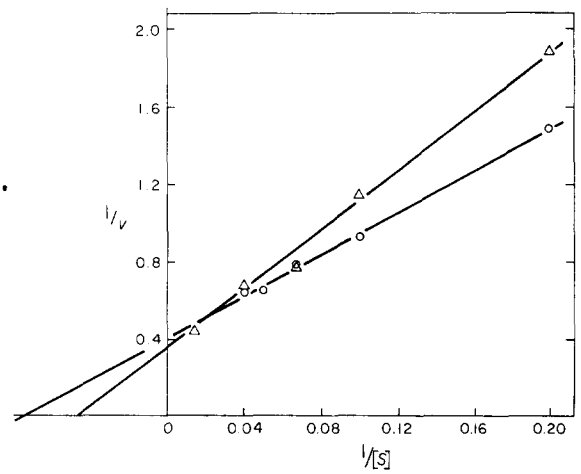

Fig. 2. Reciprocal plot of the effect of the adenosylmethionine concentration, S (μM), on the phosphatidyl-*N*-monomethylethanolamine (○—○) and phosphatidyl-*N*,*N*-dimethylethanolamine (△—△) *N*-methyltransferase activities in the initial microsomal dispersion from strain 1A. Individual assays contained the enzyme, 100 μmoles Tris–HCl (pH 7.2), either 260 mμmoles of phosphatidyl-*N*-monomethylethanolamine or 200 mμmoles of phosphatidyl-*N*,*N*-dimethylethanolamine, and from 3.5 to 17.5 mμmoles of *S*-adenosyl-L-[*Me*-^{14}C]methionine (5 μC/μmole). Final assay vol. was 0.7 ml. The velocity, V, is expressed as the mμmoles of appropriate phosphatide methylated per mg protein per 10 min at 37°.

cause the data were variable, presumably due to daily differences in the lipid dispersions. On the basis of preliminary experiments the levels of dispersed phospholipid substrates used in subsequent experiments were arbitrarily chosen at 0.37 μmoles per ml for phosphatidyl-*N*-monomethylethanolamine and 0.28 to 0.57 μmoles per ml for phosphatidyl-*N*,*N*-dimethylethanolamine.

Fig. 2 shows typical Lineweaver–Burk plots for the K_m determinations on *S*-adenosylmethionine for the phosphatidyl-*N*-monomethylethanolamine and the phosphatidyl-*N*,*N*-dimethylethanolamine *N*-methyltransferase reactions. The K_m for *S*-adenosylmethionine in the phosphatidyl-*N*-monomethylethanolamine *N*-methylation was 13 μM. Two other similar K_m determinations resulted in values of 14 and 8 μM. The K_m for *S*-adenosylmethionine in the phosphatidyl-*N*,*N*-dimethylethanolamine methylation was 18 μM. Another similar determination gave a value of 13 μM. Here again, the lipid dispersions were probably a contributing factor to the variations in the apparent K_m values. The experimental details for these experiments are given in Fig. 2. Saturating levels of *S*-adenosylmethionine (70 μM) for both *N*-methyltransfer reactions were used routinely in subsequent experiments.

Partial heat denaturation of the N-methyltransferase activity

Samples of initial microsomal dispersions were subjected to partial heat denaturation to determine if this treatment would have a selective effect on one of the two transmethylations catalyzed by these preparations. Suitable aliquots of each enzyme preparation under investigation were heated at 45° or 50° for 10 min and then assayed along with the untreated enzyme to determine the relative *N*-methyltransferase activities of each sample with respect to phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine as substrates. The results of two experiments with different initial enzyme preparations are shown in Table II where the ratio of the two activities is given for each samples. It is apparent from these data that if two enzymes catalyze the two-step conversion of phosphatidyl-*N*-monomethylethanolamine to phosphatidylcholine, they have similar heat denaturation characteristics.

Gel filtration with Sephadex

An attempt was made to fractionate by gel filtration the phosphatidyl-*N*-monomethylethanolamine *N*-methyltransferase found in the initial microsomal dispersions. A sample was applied to a 1.5 cm × 81 cm chromatographic column containing Sephadex G-150 which was equilibrated and eluted at 4° by descending chromatography with 0.05 M Tris–HCl (pH 8.0). Assays of eluate fractions disclosed that all measurable catalytic activity for both steps in the conversion of phosphatidyl-*N*-monomethylethanolamine to phosphatidylcholine is associated with material that is excluded from the stationary phase. The molecular weight of this active material is large enough to prevent its accessibility to the inner volume of the gel particles.

$(NH_4)_2SO_4$ fractionation

Preliminary experiments indicated that all of the phosphatide *N*-methyltransferase activity in microsomal dispersions precipitated at 4° between 20 and 50% satn. with $(NH_4)_2SO_4$. A fraction collected between 30 and 40% satn. was assayed

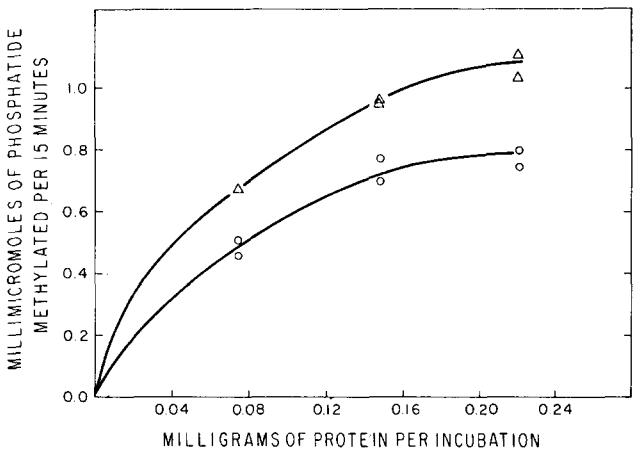

Fig. 3. Enzyme-concentration dependence of the phosphatidyl-*N*-monomethylethanolamine and -*N*,*N*-dimethylethanolamine *N*-methyltransferase activities precipitated between 30 and 40% satn. with $(NH_4)_2SO_4$. Assays contained 100 μmoles Tris–HCl (pH 7.2), 50 mμmoles *S*-adenosyl-L-[*Me*-^{14}C]-methionine (5 μC/μmole), either 260 mμmoles of phosphatidyl-*N*-monomethylethanolamine or 300 mμmoles of phosphatidyl-*N*,*N*-dimethylethanolamine, and variable amounts of the soluble enzyme preparation expressed in terms of the protein content. Final assay volume was 0.7 ml and the reactions were incubated for 10 min at 37°. ○—○, phosphatide substrate was phosphatidyl-*N*-monomethylethanolamine; △—△, phosphatide substrate was phosphatidyl-*N*,*N*-dimethylethanolamine.

for both transferase activities at different enzyme levels. The results (Fig. 3) showed that although the measured activities were not proportionate to enzyme concentration, the ratio of phosphatidyl-*N*-monomethylethanolamine to phosphatidyl-*N*,*N*-

TABLE III

FRACTIONATION OF ACTIVITY WITH $(NH_4)_2SO_4$

The proteins in a microsomal dispersion prepared by the standard procedure were fractionated by precipitation with $(NH_4)_2SO_4$ at 4°. Each fraction was allowed to precipitate for 1 h before it was recovered by centrifugation. Assay mixtures contained aliquots of the indicated enzyme fractions, 100 μmoles of Tris–HCl (pH 8.0), 50 mμmoles of *S*-adenosyl-L-[*Me*-^{14}C]-methionine, and either 260 mμmoles of phosphatidyl-*N*-monomethylethanolamine or 300 mμmoles of phosphatidyl-*N*,*N*-dimethylethanolamine. The final vol. was 0.7 ml and the assays were incubated for 15 min at 37°. II and III are used to indicate the phosphatidyl-*N*-monomethylethanolamine *N*-methyltransferase and the phosphatidyl-*N*,*N*-dimethylethanolamine *N*-methyltransferase activities, respectively.

Fraction (percent satn. with $(NH_4)_2SO_4$)	*Protein (mg per incubation)*	*Relative activity**		*Specific activity***		*Ratio**** *(II/III)*	*Phospholipid phosphorus (μmoles per mg protein)*
		II	*III*	*II*	*III*		
0	0.920	100	100	3.4	1.8	1.87	0.18
20–30	0.129	50	93	12.1	12.1	1.00	0.85
30–40	0.116	32	65	8.8	9.4	0.94	0.17
40–50	0.144	13	26	2.8	3.0	0.93	0.10
50–60	0.101	0	0	0	0		0.00

* Percent of the activity present in the crude extract.

** mμmoles of appropriate phosphatide methylated per mg protein in 15 min.

*** Ratio of phosphatidyl-*N*-monomethylethanolamine to phosphatidyl-*N*,*N*-dimethylethanolamine *N*-methyltransferase activities.

dimethylethanolamine *N*-methyltransferase activity remained constant with increasing enzyme concentration. For example, the molar ratios of these relative activities were 0.72, 0.78 and 0.72 at protein levels of 0.07, 0.15, and 0.22 mg per incubation, respectively.

Table III shows the results of an experiment in which fractions derived from precipitation with $(NH_4)_2SO_4$) were assayed with the two phospholipid substrates under investigation here. In an attempt to minimize the unknown concentration dependence of the enzyme, the protein level in the assays of these fractions was kept between 0.10 and 0.15 mg per incubation. Here again, the relative activities concerned with the two methylations were similar in the three precipitated fractions. There was no evidence for the separation of two or more enzymes which might be involved in these reactions. A suboptimal activity toward phosphatidyl-*N*,*N*-dimethylethanolamine was expressed by the enzyme in the original dispersed phase, as evidenced by its enhanced recovery in the partially purified fractions.

DISCUSSION

Microsomal preparations of *N. crassa* contain a phosphatide *N*-methyltransferase system that utilizes *S*-adenosylmethionine and endogenous phosphatidylethanolamine to make phosphatidylcholine (lecithin) by a series of three methyl transfers. The properties of a particulate-bound phosphatidylethanolamine *N*-methyltransferase in *N. crassa* that catalyzes the first methylation step in this sequence of reactions were described in a previous communication[1]. In the present work unsuccessful attempts were made to prepare this enzyme in a soluble form that would utilize the appropriate exogenous phospholipid as a substrate. The methylation of exogenous phosphatidylethanolamine by an analogous microsome-bound enzyme found in rat liver has also been difficult to attain in either microsomal or soluble preparations[7,8]. The ease with which a soluble phosphatidylethanolamine *N*-methyltransferase was recovered from extracts of *A. tumefaciens* suggests that a species difference exists in the mode of binding of this enzyme to the cell particulate[9]. The soluble enzyme from *A. tumefaciens* readily utilizes exogenous phosphatidylethanolamine as a substrate.

Early biochemical-genetic studies with *N. crassa*[10] and more recent work with cell-free systems[1] suggested that one enzyme, a phosphatidyl-*N*-monomethylethanolamine *N*-methyltransferase, may be involved in the second and third *N*-methyltransfers leading to phosphatidylcholine biosynthesis. This paper describes some properties of a soluble preparation derived from microsomes of *N. crassa* which catalyzes the two-step methylation of phosphatidyl-*N*-monomethylethanolamine to phosphatidylcholine. An effort was made in these studies to determine if this dual activity resides in a single enzyme since the soluble preparation utilizes *S*-adenosylmethionine to methylate either phosphatidyl-*N*-monomethylethanolamine or phosphatidyl-*N*,*N*-dimethylethanolamine. The two lipids used as substrates for these reactions were natural products isolated from a mutant strain of *N. crassa*. Initial studies disclosed that both the phosphatidyl-*N*-monomethylethanolamine and the phosphatidyl-*N*,*N*-dimethylethanolamine *N*-methyltransferase activities have optima near pH 8.0, and that at 37° the rates of these two reactions in the standard assay remain linear for at least 15 min. With both phospholipid substrates it was

possible to determine saturation curves for *S*-adenosylmethionine and to obtain linear Lineweaver–Burk plots. Attempts to determine Michaelis constants for phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine at saturating levels for *S*-adenosylmethionine were unsuccessful. The enzyme activity of any particular preparation with respect to both methylations was subject to some variability from one experiment to the next due to the inherent difficulty in obtaining reproducible phospholipid substrate dispersions of uniform character. The micellar nature of the lipid substrates leads to ambiguity concerning their effective concentration. Comparisons of relative enzyme activities were confined to the individual experiments in which all assays shared the same substrate preparations. Both *N*-methyltransferase activities studied here were associated with material that exceeded in molecular weight the maximum size retained by Sephadex G-150 in a gel-filtration column. The enzyme(s) may be attached to microsomal fragments since phospholipids are found in all preparations (Table III). Heat denaturation studies did not disclose a selective loss of one of the two activities. Fractionation with $(NH_4)_2SO_4$ also failed to show a preferential precipitation of either one of the *N*-methyltransferases since the relative activities with respect to the two phospholipid substrates were similar in the three fractions that were obtained in this manner. These observations are consistent with the original premise based on previous work with *N. crassa* that a single enzyme catalyzes the methylation of both phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine[1,10]. Studies by REHBINDER AND GREENBERG also indicate that a single enzyme in rat liver microsomes catalyzes both methylations of phosphatidyl-*N*-monomethylethanolamine to lecithin[8]. The resolution of this problem in *N. crassa* will require systematic studies on the behavior of purified phosphatide *N*-methyltransferases in their biphasic surface reactions with the appropriate substrate micelles. Studies are continuing on the isolation and further purification of these enzymes.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the assistance of Mrs. ANIKO MEENAN during these studies. This investigation was supported by research grants (GM 10935 and 5T1 GM 364) from the National Institutes of Health and by the Cancer Research Fund of the University of California.

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